VILNIUS UNIVERSITY

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INVESTIGATION OF TETRAMETHYLPYRAZINE DEGRADATION IN *RHODOCOCCUS* SP. TMP1 BACTERIA

Summary of doctoral thesis
Physical sciences, biochemistry (04 P)

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VILNIAUS UNIVERSITETAS

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TETRAMETILPIRAZINO SKAIDYMO *RHODOCOCCUS* SP. TMP1 BAKTERIJOSE TYRIMAS

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INTRODUCTION

Pyrazines, monocyclic aromatic rings with two nitrogen atoms in *para* position, are a class of compounds that occur almost ubiquitously in nature. They can be synthesized both chemically and biologically; however, there is very little information available on the biodegradation of these *N*-heterocyclic compounds. While bacteria strains, capable of using various alkyl-substituted pyrazines as a sole carbon and energy source, have been isolated and described (Kiener et al., 1993; Rappert et al., 2006; Rappert et al., 2007; Müller and Rappert, 2010), almost nothing is known about the degradation pathways of alkylpyrazines including tetramethylpyrazine, which is special since each of its carbon atoms carry a substituent.

Under aerobic conditions di-substituted pyrazines are metabolized via the oxidative degradation leading to the hydroxylation of the ring at a free ring position (Kiener et al., 1993). However, no enzymes involved in such bioconversion have been reported (Kiener et al., 1993). Others reported that the hydroxylation of 2,5-dimethylpyrazine was mediated by an inducible enzyme (Rappert et al., 2007). Even though this enzyme remains unidentified, it appears that the degradation of 2,5-dimethylpyrazine does not depend on molybdenum-containing enzymes, in contrast to the degradation of pyridines that are also metabolized via ring hydroxylation (Fetzner, 1998).

The degradation of tri-substituted pyrazines was demonstrated to follow the same metabolic pattern as di-substituted pyrazines (Rappert et al., 2006). The degradation involves inducible enzymes that produce hydroxylated intermediates in the initial step (Rappert et al., 2006). However, these enzymes have not been identified yet.

In contrast to partially substituted pyrazines, tetramethylpyrazine (TTMP) cannot be degraded via initial hydroxylation to form hydroxypyrazine, because its all carbons carry a substituent. Muller and Rappert (2010) suggested that the initial step of TTMP degradation may involve the ring cleavage. They used cell-free extracts from *Rhodococcus opacus* strain, which can use TTMP as a single carbon, nitrogen and energy source, and were not able to detect any intermediate forming during TTMP degradation (Müller and Rappert, 2010). However, these preliminary data were never supported by any publications following their appearance in a review. Despite the

isolation of TTMP-degrading bacteria, neither enzymes catalyzing TTMP biodegradation, nor the corresponding genes have been identified in *R. opacus*.

In pursuance to expand current fundamental knowledge about biodegradation of *N*-heterocyclic compounds and to reveal various enzymes that may be employed in the future for selective and specific bioconversion reactions, **the aim of this study** was to identify the genes responsible for bacterial degradation of TTMP and describe the catabolic pathway of alkylpyrazine metabolism in bacteria.

Objectives of this study

- To identify the genes that encode the degradation of tetramethylpyrazine in *Rhodococcus jostii* strain TMP1.
- To elucidate the expression regulation of the genes encoding the degradation of tetramethylpyrazine in *R. jostii* TMP1.
- To describe the metabolic pathway of tetramethylpyrazine degradation in *R. jostii* TMP1.
- To assess whether *R. jostii* TMP1 can be employed for selective and specific bioconversion reactions.

Scientific novelty:

This study describes the metabolic pathway of TTMP degradation in *R. jostii* strain TMP1 that is able to use TTMP as a sole carbon and energy source. For the first time bacterial genes required for alkylpyrazine biodegradation were identified. 13 kb DNA fragment was determined and designated as tetramethylpyrazine degradation (*tpd*) locus. Sequence analysis of *tpd* locus revealed eight open reading frames (ORFs) that encode seven enzymes TpdA-E, Orf1 and Orf2, as well as one transcriptional activator TpdR. Thus for the first time bacterial enzymes involved in the catabolism of alkylpyrazines were described.

Genes from *tpdABC* operon were cloned and heterologously expressed in TTMP-nondegrading *Rhodococcus erythropolis* SQ1 to investigate the function of individual Tpd proteins in TTMP biodegradation. TTMP was metabolized by SQ1 transformed with *tpdABC* and *tpdAB* genes, while individual *tpdA*, *tpdB* and *tpdC* genes were not sufficient for TTMP conversion. Reaction products of TpdABC and TpdAB were

extracted from the bioconversion media and their structure was determined by ^{1}H NMR, ^{13}C NMR and MS analysis. TpdAB metabolized TTMP to (*Z*)-*N*,*N*'-(but-2-ene-2,3-diyl)diacetamide (BDNA), whereas the product of TpdC was *N*-(3-oxobutan-2-yl)acetamide (OBNA). BDNA and OBNA are the first identified bacterial metabolites of TTMP.

Oxygen-tracer experiment revealed that TpdAB acts as monooxygenase that oxidizes and cleaves TTMP ring. While some other monooxygenases that catalyze similar reactions have been described before, these flavin monooxygenases belong to class A. In contrast, TpdAB is a flavin monooxygenase of class C and is the first enzyme in this class catalyzing the biodegradation of aromatic compounds.

In vitro enzyme analysis suggested that TpdC was an amidase, which stereospecifically hydrolyses BDNA to OBNA that is further reduced by aminoalcohol dehydrogenase TpdE, using NADPH as an electron donor. Only a few short chain dehydrogenases/reductases, such as TpdE, have been described in *Rhodococcus* spp. before, thus the identification and isolation of TpdE should help to expand current knowledge about the specificity, selectivity and mechanisms of the reactions catalyzed by the enzymes of this type.

Altogether the identification of degradation genes and the isolation of intermediate metabolites were used to characterize the reactions of aerobic TTMP degradation and allowed to reconstruct enzymatic sequence of TTMP metabolism in *R. jostii* TMP1. This is the first validated catabolic pathway of bacterial biodegradation of alkylpyrazines.

It was demonstrated that this catabolic pathway is controlled by transcription regulator TpdR that specifically binds to *tpdABC* promoter and activates the expression of *tpd* genes in the presence of TTMP. Model system with EGFP reporter gene was used to define the minimal promoter sequence and transcription initiation site of *tpdABC*. Sequence analysis of the promoter revealed two 15 bp repeats and two inverted sequences (7 bp in length) that were required for promoter to function. The transcription activator TpdR and the promoter of *tpdABC* constitute the first described system that regulates the expression of the genes involved in alkylpyrazine biodegradation.

Due to the rising interest in biocatalysis as a new mild route for the synthesis of a variety of biologically and pharmacologically active compounds, it was investigated

whether TTMP-degrading TMP1 bacteria could be used for bioconversion of various alkylpyrazines and alkylpyridines. It was found, that TTMP-induced *R. jostii* TMP1 strain was able to use or modify both alkylpyrazines and alkylpyridines, demonstrating the first case of cross-inducibility of such type. In addition, resting cells of *R. jostii* TMP1 were shown to be applicable for the synthesis of hydroxypyridines under mild conditions. For the first time biocatalytical synthesis of 2,4,6-trimethylpyridin-3-ol, which exhibits antioxidative, geroprotective and anti-ischemic activity, was demonstrated.

Thesis statements:

1. The genetic locus of tetramethylpyrazine degradation was identified in *R. jostii* strain TMP1.
2. *tpdA*, *tpdB* and *tpdC* open reading frames are organized into *tpdABC* operon.
3. The expression of *tpdABC* operon is regulated by transcription activator TpdR.
4. Monooxygenase TpdA together with TpdB oxidizes tetramethylpyrazine to (*Z*)-*N*,*N*'-(but-2-ene-2,3-diyl)diacetamide.
5. Amidase TpdC hydrolyses (*Z*)-*N*,*N*'-(but-2-ene-2,3-diyl)diacetamide to *N*-(3-oxobutan-2-yl)acetamide.
6. NADPH-dependent aminoalcohol dehydrogenase TpdE reduces *N*-(3-oxobutan-2-yl)acetamide.
7. *R. jostii* strain TMP1 can be employed for the bioconversion of alkykpyrazines and alkylpyridines as well as the biosynthesis of hydroxypyridines.

Contents of the doctoral thesis

The dissertation is written in Lithuanian and contains the following parts: Introduction, Literature Review, Materials and Methods, Results and Discussion, Conclusions, List of References (160 positions), Tables (8), Figures (31) and Annex (1). Total 117 pages.

MATERIALS AND METHODS

Reagents

Reagents and kits for molecular biology were from Fermentas (Thermo Fisher Scientific, Lithuania). DNA and RNA isolation kits were from Zymo Research Corporation (USA). PCR primers were synthesized by Metabion (Germany). Bacterial media were from Oxoid (Thermo Fisher Scientific, UK). Substituted pyrazines and pyridines, thiostrepton, $H_2{}^{18}O$ and Nessler's reagent were from Sigma-Aldrich (Germany). [γ–^{32}P]ATP was from Amersham Biosciences (USA).

Bacterial strains and molecular biology techniques

The tetramethylpyrazine (TTMP) degrading bacterium *Rhodococcus jostii* TMP1 and 2,5-dimethylpyrazine-degrading *Arthrobacter nitroguajacolicus* 25DOT1 were previously isolated from soil samples (Kutanovas, 2008). *R. erythropolis* SQ1 (Quan and Dabbs, 1993) was chosen as the host strain for the expression of recombinant genes in bioconversion experiments. *E. coli* DH5α (F$^-$ Φ80*lacZΔM15* Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (r_K^-, m_K^+) *phoA supE44* λ$^-$*thi*-1 *gyrA96 relA1*) from Pharmacia (USA) was used for cloning experiments. TpdE protein was expressed in *E. coli* BL21(DE3) (F$^-$ *ompT hsdS*$_B$(r_B^-, m_B^-) *gal dcm* (DE3)) Avidis (France). Standard techniques were used for DNA manipulations (Sambrook and Russell, 2001).

Plasmids

pUC19 (Sambrook and Russell, 2001), pTZ57R/T from Fermentas (Thermo Fisher Scientific, Lithuania), pET-21b(+) from Novagen (Germany), pART2, pART3-*gfp*, pART2-*gfp* (Sandu et al., 2005), pTip-QC1, pTip-QC2 (Nakashima and Tamura, 2004) pUC19-H1, pART3-5'UTR-*gfp*, pART2-*tpdA*, pART2-*tpdB*, pART2-*tpdC*, pART2-*tpdAB*, pART2-*tpdBC*, pART2-*tpdABC*, pET-*tpdE*, pET-*tpdC*-His$_6$, pART2-*tpdC*-His$_8$, pTipQC-*tpdC*, pTipQC-His$_6$-*tpdC* were constructed during this work.

Bacterial growth medium and conditions

Rhodococcus spp. strains were grown at 30 °C with aeration, *E. coli* strains were grown at 37 °C with aeration. *R. jostii* TMP1 was cultivated either in Nutrient broth (NB, Oxoid) medium or in minimal medium (5 g/l NaCl, 1 g/l K_2HPO_4, 1 g/l $NH_4H_2PO_4$, 0.1 g/l $MgSO_4$, pH 7.2) supplemented with tetramethylpyrazine (0.05%) or pyridine

(0.05%). For cell suspension and bioconversion experiments *R. erythropolis* SQ1 was grown in 1 l flasks containing 250 ml of NB medium until the culture reached an OD_{600} of 1.6–2.0. Then cells were collected by centrifugation, washed twice and resuspended in the 10 mM potassium phosphate, pH 7.2, at four fold higher density. *E. coli* strains were cultivated in NB medium. *E. coli* strains transformed with recombinant plasmids were grown with either 50 μg/ml ampicillin or 40 μg/ml kanamycin, as required. *R. erythropolis* SQ1 transformed with recombinant plasmids was grown with 60 μg/ml kanamycin or 30 μg/ml chloramphenicol.

Construction of plasmids

Total DNA was isolated as described previously (Woo et al., 1992) from *R. jostii* TMP1 strain cultivated in NB medium. Genomic library was created by digesting DNA with HindIII and cloning the resulting fragments into the HindIII site of pUC19. To construct tetramethylpyrazine-inducible GFP-encoding plasmids as well as recombinant plasmids encoding different genes of tetramethylpyrazine degradation (*tpd*) locus, primers with required restriction sites were designed, fragments were amplified by PCR using total DNA as a matrix and cloned into a required plasmid. *R. erythropolis* SQ1 and *E. coli* strains were transformed with plasmid DNA by electroporation.

Analysis of protein expression profile induced by TTMP

R. jostii TMP1 was cultivated with TTMP or pyridine; cells were collected by centrifugation and suspended in 50 mM potassium phosphate, pH 7.2. Silica beads (0.1 mm diameter, 0.5 g/ml) were added and cells were disrupted with ultrasound processor VC750 (Sonics & Materials, Inc. USA) at 750 W for 10 min. Cell debris was removed by centrifugation at 16 000×*g* for 10 min. Remaining protein extracts were fractionated in 14% SDS-PAGE gels and visualized by Coomassie Blue protein staining.

MS/MS analysis

R. jostii TMP1 was cultivated in minimal medium supplemented with TTMP. Cells were collected and proteins were separated on SDS-PAGE gel. The band corresponding to the induced 40 kDa protein was excised and subjected to *de novo* sequencing based on MALDI TOF/TOF mass spectrometry and subsequent computational analysis at the Proteomics Centre of the Institute of Biochemistry, Vilnius University (Vilnius, Lithuania). The sample was purified as described before (Hellman et al., 1995). Tryptic-

digest from gel slice was analyzed by 4000 QTRAP (AB Sciex, Framingham, USA) mass spectrometer in linear ion trap mode using information dependent acquisition (IDA) and dynamic exclusion protocol. The acquisition method consisted of an IDA scan cycle including the enhanced mass scan (EMS) as the survey scan, enhanced resolution scan (ER) to confirm charge state and six dependent enhanced product ion (EPI) scans (MS/MS). With the threshold of the ion intensity at 100000 counts per second (cps), the IDA criteria were set to allow the most abundant ions in the EMS scan to trigger EPI scans. Survey MS scan was set to mass range from 400 m/z to 1400 m/z. Dynamic ion exclusion was set to exclude precursor ions after their two occurrences during 60 s interval. Peak lists were generated using Analyst software 1.4.2 (AB Sciex, Framingham, USA).

Illumina sequencing, contig assembly and inducible gene locus identification

R. jostii TMP1 DNA was sequenced using Illumina GA2 platform (Macrogen, Korea) and contigs were assembled using CLC-Genomics Workbench software (CLC bio, Denmark). To identify the TTMP-inducible gene, a search of the mass spectrometry-derived data against *R. jostii* TMP1 genome was performed.

Gene sequence analysis

The deduced amino acid sequences of the proteins encoded by the *tpd* locus were searched against the NCBI database using BLAST (Pearson and Lipman, 1988). Protein functions were assigned based on sequence similarity search against NCBI Conserved Domain Database (Marchler-Bauer et al., 2011). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura et al., 2011).

Qualitative and quantitative RT-PCR

R. jostii TMP1 was cultivated in minimal medium containing glucose (0.1%), TTMP (0.05%) or pyridine (0.05%) as the sole carbon source until culture reached the OD_{600} of 0.5. Total RNA was isolated using ZR Soil/Fecal RNA MicroPrep™ kit (Zymo Research Corporation, USA). Quantitative PCR amplification was performed using Rotor-Gene Q 5-plex HRM (Qiagen, Germany). qPCR was conducted in 15 µl of reaction mixture containing 1.5 µM Syto9, 7.5 µl of Verso 1-step QRT-PCR mix, 0.15 µl of Verso Enzyme, 0.75 µl of RT enhancer (all from Verso 1-step QRT-PCR Kit, Thermo Scientific, USA), 200 nM of each primer and 1 µl of RNA tested. The qPCR was

initiated with reverse transcription at 50 °C for 15 min, followed by initial denaturation at 95 °C for 15 min and either subsequent 45 cycles of 95 °C for 15 s, 58 °C for 1 min and 72 °C for 10 s (for tpdA, tpdB, tpdC, tpdD and tpdE) or subsequent 35 cycles of 95 °C for 20 s, 50 °C for 1 min and 72 °C for 1 min (for 16S rRNA). For qualitative evaluation, end-point PCR products were analysed using electrophoresis. For quantitave analysis, fluorescence data were recorded after the annealing step. All experiments were carried out in duplicate. To verify the absence of DNA in the RNA samples, the procedure without reverse transcriptase step was performed. The threshold cycle (C_T, threshold value of 0.05) values were obtained using Rotor-Gene Q Series Software 2.1.0 (Build 9). Relative target RNA analysis was performed using 2^-dC_T algorithm and 16S rRNA as a reference for normalization.

EGFP fluorescence measurement

R. jostii TMP1 strains was transformed with either pART3-*gfp* or pART3-5'UTR-*gfp* plasmid and cultivated in 25 ml of minimal medium supplemented with either 0.05% TTMP, 0.05% pyridine or 0.1% glucose for 48 hours until the OD_{600} reached 1.0 – 1.6. Cells were collected by centrifugation, washed three times and resuspended in 10 mM potassium phosphate, pH 7.2, to yield a final OD_{600} = 10. The suspension (200 µl/well) was transfered to black 96-well plate (Costar) and fluorescence was measured in FlexStationII^{384} fluorimeter (Molecular Devices, USA) at λ_{ex} = 485nm; λ_{em} = 510 nm. The measurements of three independent experiments were repeated in triplicates. Bacterial fluorescence was imaged under UV light using UVT-28 ME transillumitor (Herolab GmbH Laborgeraete, Germany) and Canon Eos 500D camera.

Cell suspension experiments

R. erythropolis SQ1 transformed with pART2 recombinant plasmids encoding different genes of *tpd* locus was cultivated as described above. Cells suspension was supplemented with either 0.1 mM tetramethypyrazine or 0.1 mM (*Z*)-*N*,*N*'-(but-2-ene-2,3-diyl)diacetamide and incubated at 30 °C for an hour. Bacteria were removed by centrifugation at 16 000×*g* for 1 min and UV absorption spectra of supernatants were recorded in PowerWave™ XS plate reader (BioTek Instruments, Inc. USA).

Bioconversion of TTMP

R. erythropolis SQ1 transformed with pART2-*tpdABC* or pART2-*tpdAB* recombinant plasmids was cultivated as described above. Bioconversion reactions were carried out in 250 ml volume at 30 °C with shaking at 180 rpm. Tetramethylpyrazine and glucose was added to the reaction mixture in portions of 20 mg and 125 mg, respectively, monitoring the progress of the conversion by UV absorption spectrum in 200–320 nm range. The reaction was performed for 6 days; total amount of tetramethylpyrazine added was 240 mg for pART2-*tpdABC* and 140 mg for pART2-*tpdAB*. Accumulation of bioconversion products was monitored by thin layer chromatography in chlorophorm:methanol 9:1 and using substrate compound as a reference.

Intermediate metabolite isolation

Bacteria were removed from the bioconversion reaction mixtures by centrifugation at 4000×g for 20 min, and supernatants were evaporated under reduced pressure. The product of TpdABC was extracted from concentrated aqueous solution with chloroform. The product of TpdAB was isolated by sequential dissolving in acetonitrile and chloroform. Isolated intermediate metabolites were used for structural analysis and for the whole cell and enzyme experiments.

Metabolite structural analysis

Product structures were determined using ^{1}H NMR, ^{13}C NMR and MS analyses. ^{1}H and ^{13}C NMR spectra were recorded on Varian Unity INOVA 300 spectrometer (300 and 75 MHz, respectively). TpdABC product was dissolved in DMSO-d_6, TpdAB product was dissolved in $CDCl_3$. Spectra were calibrated with respect to the solvent signal ($CDCl_3$: ^{1}H δ = 7.26; ^{13}C δ = 77.2; DMSO-d_6: ^{1}H δ = 2.50; ^{13}C δ = 39.5). HRMS were recorded on a Dual-ESI Q-TOF 6520 mass spectrometer (Agilent Technologies).

OBNA biosynthesis using $H_2{}^{18}O$

R. erythropolis SQ1 transformed with pART2-*tpdAB* recombinant plasmid was cultivated as described above. Cells were collected by centrifugation, washed three times in 10 mM potassium phosphate, pH 7.2, and resuspended in 10 mM potassium phosphate, pH 7.2, with 0.05% glucose in $H_2{}^{18}O$. Bioconversion reaction was carried out in 0.5 ml volume (in 15 ml tube) at 30 °C with shaking at 180 rpm. Reaction mixture was allowed to reach equilibrium for one hour and then tetramethylpyrazine was added

to final concentration of 0.5 mM. Bioconversion reaction was performed for 16 hours, following the conversion by UV absorption spectrum in 200–320 nm range. Upon exhaustion of the substrate (when absorption peak at 290 nm was lost), bacteria were removed by centrifugation, reaction mixture was lyophilized and reaction product was dissolved in acetonitrile. HPLC-MS analysis of reaction product was performed on LC2020 mass spectrophotometer (Shimadzu) after separation on the YMC-Pack Pro C18/S-0.3 μm (150×3 mm) column and water:acetonitrile gradient.

TpdC expression and purification

R. erythropolis SQ1 transformed with pTipQC-His_6-*tpdC* plasmid were incubated with chloramphenicol until the OD_{600} reached 0.4. The expression of recombinant protein was induced with thiostrepton (1 μg/ml) for 17 h. Cells were collected by centrifugation, washed with 25 mM potassium phosphate, pH 7.2, resuspended in 9 ml of 25 mM photassium phosphate with 100 mM NaCl and 5 mM imidazole, pH 7.2, and disrupted by sonication. Obtained cell-free extracts were loaded onto a HiTrap IMAC FF, 1 ml nickel column (GE Healthcare) and proteins were eluted with 25 mM potassium phosphate, containing 100 mM NaCl and 1 M imidazole, pH 7.2. The purity of TpdC was confirmed by electrophoresis in 14% SDS-PAGE.

TpdC activity measurements

Amidase activity of TpdC was determined at 20 °C, by measuring the decrease in A_{226} resulting from the hydrolysis of BDNA ($\varepsilon_{226} = 7100\ M^{-1}cm^{-1}$). Reaction mixture consisted of 50 mM potassium phosphate, pH 7.5, with 0.1 mM BDNA and TpdC. Optimal pH for TpdC activity was determined in the range of pH 6.2–9.0, using potassium phosphate and Tris-HCl buffered solutions.

Ammonia detection with Nessler's reagent

Ammonia levels were measured in the reaction mixture consisting of 700 μl 10 mM potassium phosphate, pH 7.5, 100 μl Nessler's reagent (0,09 mol/l potassium tetraiodomercurate (II) and 2,5 mol/l potassium hydroxide solution) and 200 μl of sample. The absorbtion of the reaction product was measured at 490 nm. NH_4^+ concentration was determined from the standard curve that was obtained using 0.1–1.0 mM NH_4Cl solutions.

TpdE expression and purification

tpdE gene was fused with 3'-polyhistidine sequence of pET21b(+) expression vector. *E. coli* BL21(DE3) bacteria were transformed with resulting pET21-*tpdE* plasmid and cultured aerobically at 30 °C in 1 l conical flasks with 200 ml of BHI medium (Oxoid) supplemented with 50 mg/ml ampicillin. When the cultures reached OD_{600} = 1.2, 0.5 mM IPTG was added to induce *tpdE* gene expression and the cultures were incubated for 4 h. Cells were collected by centrifugation, washed with 50 mM potassium phosphate, pH 7.2, resuspended in 8 ml of the same solution and disrupted with sonicator VC750 (Sonics & Materials, Inc. USA) at 750 W for 5 min. Cell debris was removed by centrifugation at 16 000×*g* for 10 min. Obtained cell-free extracts were loaded onto a HiTrap IMAC FF, 5 ml nickel column (GE Healthcare) and proteins were eluted with 50 mM potassium phosphate with 1 M imidazole, pH 7.2. The purity of TpdE was confirmed by electrophoresis in 14% SDS-PAGE.

TpdE activity measurements

NADPH-dependent ketoreductase activity of TpdE was determined in a Helios gamma UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) by measuring the decrease in A_{340} resulting from the oxidation of NADPH (ε_{340} = 6220 $M^{-1}cm^{-1}$) after the addition of the substrate OBNA. TpdE activity was assayed in buffer containing 0.2 mM NADPH and 5 μM OBNA. One unit of activity was defined as the amount of the enzyme that catalyzed the oxidation of 1 μmol of NADPH per minute. The optimal pH for TpdE activity was determined to be in the range of pH 6.0 to 8.5, using potassium phosphate and Tris-HCl buffers. The apparent TpdE Km values for NADPH and OBNA were determined by varying concentrations of these substrates from 50 to 500 μM and from 0.1 to 10 μM, respectively. Data from at least three independent experiments were combined. For data fitting, GraFit (Erithacus Software Ltd.) software was used.

Bioconversion of 2,4,6-trimethylpyridine

R. jostii TMP1 was grown for 2 days in 1 l flasks containing 200 ml of medium as described above. Then cells were collected by centrifugation, washed twice and resuspended in 10 mM potassium phosphate, pH 7.2, at four-fold higher density. Bioconversion reactions were carried out in 200 ml volume at 30 °C with shaking at 180 rpm. 2,4,6-trimethylpyridine and glucose were added to the reaction mixture in

portions of 20 mg and 125 mg, respectively, following the conversion by the changes in UV absorption spectrum in 200–320 nm range. The reaction was performed for 96 hours. Accumulation of bioconversion product was monitored by thin layer chromatography in chlorophorm:methanol 9:1 and using substrate compound as a reference. To isolate bioconversion product bacteria were removed from the reaction mixtures by centrifugation and supernatant was evaporated under reduced pressure. The product was isolated by sequentially dissolving it in acetonitrile and chloroform. Total yield of 70% was achieved. Product structure was determined using ^{1}H NMR, ^{13}C NMR and MS analyses. ^{1}H and ^{13}C NMR spectra were recorded on Varian Unity INOVA 300 spectrometer (300 and 75 MHz, respectively). Spectra were calibrated with respect to the solvent signal ($CDCl_3$: ^{1}H $\delta = 7.26$; ^{13}C $\delta = 77.2$). MS was recorded on LC2020 mass spectrometer (Shimadzu) after the separation on the YMC-Pack Pro C18/S-0.3 μm column (150×3 mm) using a water:acetonitrile gradient.

RESULTS AND DISCUSSION

Identification of *tpd* gene locus in *R. jostii* TMP1

R. jostii TMP1 bacteria were previously isolated from soil as strain capable to use tetramethylpyrazine as a sole source of carbon and energy (Kutanovas, 2008). To elucidate the metabolic pathway of tetramethylpyrazine (TTMP) tetramethylpyrazine degradation (*tpd*) gene locus was identified and intermediate metabolites of TTMP were determined.

To detect TTMP catabolism-related enzymes we investigated whether TMP1 cultivation with TTMP causes the up-regulation of proteins that may be involved in the TTMP metabolic pathway. In the SDS-PAGE gels of cell-free extracts several TTMP-inducible protein bands were observed, including a dominant 40 kDa protein (Fig. 1A). This inducible protein expression was specific to TTMP adaptation rather that shared with other *N*-heterocyclic compounds, as TMP1 cultivation with pyridine induced different protein expression profile and did not cause up-regulation of the 40 kDa protein (Fig. 1B). This suggested that the 40 kDa protein plays an important and specific role in TTMP degradation. Therefore this protein was chosen for further analysis in order to elucidate TTMP metabolic pathway.

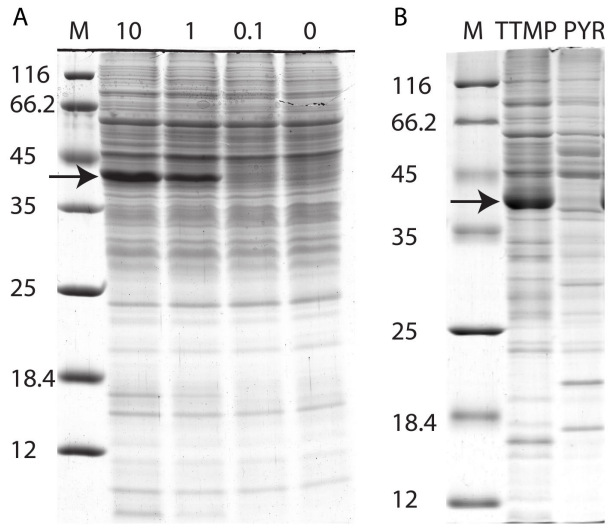

Fig. 1. Tetramethylpyrazine-inducible protein. The cultivation with tetramethylpyrazine induced the expression of 40 kDa protein (arrows) in *R. jostii* TMP1. *A*, Cultures of TMP1 were cultivated for 18 hours in NB medium with different concentrations of tetramethylpyrazine (0.1–10 mM). *B*, TMP1 was cultivated for 48 hours in minimal medium with 0.05% tetramethylpyrazine (TTMP) or 0.05% pyridine (PYR) as a single source of carbon. SDS-PAGE gels were stained with Coomassie Blue.

To determine the identity of the TTMP-inducible protein, the 40 kDa band was excised from SDS-PAGE gel and analyzed by MS/MS sequencing. The sequences of possible candidate peptides were aligned with the genomic Illumina database, resulting in the identification of the contig with the locus of 40 kDa protein-encoding gene. To verify the sequence of this tetramethylpyrazine degradation (*tpd*) locus, HindIII genomic library was created and the clone with sequence of interest was identified by the PCR screening with the primers specific to 40 kDa protein-encoding gene. The sequencing of this clone provided the sequence of 7 kb fragment of *tpd* locus. The remaining part of *tpd* locus was verified by sequencing the overlapping fragments obtained by PCR using total DNA as a matrix and primers designed according to the contig sequence. In total 13 kb locus containing the genes of TTMP degradation was sequenced. Sequence analysis of *tpd* locus revealed eight open reading frames (ORFs) that were denominated *tpdA*, *tpdB*, *tpdC*, *tpdR*, *tpdD*, *tpdE, orf1* and *orf2* (Fig. 2). The 40 kDa inducible protein is encoded by the *tpdA* gene. Three of the ORFs are organized into the predicted operon *tpdABC* (Fig. 2).

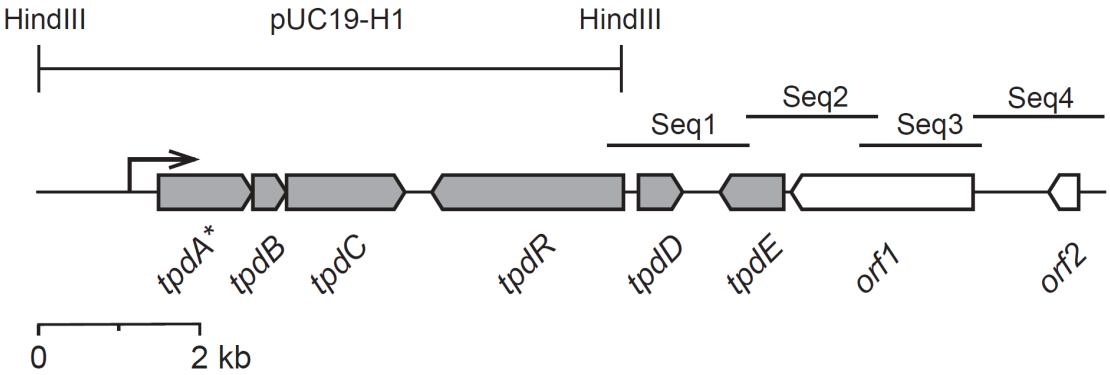

Fig. 2. Gene locus of *R. jostii* TMP1 involved in the metabolism of tetramethylpyrazine. The markings on the top denote separate fragments that were sequenced to verify *tpd* locus: the clone from HindIII genomic library (pUC19-H1) and four fragments obtained by PCR (Seq1–Seq4). Grey arrows represent genes that are known to be involved in TTMP degradation, white arrows indicate other ORFs in the locus. **tpdA* gene encodes 40 kDa protein inducible by tetramethylpyrazine.

Gene sequence analysis of *tpd* gene locus

Sequence analysis revealed that TpdA shows high similarity to luciferase-like monooxygenases and contains conserved domains characteristic to the cl07892 superfamily of flavin-utilizing monoxygenases and has some common domains with coenzyme F420-dependent flavin oxidoreductases (Table 1). Similarly like TpdA

requires TpdB to oxidize TTMP, bacterial luciferases act as a complex of α and β subunits. However, these two subunits are homologous to each other (Ellis, 2010), while TpdB protein is considerably smaller than TpdA and does not share the homology with luciferase-like monooxygenases. The deduced product of *tpdB* belongs to the nuclear transport factor 2 (NTF2-like) superfamily, which contains proteins that diverge greatly in their function despite many common structural details, including ketosteroid isomerases and the beta-subunit of the ring hydroxylating dioxygenses (Table 1).

Flavin-utilising monooxygenases usually act in two-component enzyme systems, composed of a larger component (an oxidase), which uses the reduced flavin nucleotides to hydroxylate substrates, and a smaller component (flavin reductase), which uses NAD(P)H to reduce FAD or FMN (Ellis, 2010). The flavin reductase of this type is encoded by *tpdD* gene (Table 1).

TpdC belongs to the superfamily of amidases (Table 1) and shares very high homology with omega-octalam hydrolase, whose function has been confirmed at the protein level (Fukuta et al., 2010).

Table 1. Functional annotations of deduced Tpd proteins. The conserved domains of putative *tpd* locus proteins were analyzed against the Conserved Domain Database via NCBI website.

Protein	Size, aa/kDa	Putative function	Superfamily designation		
			Superfamily (Access. No)	E-Value	Conserved domain
TpdA	387/42.9	Flavin-utilizing monoxygenase	Flavin-utilizing monooxygenases (cl07892)	5.3e-16	Non-described
TpdB	136/15.2	Unknown	NTF2 like (cl09109)	7.4e-09	Non-described
TpdC	484/51.4	Amidase	Amidase (cl11426)	2.3e-63	Non-described
TpdR	787/86.2	LuxR family transcriptional regulator	LuxR_C-like (cl17315)	1.4e-14	Sequence-specific DNA binding domains
			AAA* (cl17189)	1.2e-4	Nucleotide phosphate-binding motif
TpdD	179/19.3	Flavin reductase	Flavin reductases (cl00801)	5.7e-26	FMN-binding domain of NAD(P)H-flavin oxidoreductases
TpdE	260/26.9	Short-chain dehydrogenase/ reductase	NADB_Rossmann (cl09931)	2.4e-73	Rossmann-fold NAD(P)H/NAD(P)(+) binding domain
Orf1	747/81.2	Large subunit of *N,N*-dimethyl-formamidase	-		
Orf2	123/13.6	Peptidase	GAT_1 (cl00020)	8.9e-16	Non-described

*AAA, ATPases associated with a wide variety of cellular activities

TpdE shares the strong similarity with classical short-chain dehydrogenases/reductases (SDRs) that have a Rossmann-fold NAD(P)H/NAD(P)(+) binding (NADB) domain (Table 1). This domain is found in numerous dehydrogenases and other redox enzymes, which can use a wide variety of substrates, including alcohols, glucose and steroids (Oppermann et al., 2003).

orf1 and *orf2* sequences that were found upstream from *tpdE* also may contribute to TTMP degradation. *orf1* encodes the large subunit of *N,N*-dimethylformamidase, while *orf2*-encoded protein is homologous to peptidases (Table 1).

tpdR encodes a putative regulator of *tpd* locus. Deduced protein of *tpdR* contains domains specific to the luxR family transcriptional regulators.

Regulation of *tpd* gene expression

To confirm that *tpdA*, *tpdB* and *tpdC* genes were co-transcribed, RT-PCR analysis was performed using primers designed to amplify the intergenic regions. End-point PCR revealed that gene pairs *tpdA*-*tpdB* and *tpdB*-*tpdC* were transcribed as a contiguous transcript, confirming *tpdABC* operon (Fig. 3A). The transcription of *tpdABC* was specifically induced by TTMP but not by glucose or pyridine (Fig. 3A).

To verify that the expression of *tpdABC* operon was regulated by TTMP, the fragment of the up-stream region of *tpdA* gene was amplified by PCR and fused to a green fluorescent protein (EGFP) gene from pART3-*gfp* vector. TMP1 strain, transformed with pART3-5'UTR-*gfp* plasmid, expressed EGFP when cultivated on TTMP as determined by the developed bacterial fluorescence (Fig. 3B). The level of EGFP fluorescence increased 10-fold in TTMP-induced bacteria carrying pART3-5'UTR-*gfp* compared to that in glucose-induced bacteria. In contrast, no EGFP fluorescence above the background level was detected upon the induction with pyridine. This confirmed that the upstream region of *tpdA* contained a promoter, which was specifically activated upon the exposure to TTMP.

To investigate whether the expression of *tpdD* and *tpdE* genes were also dependent on TTMP, real-time RT-PCR analysis was performed. The results revealed

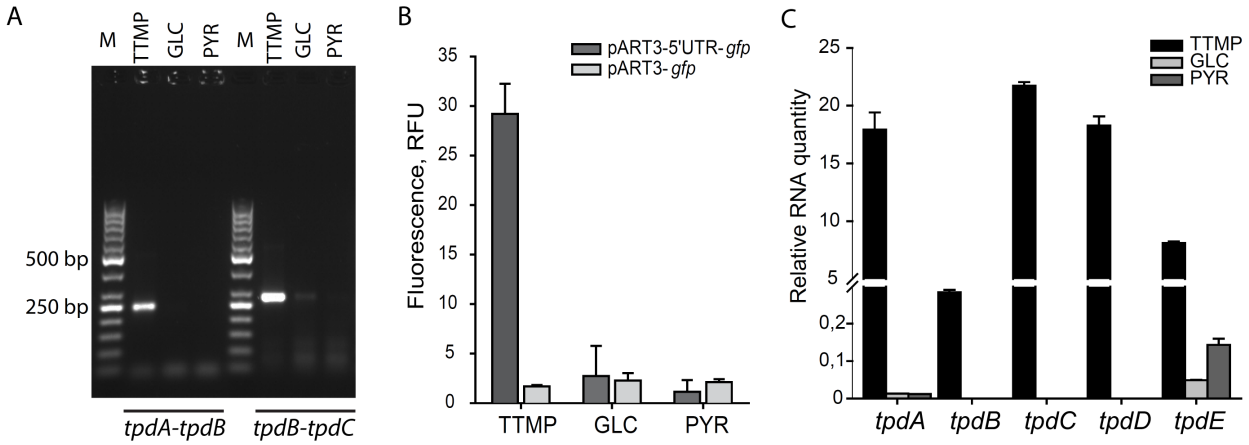

Fig. 3. Induction of *tpd* locus expression by tetramethylpyrazine in *R. jostii* TMP1. TMP1 strain was cultivated in liquid minimal medium supplemented either with 0.05% tetramethylpyrazine (*TTMP*), 0.1% glucose (*GLC*) or 0.05% pyridine (*PYR*) as a single source of carbon. *A*, RT-PCR analysis using primers designed to amplify the intergenic regions between *tpdA-tpdB* and *tpdB-tpdC*. *B*, TMP1 was transformed with pART3-*gfp* plasmid containing *tpdA* upstream region inserted as a promoter (pART-5'UTR-*gfp*). Bacterial EGFP fluorescence was measured in a plate reader (λ_{ex} = 485nm; λ_{em} = 510 nm); the data are presented as averages of three independent experiments ± standard deviation. *C*, quantitative RT-PCR analysis of the transcription of *tpdA-E* genes. The data are presented as a relative RNA amount calculated from the threshold cycles using the threshold cycle of 16S RNA as a reference; averages of duplicate runs are presented ± standard deviation.

that the expression of each of the *tpdA-E* genes was specifically induced when *R. jostii* TMP1 was cultivated in the presence of TTMP (Fig. 3C), suggesting that proteins TpdD and TpdE participate in TTMP catabolism.

Metabolic pathway of TTMP degradation in *R. jostii* TMP1

To investigate the metabolic reactions involved in TTMP degradation by the proteins encoded in *tpd* locus, pART2 expression vectors with different genes of *tpdABC* operon and their combinations were constructed. The involvement of separate genes in the metabolism of TTMP was analyzed in *Rhodoccocus erythropolis* SQ1, since it does not metabolize TTMP itself. The transformation of SQ1 strain with pART2 vector led to an effective protein expression as confirmed by EGFP fluorescence developed in the cells transformed with pART2-*gfp*. TTMP metabolism was evaluated in resting cell suspension observing the changes of absorbance in UV spectrum. While transformation with pART2 vector itself did not cause TTMP conversion, TTMP was metabolized by SQ1 transformed with *tpdABC* and *tpdAB* constructs (Fig. 4). Meanwhile, *tpdA*, *tpdB*

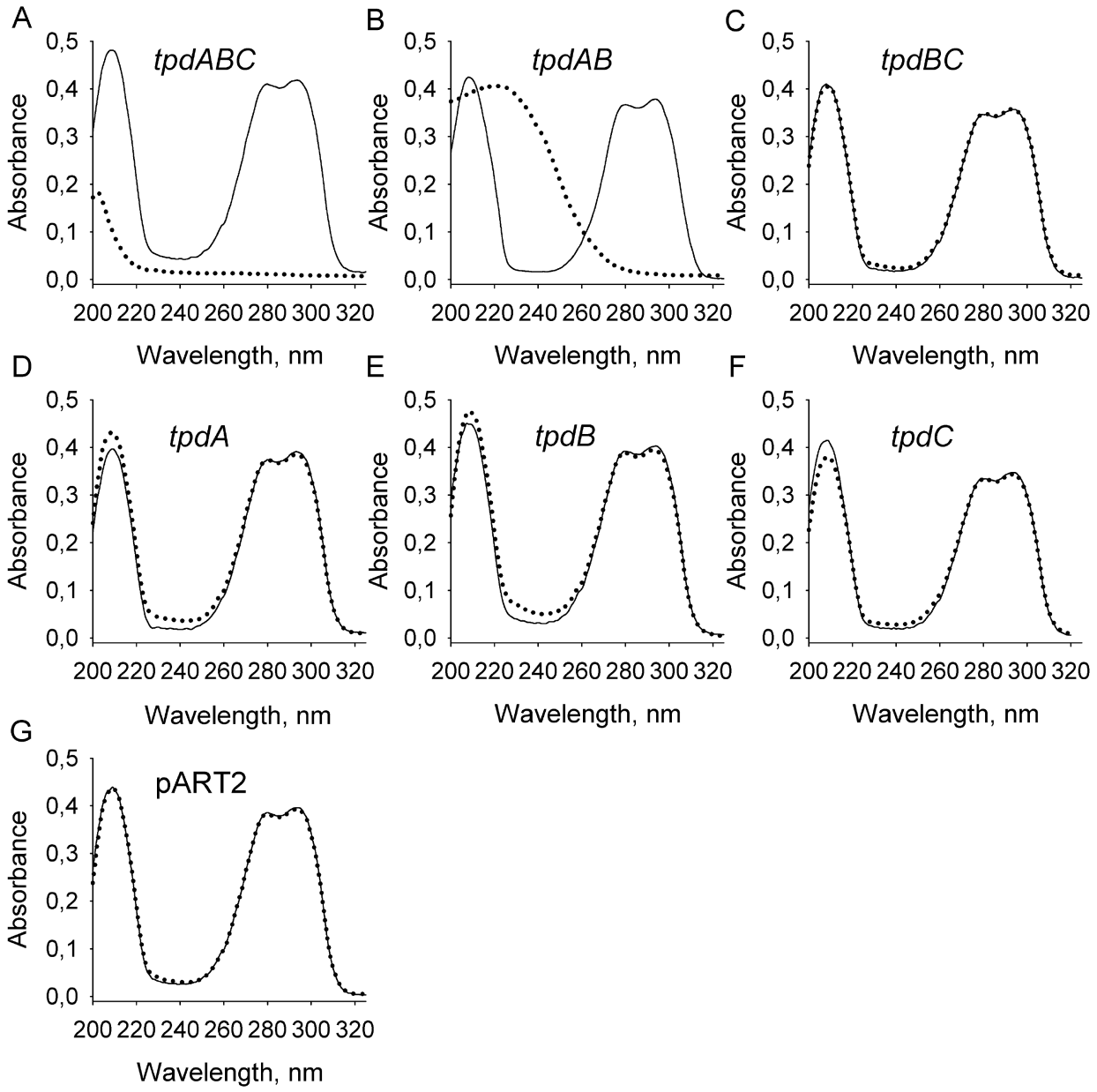

Fig. 4. Tetramethylpirazine metabolism in *R. erythropolis* SQ1 transformed with pART2 plasmid containing different combinations of genes from *tpd* locus. Cultures of *R. erythropolis* SQ1 were incubated in potassium phosphate with 0.1 mM tetramethylpyrazine for 17 hours and UV absorption spectra were recorded. Solid line represents the initial spectrum of tetramethylpyrazine; dotted line indicates the final spectrum of the bioconversion products.

and *tpdC* separately were not sufficient for TTMP conversion (Fig. 4). Furthermore, TTMP degradation by TpdABC gave rise to different metabolites than TpdAB as evaluated by their UV spectra (Fig. 4) and thin layer chromatography.

TpdABC- and TpdAB-generated metabolites were extracted from the bioconversion media and their structure was determined by ^{1}H NMR, ^{13}C NMR and MS analyses. The ^{1}H NMR spectrum of TpdABC product showed five peaks in ^{1}H NMR spectrum: δ 6.37 (br s, 1H, N*H*), 4.58 (p, *J* = 7.1 Hz, 1H, C*H*), 2.21 (s, 3H, COCH_3),

1.99 (s, 3H, NHCOCH_3), 1.36 (d, J = 7.1 Hz, 3H, CH_3); and six peaks in ^{13}C NMR spectrum: δ 207.1, 169.7, 54.8, 26.8, 23.4, 17.8, identifying TpdABC product as *N*-(3-oxobutan-2-yl)acetamide (OBNA). These NMR spectra were in full agreement with previously published results (Peach et al., 2006). Meanwhile, the product of TpdAB showed three peaks with δ 8.67 (s, 1H, N*H*), 1.88 (s, 3H, CH_3), 1.82 (s, 3H, CH_3), while ^{13}C NMR spectrum contained four peaks with δ 167.7, 121.2, 23.2, 16.4. High resolution MS analysis confirmed the molecular formula of the compound as $C_8H_{15}N_2O_2$ (calculated M+H$^+$ mass was 171.11335, found mass was 171.1134). These spectra revealed that TpdAB metabolized TTMP to (*Z*)-*N*,*N*'-(but-2-ene-2,3-diyl)diacetamide (BDNA).

Next, intermediate metabolite BDNA was used as a substrate for SQ1 transformed with separate genes of *tpdABC* operon and their combinations. TpdABC, TpdBC and TpdC were able to metabolize BDNA, indicating that TpdC alone was sufficient to

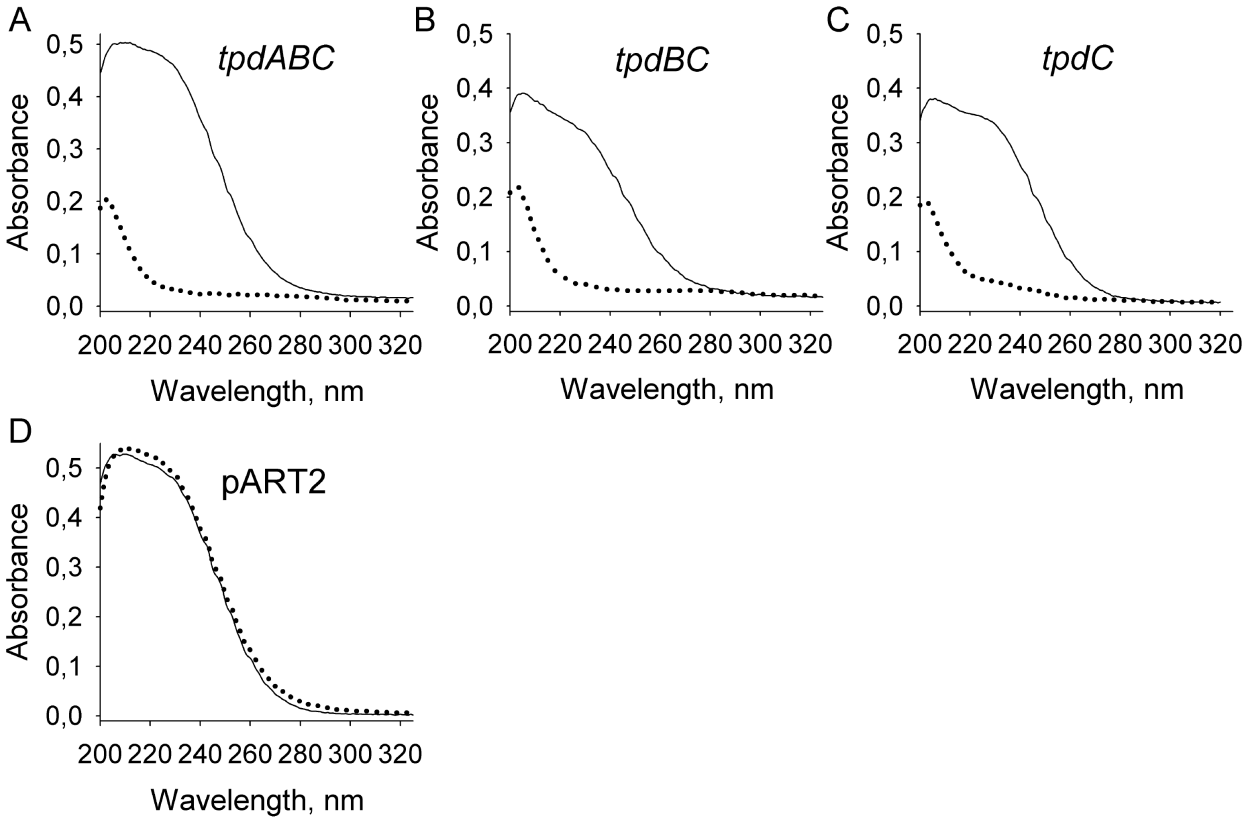

Fig. 5. (*Z*)-*N*,*N*′-(but-2-ene-2,3-diyl)diacetamide metabolism in *R. erythropolis* SQ1 transformed with pART2 plasmid containing different combinations of genes from *tpd* locus. Cultures of *R. erythropolis* SQ1 were incubated in potassium phosphate with 0.1 mM (*Z*)-*N*,*N*′-(but-2-ene-2,3-diyl)diacetamide for one hour and UV absorption spectra were recorded. Solid line represents the initial spectrum of (*Z*)-*N*,*N*′-(but-2-ene-2,3-diyl)diacetamide; dotted line indicates the final spectrum of the bioconversion products.

Table 2. Products of resting-cell reactions of *R. erythropolis* SQ1 transformed with the genes of *tpd* locus. Cultures of transformed bacteria were incubated with 0.1 mM tetramethylpyrazine (TTMP) or 0.1 mM (*Z*)-*N*,*N*'-(but-2-ene-2,3-diyl)diacetamide (BDNA). Substrate degradation was observed by UV absorption spectra. Reaction products BDNA and OBNA were determined either by H^1-NMR, C^{13}-NMR and MS analyses (TTMP conversion) or by UV spectra comparison and thin layer chromatography (BDNA conversion).

Plasmid	Expressed gene(s)	Substrate	
		TTMP	BNDA
pART2	-	*nd*	*nd*
pART2-*tpdA*	*tpdA*	*nd*	*na*
pART2-*tpdB*	*tpdB*	*nd*	*na*
pART2-*tpdC*	*tpdC*	*nd*	OBNA
pART2-*tpdAB*	*tpdA, tpdB*	BNDA	*na*
pART2-*tpdBC*	*tpdB, tpdC*	*nd*	OBNA
pART2-*tpdABC*	*tpdA, tpdB, tpdC*	OBNA	OBNA

nd, bioconversion not detected;
na, bioconversion not analysed

catalyze BDNA conversion (Fig. 5). The results of bioconversion experiments (summarized in Table 2) demonstrated that TTMP metabolism by the enzymes encoded in *tpdABC* locus is a multi-stage process, involving TTMP oxidation by TpdAB to BDNA and then BDNA hydrolysis by TpdC to OBNA.

Even though the structure of the product of TTMP oxidation by TpdAB resembles that of dioxygenase reactions (Fetzner, 2012), similar products may also arise from aromatic ring cleavage catalyzed by flavoprotein monooxygenases, such as 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase or 5-pyridoxic acid oxygenase (Chaiyen, 2010). These monooxygenases open aromatic ring of the substrate and incorporate only one oxygen atom from O_2, whereas another one comes from a H_2O molecule. To determine the exact mechanism of the ring oxidation catalyzed by TTMP oxygenase TpdAB ^{18}O-tracer experiments were performed. TTMP bioconversion to BDNA was carried out in $H_2{}^{18}O$ and product was analyzed by MS. The molecular mass of ^{18}O-labelled BDNA was 172 (compared to $M_r = 170$ obtained in H_2O), indicating that only one ^{18}O atom was incorporated during TTMP oxidation by TpdAB. This suggests that the second oxygen atom arose from molecular oxygen and reveals TpdAB as TTMP monooxygenase.

Flavin-utilizing monooxygenases of TpdA type act as a part of a two-component enzyme system that includes flavin reductase. The flavin reductase in *tpd* locus is encoded by *tpdD* (Table 1). Therefore, the oxidation of TTMP should require both TpdA

and TpdD acting together. However, in *R. erythropolis* SQ1 *tpdD* was not necessary to metabolize TTMP (Fig. 4). This is unsurprising, as *Rhodococcus* strains carry many flavin reductases (e.g. in genomes published by McLeod et al., (2006); Sekine et al., (2006)) that may have substituted *tpdD*.

Product of TTMP oxidation is further hydrolyzed by TpdC and gives rise to optically active product that exhibits specific rotation of polarized light a_D^{20} = +102° (1.16 g in 100 ml $CHCl_3$), indicating that reaction occurs stereospecifically. This suggests that TpdC acts as a BDNA hydrolase. *In vitro* experiments using purified TpdC and Nessler's reagent revealed that ammonia is released during BDNA hydrolysis, indicating that TpdC is BDNA amidase. ^{1}H NMR spectra (in $CDCl_3$, data not shown) revealed that BDNA may exist in different tautomeric forms. Since, BDNA hydrolysis produces optically active OBNA rather than a racemate, suggesting that the imine form of BDNA (Fig. 6) rather than amine is a true substrate for TpdC.

To demonstrate that *R. jostii* TMP1 *tpdE* gene product was an SDR, as predicted by the sequence analysis, His_6-tagged TpdE protein was overproduced in *E. coli* and purified by affinity chromatography. A protein with an apparent molecular mass of ~30 kDa was overproduced, which agrees with the expected size of the recombinant His_6-TpdE protein (27.9 kDa). The TpdE demonstrated specific ketoreductase activity using OBNA as a substrate and NADPH as an electron donor. The optimum pH of 7.2 for activity was determined. The apparent K_m for NADPH calculated from steady-state analysis was 379±73 μM in the presence of 6 mM OBNA, and the apparent K_m for OBNA was 5.2±1.5 μM in the presence of 500 μM NADPH (at pH 7.2 and 30 °C), with k_{cat} of 2.5±0.1 s^{-1}. The enzyme has an absolute requirement for NADPH, yielding no detectable OBNA reduction in the presence of NADH. These experiments confirmed that TpdE acts as an aminoalcohol dehydrogenase.

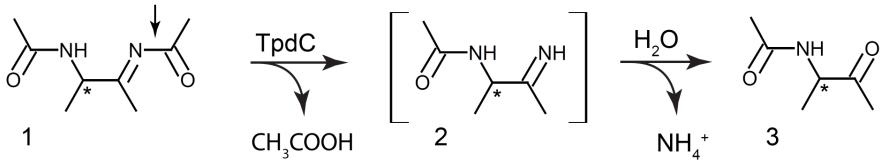

Fig. 6. (*Z*)-*N*,*N*'-(but-2-ene-2,3-diyl)diacetamide (BDNA) amidase TpdC hydrolyses BDNA (*1*) to *N*-(3-oxobutan-2-yl)acetamide (OBNA) (*3*). TpdC cleaves amidic bond (an arrow) in tautomeric BDNA (*1*) imine. Spontaneous hydrolysis of intermediate imine (*2*) to OBNA (*3*) is accompanied by the release of ammonia. * – chiral centre.

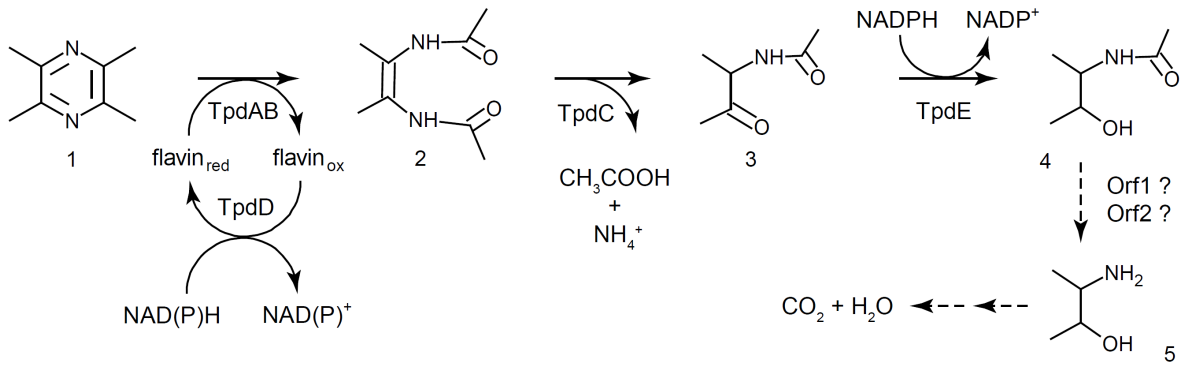

Fig. 7. Proposed tetramethylpyrazine catabolic pathway in *R. jostii* TMP1. 1 – tetramethylpyrazine, 2 – (*Z*)-*N*,*N*′-(but-2-ene-2,3-diyl)diacetamide (BDNA), 3 – *N*-(3-oxobutan-2-yl)acetamide (OBNA), 4 – *N*-(3-hydroxybutan-2-yl)acetamide 5 – 3-amino-2-butanol. Dashed arrows indicate hypothetical reactions. TTMP oxygenase – TpdAB, flavin reductase – TpdD, BDNA hydrolase – TpdC, aminoalcohol dehydrogenase – TpdE.

Both Orf1 and Orf2 may recognise the amidic bond in the TpdE product, as predicted by sequence analysis, and may hydrolyse it to 3-amino-2-butanol, thus contributing to subsequent steps of TTMP metabolism (Fig. 7).

Thus TTMP degradation was demonstrated to be a multi-stage process, involving oxidative aromatic ring-cleavage by TpdA and TpdB complex, subsequent hydrolysis step catalyzed by TpdC and keto group reduction by TpdE (Fig. 7). In summary, we identified here for the first time the genetic locus responsible for bacterial degradation of pyrazines, namely tetramethylpyrazine. Our data revealed that the initial step of TTMP degradation is the ring oxidation and cleavage, which is in accordance with previous reports on TTMP metabolism by *Rhodococcus* sp. strains (Müller and Rappert, 2010). However, in contrast with Muller and Rappert (2010) data, suggesting that TTMP is oxidized by the enzyme of cytochrome P450 type, we determined that TTMP oxidase is a putative flavo-enzyme.

Biosynthetic application of *R. jostii* TMP1

The ability of *R. jostii* TMP1 to grow on *N*-heterocyclic compounds as a sole source of carbon was tested using the following compounds: pyrazine, 2,5-dimethylpyrazine (2,5-DMP), 2,3-dimethylpyrazine (2,3-DMP), 2,6-dimethylpyrazine (2,6-DMP), 2,3,5-trimethylpyrazine (TMP), TTMP, 2,3-diethyl-5-methylpyrazine, 2-, 3-, and 4-methylpyridine, 2,3-, 2,4- 2,5-, 2,6-, 3,4- and 3,5-dimethylpyridine and 2,3,5-trimethylpyridine. It appears that only TTMP and pyridine, supported the growth of

TMP1 strain. The strain also utilized succinate, therefore it was further used as a control substrate in resting cells reactions. Eventhough these coumpounds were not used as sole source of carbon it was tested if TTMP-induced TMP1 strain could modify them.

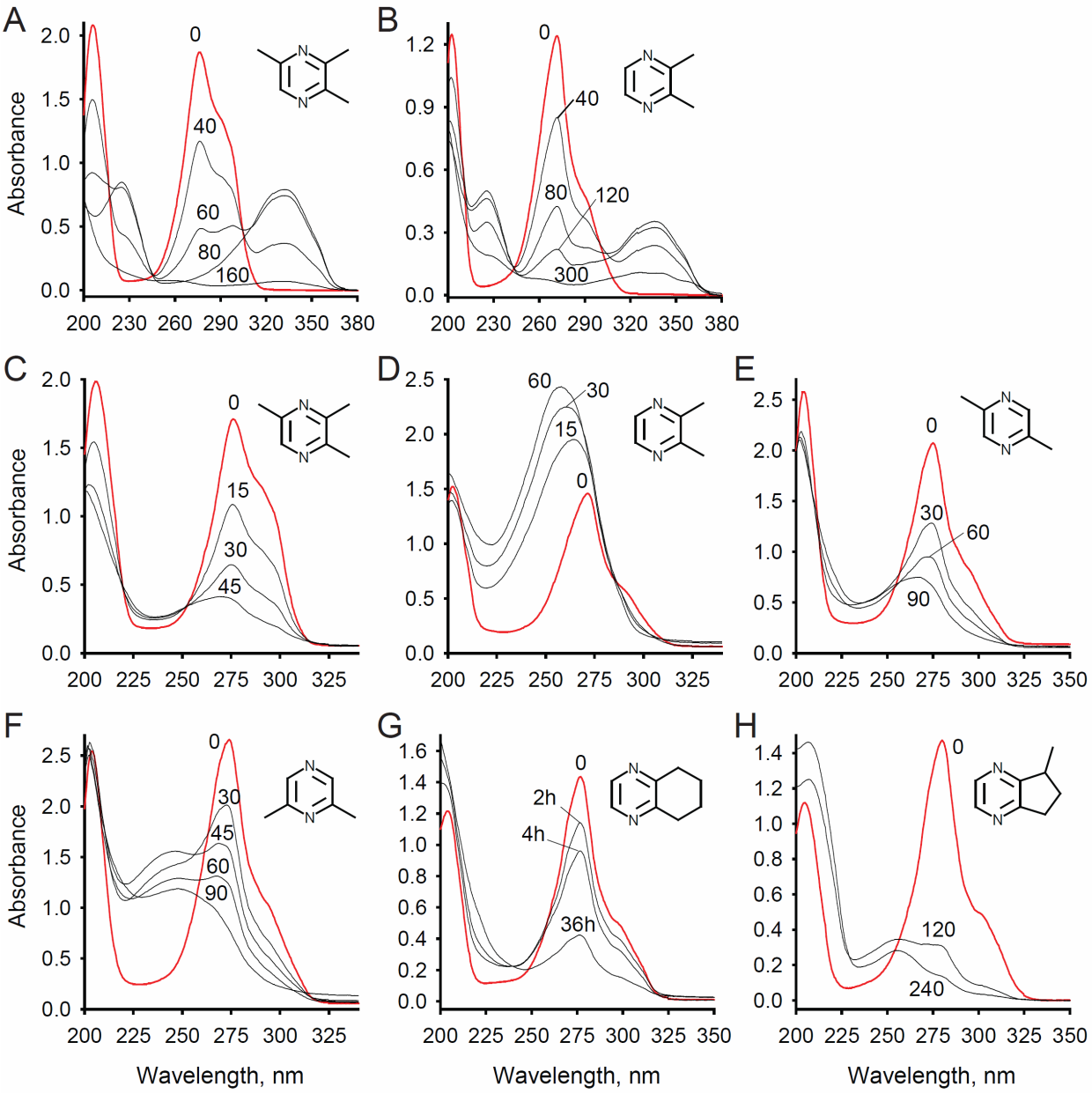

Fig. 8. Spectral changes during aerobic conversion of pyrazine compounds by resting cells. *Arthrobacter nitroguajacolicus* 25DOT1 (A, B) and *R. jostii* TMP1 (C–H) in 50 mM potassium phosphate, pH 7.2, at 20 °C. Cells of *A. nitroguajacolicus* 25DOT1 were induced with 2,5-DMP and cells of *R. jostii* TMP1 were cultivated in the presence of TTMP. The reaction mixture was scanned after centrifugation (1 min, 16,000×*g*). Incubation time is shown in min. or hours (h). *A*,*C* – 2,3,5-trimemethylpyrazine; *B*,*D* – 2,3-dimethylpyrazine; *E* – 2,5-dimethylpyrazine; *F* – 2,6-dimethylpyrazine; *G* – 5,6,7,8-tetrahydroquinoxaline (2,4-ciklohexapyrazine); *H* – 5-methyl-6,7-dihydro-5H-ciklopenta (b)pyrazine.

The changes in the UV-VIS spectrum during the typical conversion of methylpyrazines by resting cells are illustrated in Fig. 8. *R. jostii* TMP1 consumed TTMP only when it was previously induced by this substrate. Uninduced cells grown in the presence of succinic acid did not catalyze any conversion. Moreover, the cells pre-cultivated in the presence of pyridine did not consume TTMP and *vice versa*.

Generally, in biotransformations of alkylpyrazine compounds by pyrazines-degrading bacteria UV absorption maxima shifts to the longer wavelength area, indicating the introduction of a hydroxyl group (Taylor and King, 1987). However, the formation of hydroxylated compounds was not observed during the conversion of alkylpyrazines by TMP1 (Fig. 8, C–H). In comparison, 2,5-dimethylpyrazine degrading strain *Arthrobacter nitroguajacolicus* 25DOT1 induced in the presence of 2,5-DMP converted both 2,3-DMP and TMP (Fig. 8A, B). New maxima were observed at 225 nm and 340 nm. The latter indicated the formation of hydroxylated intermediates.

R. jostii TMP1 cells induced in the presence of TTMP also consumed or converted 2,3-DMP, 2,5-DMP, 2,6-DMP and TMP (Fig. 8, C–F). Compared with *A. nitroguajacolicus* 25DOT1, the formation of hydroxylated intermediates was not observed, but the new maximum in the shorter wavelength area occurred in the case of 2,3-DMP and 2,6-DMP (Fig. 8D, F). That may indicate the aromatic ring opening without hydroxylation step, similarly to TTMP.

Moreover, for the first time the similar conversion of various methyl- and ethylpyridines by the TMP1 cells induced in the presence of TTMP was observed (Fig. 9). Although majority of these compounds were modified similarly to pyrazines, the formation of hydroxylated intermediates might be expected in the bioconversion of 2,4-dimethylpyridine, 2,6-dimethylpyridine and 2,4,6-trimethylpyridine (Fig. 9E, G, K).

The capability of *R. jostii* TMP1 to hydroxylate methylpyridines was further analysed using 2,4,6-trimethylpyridine as a substrate. The time-course analysis of bioconversion of this compound showed UV absorption maxima shift to the longer wavelength area. A new maximum at 317 nm was observed indicating the formation of a hydroxylated intermediate (Fig. 9K). To elucidate the structure of the formed compound, the product of 2,4,6-trimethylpyridine bioconversion was purified. In total, 200 mg of 2,4,6-trimethylpyridine was converted using the resting cells of *R. jostii*

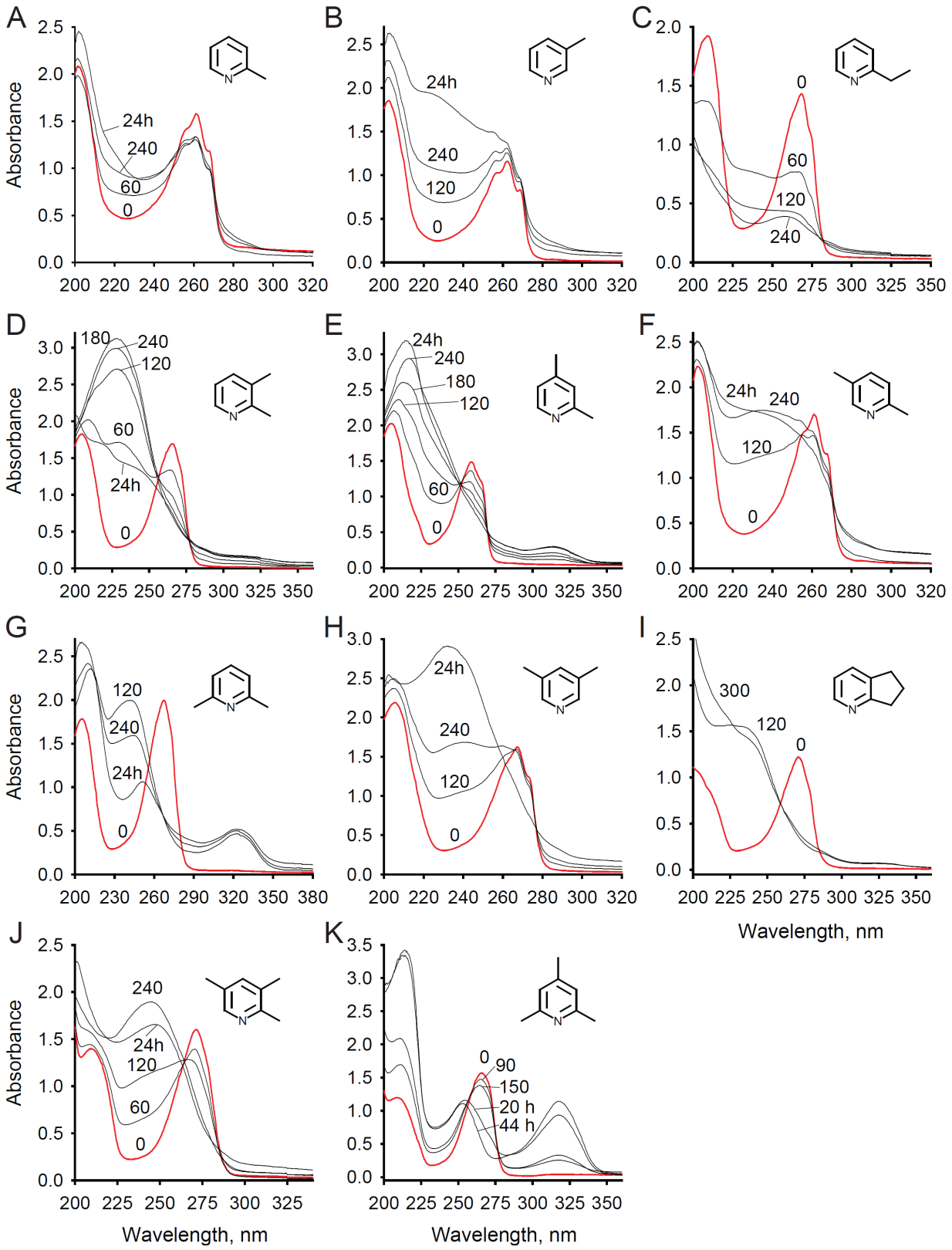

Fig. 9. Spectral changes during aerobic conversion of pyridine compounds by *R. jostii* TMP1 resting cells in 50 mM potassium phosphate, pH 7.2, at 20 °C. Cells were cultivated in the presence of TTMP. The reaction mixture was scanned after centrifugation (16,000×*g*, 1 min). Incubation time is shown in min. or hours (h). *A* – 2-methylpyridine; *B* – 3-methylpyridine; *C* – 2-ethylpyridine; *D* – 2,3-dimethylpyridine; *E* – 2,4-dimethylpyridine; *F* – 2,5-dimethylpyridine; *G* – 2,6-dimethylpyridine; *H* – 3,5-dimethylpyridine; *I* – 2,3-cyclopentenopyridine; *J* – 2,3,5-trimethylpyridine; *K* – 2,4,6-trimethylpyridine.

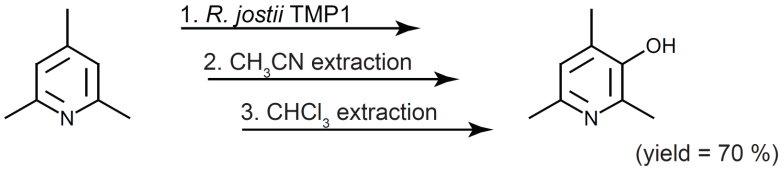

Fig. 10. Synthesis of 2,4,6-trimethylpyridin-3-ol from 2,4,6-trimethylpyridine using resting cells of *Rhodococcus jostii* TMP1.

TMP1. After purification 159 mg of product was obtained, resulting in bioconversion yield of 70%. The ^{1}H NMR ($CDCl_3$, 300 MHz) analysis of obtained compound showed five peaks with δ 6.90 (br s, 1H, OH), 6.78 (s, 1H, C*H*), 2.36 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 2.21 (s, 3H, CH_3); and eight peaks in ^{13}C NMR ($CDCl_3$, 75 MHz) spectrum: δ 148.6, 147.4, 144.7, 135.8, 123.9, 22.5, 18.6, 16.3. MS analysis confirmed the molecular formula of the compound as $C_8H_{11}NO$ (calculated $M+H^+$ mass was 138.08, found mass was 138.15). The analysis confirmed that *R. jostii* TMP1 metabolized 2,4,6-trimethylpyridine to 2,4,6-trimethylpyridin-3-ol (Fig. 10).

We assume that pyrazines and pyridines are modified by TpdAB flavin monooxygenase, since uninduced TMP1 strain did not convert these compounds.

RESUMÉ

This study revealed the bacterial genetic locus responsible for alkylpyrazine metabolism for the first time. The enzymes, encoded by *tpd* genes, were identified and intermediate metabolites of TTMP catabolism were isolated. It was demonstrated that the initial steps of TTMP biodegradation in *R. jostii* TMP1 follow a series of enzymatic reactions: TTMP is oxidized by TpdAB monooxygenase, then hydrolysed by TpdC amidase and further reduced by aminoalcohol dehydrogenase TpdE. This enzymatic sequence provides the first validated pathway for pyrazine degradation. Selective and specific Tpd enzymes may serve in the future as the catalysts for the biosynthesis of biologically and pharmacologically active compounds. Indeed, it was shown that *R. jostii* TMP1 can be employed to modify a variety of alkylpyrazines and alkylpyridines and is applicable for the synthesis of hydroxypyridines under mild conditions.

CONCLUSIONS

1. The identified 13 kb genetic locus in *Rhodococcus jostii* TMP1 strain encodes the genes required for tetramethylpyrazine degradation.
2. *tpdA*, *tpdB* and *tpdC* open reading frames are organized into *tpdABC* operon.
3. The expression of *tpdABC* operon is regulated by transcription activator TpdR.
4. Monooxygenase TpdA together with TpdB oxidizes tetramethylpyrazine to (*Z*)-*N*,*N*'-(but-2-ene-2,3-diyl)diacetamide.
5. Amidase TpdC hydrolyses (*Z*)-*N*,*N*'-(but-2-ene-2,3-diyl)diacetamide to *N*-(3-oxobutan-2-yl)acetamide.
6. NADPH-dependent aminoalcohol dehydrogenase TpdE reduces *N*-(3-oxobutan-2-yl)acetamide.
7. *Rhodococcus jostii* strain TMP1 can be employed for the bioconversion of alkylpyrazines and alkylpyridines as well as the biosynthesis of hydroxypyridines.

LIST OF PUBLICATIONS

Articles

- **Kutanovas S**, Stankeviciute J, Urbelis G, Tauraite D, Rutkiene R, Meskys R. Identification and characterization of tetramethylpyrazine catabolic pathway in *R. jostii* TMP1. (2013). *Appl Environ Microbiol.* doi:10.1128/AEM.00011-13.
- **Kutanovas S**, Rutkiene R, Urbelis G, Tauraite D, Stankeviciute J, Meskys R. (2013) Bioconversion of methylpyrazines and pyridines using novel pyrazines-degrading microorganisms. *Chemija* 24, 67-73.

Conference posters

- Stankevičiūtė J, **Kutanovas S**, Rutkienė R, Meškys R. (2012) Purification and characterization of alcohol dehydrogenase from *R. jostii* TMP1. 12th International Lithuanian Biochemical Society Conference, Tolieja, Lithuania.
- **Kutanovas S**, Karvelis L, Michailova K, Časaitė V, Stankevičiūtė J, Meškys R. (2010) Biodegradation of carboxypyridines and carboxypyrazines. COST Action CM0701 „Cascade Chemoenzymatic Processes – New Synergies Between Chemistry and Biochemistry“ (CASCAT), Vilnius, Lithuania.

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SANTRAUKA

Šio darbo metu buvo nustatytas ir aprašytas tetrametilpirazino (TTMP) skaidymo metabolinis kelias anksčiau išskirtose *R. jostii* TMP1 bakterijose, kurios gali naudoti TTMP kaip vienintelį anglies ir energijos šaltinį (Kutanovas, 2008). TMP1 bakterijas kultivuojant su TTMP buvo indukuojama 40 kDa baltymo sintezė. Kaip potencialus TTMP skaidymo fermentas šis baltymas buvo išskirtas ir nustatytos jo peptidų aminorūgščių sekos. Peptidų sekos buvo sulygintos su genomine Illumina duomenų baze ir nustatytas 40 kDa baltymą koduojantis genas ir genų sankaupa, kuri pavadinta tetrametilpirazino degradavimo (*tpd*) sankaupa. Iš viso buvo nustatyta 13 kb DNR fragmento seka, koduojanti TTMP skaidymo genus. Analizuojant *tpd* genų sankaupos seką, nustatyti aštuoni atviri skaitymo rėmeliai (ORF), kurie pavadinti *tpdA* (koduoja 40 kDa indukuojamą baltymą), *tpdB*, *tpdC*, *tpdR*, *tpdD*, *tpdE, orf1* ir *orf2*. Kiekybine AT-PGR analize nustatyta, kad trys iš šių rėmelių sudaro *tpdABC* operoną.

AT-PGR analizė parodė, kad *tpdA-E* genų raiška yra specifiškai indukuojama, auginant *R. jostii* TMP1 bakterijas su TTMP. Esant TTMP, klonuotas *tpdABC* promotorius indukuoja reporterinio EGFP geno raišką. Nustačius *tpdABC* operono transkripcijos pradžios tašką ir atlikus minimalios promotoriaus sekos analizę, buvo aptiktos 15 bp pasikartojančios ir 7 bp invertuotos sekos, būtinos promotoriaus veikimui. Šias sekas potencialiai atpažįsta *tpdR* geno koduojamas transkripcijos aktyvatorius, kurį klonavus kartu su *tpdABC* promotoriumi buvo gauta sistema, kurią galima pritaikyti TTMP indukuojamos rekombinantinių baltymų raiškos vektoriams.

tpdABC operono genai buvo klonuoti ir perkelti į *R. erythropolis* SQ1 bakterijas, taip suteikiant joms gebėjimą metabolizuoti TTMP. TTMP buvo metabolizuojamas SQ1 bakterijose, transformuotose plazmidėmis, turinčiomis *tpdABC* ir *tpdAB* genus, tačiau atskirų *tpdA*, *tpdB* ir *tpdC* genų TTMP konversijai nepakako. TpdABC ir TpdAB reakcijos produktai buvo išgryninti, o jų struktūros nustatytos ^{1}H NMR, ^{13}C NMR ir MS analizės metodais. TpdAB metabolizavo TTMP iki (*Z*)-*N*,*N*'-(but-2-ene-2,3-diil)diacetamido (BDNA), o TpdC produktas buvo *N*-(3-oksobutan-2-il)acetamidas (OBNA). Eksperimentais su ^{18}O izotopu žymėtu vandeniu nustatyta, kad skaidydamas TTMP žiedą TpdA baltymas veikia kaip monooksigenazė. *In vitro* fermentinės analizės rezultatai pademonstravo, kad TpdC yra amidazė, specifiškai hidrolizuojanti BDNA iki

OBNA. Be to, nustatyta, kad OBNA nuo NADPH priklausomos dehidrogenazės TpdE yra redukuojamas iki aminoalkoholio. Skaidymo genų nustatymas ir tarpinių metabolitų išskyrimas suteikė galimybę pirmą kartą aprašyti aerobinio TTMP skaidymo reakcijas ir rekonstruoti tetrametilpirazino katabolizmo kelią *R. jostii* TMP1 bakterijose.

Dėl pastaruoju metu didėjančio susidomėjimo biokatalize kaip nauju švelniu būdu sintetinti biologiškai ir farmakologiškai aktyvius junginius, buvo ištirta, ar TTMP skaidančios *R. jostii* TMP1 bakterijos galėtų būti naudojamos alkilpirazinų ir alkilpiridinų biokonversijai. Buvo pademonstruota, kad *R. jostii* TMP1 bakterijos, indukuotos TTMP, taip pat skaido arba modifikuoja 2,3-dimetilpiraziną, 2,5-dimetilpiraziną ir 2,6-dimetilpiraziną bei trimetilpiraziną. Esant TTMP, *R. jostii* TMP1 bakterijos taip pat kartu metabolizuoja įvairius metil- ir etilpiridinus. Be to, šios bakterijos hidroksilina kai kuriuos metilpiridinus, įskaitant 2,4,6-trimetilpiridiną. Pirmą kartą buvo pademonstruota 2,4,6-trimetilpiridin-3-olio, pasižyminčio antioksidantinėmis, stabdančiomis senėjimą ir saugančiomis nuo išemijos savybėmis, biokatalizinė sintezė. Tad indukuotos *R. jostii* TMP1 bakterijos galėtų būti taikomos hidroksipiridinų sintezei švelniomis sąlygomis.

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Scientific publications:

1. **Kutanovas S**, Stankeviciute J, Urbelis G, Tauraite D, Rutkiene R, Meskys R. Identification and characterization of tetramethylpyrazine catabolic pathway in *R. jostii* TMP1. (2013). *Appl Environ Microbiol.* doi:10.1128/AEM.00011-13.
2. **Kutanovas S**, Rutkienė R, Urbelis G, Tauraitė D, Stankevičiūtė J, Meškys R. (2013) Bioconversion of methylpyrazines and pyridines using novel pyrazines-degrading microorganisms. *Chemija* 24, 67-73.

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