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ORIGINAL PAPER

Degradation of 2,5-dimethylpyrazine by *Rhodococcus erythropolis* strain DP-45 isolated from a waste gas treatment plant of a fishmeal processing company

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Abstract A bacterium, strain DP-45, capable of degrading 2,5-dimethylpyrazine (2,5-DMP) was isolated and identified as *Rhodococcus erythropolis.* The strain also grew on many other pyrazines found in the waste gases of food industries, like 2,3-dimethylpyrazine (2,3-DMP), 2,6-dimethylpyrazine (2,6-DMP), 2-ethyl-5(6)-dimethylpyrazine (EMP), 2-ethylpyrazine (EP), 2-methylpyrazine (MP), and 2,3,5-trimethylpyrazine (TMP). The strain utilized 2,5-DMP as sole source of carbon and nitrogen and grew optimally at 25°C with a doubling time of 7.6 h. The degradation of 2,5-DMP was accompanied by the growth of the strain and by the accumulation of a first intermediate, identified as 2-hydroxy-3,6-dimethylpyrazine (HDMP). The disappearance of HDMP was accompanied by the release of ammonium into the medium. No other metabolite was detected. The degradation of 2,5-DMP and HDMP by strain DP-45 required molecular oxygen. The expression of the first enzyme in the pathway was induced by 2,5-DMP and HDMP whereas the second enzyme was constitutively expressed. The activity of the first enzyme was inhibited by diphenyliodonium (DPI), a flavoprotein inhibitor, methimazole, a competitive inhibitor of flavin-containing monooxygenases, and by cytochrome P450 inhibitors, 1-aminobenzotriazole (ABT) and phenylhydrazine (PHZ). The activity of the second enzyme was inhibited by DPI, ABT, and PHZ. Sodium tungstate, a specific antagonist of molybdate, had no influence on growth and consumption of 2,5-DMP by strain DP-45. These results led us to propose that a flavin-dependent monooxygenase or a cytochrome P450-dependent monooxygenase rather than a molybdenum hydroxylase catalyzed the initial hydroxylation step and that a cytochrome P450 enzyme is responsible for the transformation of HDMP in the second step.

Keywords Biodegradation · odor · pyrazine · *Rhodococcus erythropolis*

Abbreviations

ABT	(1-aminobenzotriazole)
AP	(Acetylpyrazine)
DEP	(2,3-diethylpyrazine)
DM	(2,3-diethyl-5-methylpyrazine)
2,3-DMP	(2,3-dimethylpyrazine)
2,5-DMP	(2,5-dimethylpyrazine)
2,6-DMP	(2,6-dimethylpyrazine)
DPI	(Diphenyliodonium)

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EP	(2-ethylpyrazine)
EMP	(2-Ethyl-5(6)-methylpyrazine)
HDMP	(2-hydroxy-3,6-dimethylpyrazine)
IP	(2-isobutyl-3-methoxypyrazine)
MP	(2-methylpyrazine)
P	(Pyrazine)
PHZ	(Phenylhydrazine)
TMP	(2,3,5-trimethylpyrazine)
TTMP	(2,3,5,6-tetramethylpyrazine)

Introduction

Pyrazines are heterocyclic nitrogen-containing compounds found mainly in processed food, where they are formed during heating processes. Some pyrazines show bactericidal (MacDonald 1973; Van Scoy 1992) or chemoprotective (Kim et al. 1997) activities. Therefore, corresponding structures have been frequently incorporated in biologically active compounds such as antiseptics, disinfectants, herbicides, fungicides, and insecticides as well as in pharmaceuticals with different fields of application (Baker et al. 1989; Street et al. 1992; Wieser 1997; Tinschert et al. 2000; Schmid et al. 2001; Brown 2002;).

Alkylpyrazines are generally found in a wide variety of foods, beverages and in the air of food processing factories (Maarse 1991; Rappert and Müller 2005a). These compounds have very low odor thresholds. 2,5-DMP is the main pyrazine detected in cocoa bean- or soybean-based fermented foods, where they are recognized as important contributors to their flavor (Besson et al. 1997; Rappert and Müller 2005a). 2,5-DMP was also used as a substrate to produce 5-methylpyrazine-2-carboxylic acid, an intermediate in the synthesis of an antilipolytic drug (Schmid et al. 2001).

Despite the fact that pyrazines are smelling more or less pleasantly, their relatively high concentration as well as their continuous production and permanent presence renders them pollutants that bother neighbors and passers-by. Pyrazines are considered as one of the major malodorous class of compounds in the exhaust gas stream of various food industries (Ranau and Steinhart 2004; Nagorny and Francke 2005; Ranau et al. 2005; Rappert and Müller 2005a, b). 2,5-DMP was found to be one of the alkylated pyrazines which are the most intensively smelling compounds among the volatiles emanating from chocolate and coffee producing factories (Nagorny and Francke 2005; Ranau and Steinhart 2004; Ranau et al. 2005). Therefore, it is important to find methods to degrade these compounds before they reach the environment.

There are several reports of bioremediation of heteroaromatic compounds; however, little is known about microbial degradation of pyrazines. In a previous paper we described the isolation of *Mycobacterium* sp. strain DM-11, which can utilize 2,3-diethyl-5-methylpyrazine (DM) as the sole source of carbon, nitrogen, and energy. However, this strain could not grow on 2,5-DMP (Rappert et al. 2004, 2006). Since 2,5-DMP is one of the key compounds that cause odor problems, it was important to obtain microorganisms that can degrade this compound. Very few reports indicate transformation of 2,5-DMP by bacterial strains. Kiener (1992) showed that *Pseudomonas putida*, metabolizing toluene via benzyl alcohol, produced 5-methylpyrazinecarboxylic acid from 2,5-DMP. Though two bacterial strains (i.e., *Rhodococcus erythropolis* strain DSM 6138 and *Arthrobacter* sp. strain DSM 6137) that can grow on 2,5-DMP as a sole substrate were reported (Kiener et al. 1993, 1994), not much information relating to the mechanism of 2,5-DMP degradation was provided.

In order to understand the mechanism of microbial degradation of 2,5-DMP, in this study, a bacterial strain capable of using 2,5-DMP as the sole carbon and energy source was isolated and characterized. The identification of HDMP as a key metabolic intermediate allows the proposal of the first reaction in the degradation pathway. Finally, the inducibility of the pathway, effects of molecular oxygen, effects of inhibitors, as well as the transformation or use of other pyrazines by the newly isolated bacteria was examined.

Materials and methods

Chemicals

Chemicals were purchased from Sigma–Aldrich, Fluka or Merck (Germany) and were of the highest purity available.

The metabolite, HDMP, was synthesized according to the method described by Ohta et al. (1979). Spectroscopic data of HDMP are given in Table 1.

Media and culture conditions

The mineral medium M1 and a nitrogen free mineral medium M1 (NF-M1) were used to cultivate the isolated strain. The cultivation was done according to Rappert et al. (2006). In addition to 2,5-DMP, pyrazine (P), MP, 2,3-DMP, 2,6-DMP, TMP, 2,3,5,6-tetramethylpyrazine (TTMP), EP, 2,3-diethylpyrazine (DEP), DM, EMP, acetylpyrazine (AP), and 2-isobutyl-3-methoxypyrazine (IP) were used as substrates. Soluble substrates were sterilized by filtration. All substrates were provided at 0.5 mM, unless otherwise indicated, as the sole source of carbon and energy in the mineral medium.

All bacterial biodegradation tests under anaerobic conditions were carried out as described by Rappert et al. (2006). 10% (v/v) inoculum and 3 mM substrate were used. To test nitrate as alternative electron acceptor 10 mM, sodium nitrate was added. All the tests were done in duplicates.

To test for cometabolism, NF-M1 medium was used containing both the substrates, 2,5-DMP (0.5 mM) and the test compound (different pyrazines, concentration 0.5 mM). Flasks were inoculated with 10% (v/v) of a liquid culture of strain DP-45, which had been grown on 2,5-DMP. Growth and transformation of the chemicals were determined. Cometabolism experiments were performed in duplicates.

Table 1 Spectroscopic data of synthetic HDMP

Analysis type	Spectral data
Mass spectrum (EI, 70 eV)	124 (M, 75); 96 (37); 95 (100); 81 (10); 55 (25); 54 (34); 42 (52)
^{1}H-NMR (400 MHz, $CDCl_3$)	δ = 2.22(s, 3H, CH_3); 2.35(s, 3H, CH_3); 1.56 (1H, –OH)
^{13}C-NMR (101 MHz, $CDCl_3$)	δ = 16.43 (CH_3); 20.24 (CH_3); 123.11 (C_5); 134.82 (C_3, C_6); 154.51 (C_2)

Enrichment, isolation and identification of 2,5-DMP-degrading strain

Enrichment and isolation of 2,5-DMP-degrading strain was done according to Rappert et al. (2006), except that 0.5 mM 2,5-DMP was used as the sole carbon source. The isolate was characterized and identified at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany). A variety of physiological tests were done according to Goodfellow (1989) and Klatte et al. (1994). Analysis of mycolic acids was done as described (Kämpfer et al. 1990; Miller and Berger 1985). Analysis of ribotyping was done according to Bruce (1996). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA and purification of the PCR product were carried out as described by Rainey et al. (1996). Purified PCR products were sequenced using the CEQ^{TM} DTCS-Quick Start Kit (Beckmann Coulter). The sequences were compared to the 16S rRNA sequences in the EMBL database (EMBL Outstation, Cambridge, UK), DSMZ database, and those of the Ribosomal Database Project (Maidak et al. 1999).

Resting cells assays

Resting cell suspensions with 100 μg ml^{-1} of chloramphenicol (this concentration was experimentally confirmed to inhibit growth of the strain DP-45 effectively) were prepared according to Rappert et al. (2006). Test compounds were used in 3 mM concentrations.

To determine whether the enzyme systems responsible for the biotransformation of 2,5-DMP and HDMP were induced or constitutively expressed, two batches of cells were prepared. For induced cells, cells were grown in NF-M1 containing 3 mM 2,5-DMP or HDMP, respectively. For non-induced cells, cells were grown in

LB (Luria-Bertani) medium at 120 rpm and 25°C over night. The cells were harvested by centrifugation, washed three times with 50 mM sodium phosphate buffer pH 6.75. The washed cells were tested for their ability to transform 2,5-DMP or HDMP in the presence of chloramphenicol.

Inhibition assays

Effects of selected inhibitors like DPI, methimazol, sodium tungstate, ABT, and PHZ on growth and substrates degradation by strain DP-45 were determined. Strain DP-45 was grown in NF-M1 containing 3 mM 2,5-DMP or HDMP as the sole substrate with different concentrations of the selected inhibitors. During cultivation, growth and substrate concentration in the culture medium were determined. To confirm that the effect of the inhibitor was due to the inhibition of substrate degradation, the effect of inhibitor on the growth of strain DP-45 in LB medium was also tested. Effects of the selected inhibitors were also tested in non-growing resting-cells. 2,5-DMP-grown cells were collected by centrifugation, washed, and suspended in 50 mM phosphate buffer (pH 6.75) to an OD_{550} of 15 (corresponding to a cell density of approximately 3.2 mg dry weight ml^{-1}). About 2 ml of the cell suspension was then placed in 20-ml serum vials and different concentrations of the inhibitors were added. The reaction mixtures were incubated at 25°C for 1 h before 2,5-DMP or HDMP was added to a final concentration of 3 mM. At appropriate time intervals, aliquots of the cell suspension were withdrawn and analyzed for substrate concentration. For control reaction mixtures without inhibitor were used. All experiments were carried out in duplicates.

Analytical procedures

Bacterial growth was monitored routinely by recording the optical density at 550 nm, and the total cell count was determined by counting under the microscope using a Thoma chamber.

Substrate concentrations of the volatile compounds were measured by headspace gas chromatography (Rappert et al. 2006) or by recording the UV–Vis absorption spectra.

UV–Vis absorption spectra were recorded with an Uvikon 930 spectrophotometer (Kontron Instruments, Le-Mont-sur-Lausanne, Switzerland). Pyrazines and their metabolites were identified by their characteristic UV absorption spectra between 200 and 400 nm.

For isolation and identification of 2,5-DMP metabolites, compounds were isolated either from growing cultures of strain DP-45 in NF-M1 medium with 2,5-DMP (3 mM) as a sole source of carbon, nitrogen, and energy or by the preparative bioconversion of 2,5-DMP by freshly prepared resting cells of strain DP-45. Isolation and identification of the metabolite were done according to Rappert et al. (2006).

Ammonium (NH_4^+) was analyzed using ammonium test kit (Spectroquant, Merck, Germany) according to the instructions of the manufacturer.

Results

Isolation and identification of 2,5-DMP-degrading strain DP-45

Strain DP-45 was enriched and isolated from a liquid sample from a waste gas treatment plant of a fishmeal producing industry in Germany by using 2,5-DMP as the sole source of carbon and energy. This organism produced creamy beige-red to orange-red circular colonies on LB medium. The isolate was aerobic, gram-positive and non-motile. Its morphology changed from cocci to rods, which could elongate to longer ones or to branched filaments. The physiological tests for species-identification (substrate utilization value) were 99% similar to that of *Rhodococcus erythropolis* DSM 43066. The mycolic acid pattern was compared with the pattern stored in the *Rhodococcus* mycolic acid database. The pattern of strain DP-45, which a chain-length of $C_{32–42,}$ showed a high similarity to *R. erythropolis.* The fatty acid pattern revealed unbranched saturated and unsaturated fatty acids plus tuberculostearic acid. Fatty acid composition analysis (MIDI software) identified a high similarity (0.73) to *R. erythropolis.* The correlation factor of the riboprint pattern of strain DP-45 to that of *R. erythropolis* DSM 43066 was 0.77. Sequence

analysis of the 16S rRNA gene revealed 99.7% similarity to *R. erythropolis* DSM 43066. Based on conventional markers (morphology), physiology (substrate utilization), chemotaxonomic markers (fatty acid and mycolic acid patterns), and molecular biological results (riboprint patterns and comparative 16S rRNA gene sequencing), it was concluded that strain DP-45 belongs to the species *R. erythropolis*.

Growth of *R. erythropolis* strain DP-45 on 2,5-DMP as carbon and nitrogen source

R. erythropolis strain DP-45 was enriched, isolated and routinely grown in mineral medium M1 containing 2,5-DMP as a sole source of carbon and energy. Strain DP-45 grew optimally at 25°C, temperatures above 40°C completely inhibited growth. In order to check whether strain DP-45 can also utilize 2,5-DMP as a sole source of nitrogen, its growth in a nitrogen free mineral medium containing 3 mM 2,5-DMP as a sole substrate was examined. During cultivation, after a lag phase of 6 h, 2,5-DMP quickly decreased and disappeared from the medium within 28 h (Fig. 1). Cell growth began with a lag phase of 6 h then increased quickly (t_d: 7.6 h) and reached a stationary phase after 24 h of cultivation. The amount of ammonium in the culture medium increased parallel to cell growth and reached the maximum of 5.2 mM after 24 h of cultivation. Accumulation of ammonium was not found in the control samples without DP-45 cells. The pH of the medium increased slightly from 6.75 to 6.82 after 2,5-DMP was completely utilized. The omission of ammonium sulfate from the mineral medium did not influence growth and final cell counts. The rate of 2,5-DMP degradation in the medium without ammonium sulfate was slightly higher than in the medium with $(NH_4)_2SO_4$. The final cell counts correlated with the amount of 2,5-DMP in the culture medium up to 10 mM. Further increase of 2,5-DMP caused inhibition of growth and 2,5-DMP degradation. These results show that strain DP-45 utilized 2,5-DMP as a sole source of carbon and nitrogen. In the absence of molecular oxygen neither growth nor 2,5-DMP degradation or ammonium accumulation was observed (Fig. 1). Nitrate could not replace molecular oxygen.

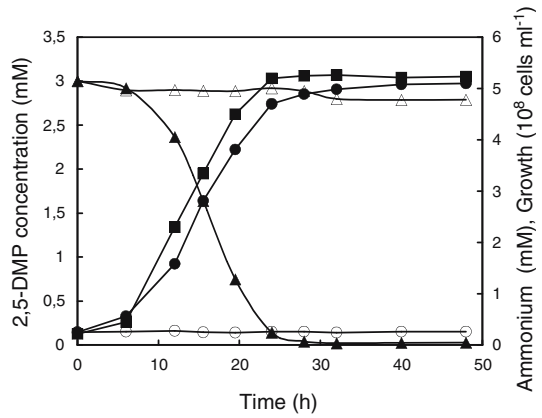

Fig. 1 Time course of utilization of 2,5-DMP by *R. erythropolis* strain DP-45 in liquid NF-M1 medium with 3 mM 2,5-DMP as the sole carbon and nitrogen source in the presence (closed symbols) and absence (open symbols) of molecular oxygen. Symbols: circles, growth; triangles, 2,5-DMP concentrations; squares, ammonium concentrations. Data are means of results of duplicate experiments, and the standard deviations were within 5% of the mean values ($n = 2$)

Growth of *R. erythropolis* strain DP-45 with other pyrazines, biotransformation by resting cells, and cometabolism by cells grown on 2,5-DMP

A total of 13 pyrazines, which are frequently found in the waste gas from various food industries, were tested as substrates for growth, as substrates in biotransformations by resting cells of strain DP-45, and in cometabolism tests with the cells growing on 2,5-DMP. The results obtained are shown in Table 2. It was found that in addition to 2,5-DMP, strain DP-45 could utilize 2,3-DMP, 2,6-DMP, EMP, EP, MP, and TMP as a sole carbon and nitrogen source for growth. In the cometabolic culture with 2,5-DMP, the degradation rate of these compounds, except for 2,6-DMP and EMP, increased 2–9 times. Degradation of DEP, DM and P was only possible with 2,5-DMP-induced cells or in cometabolic degradation during growth with 2,5-DMP. For AP, IP, and TTMP no degradation was observed. The degradation of 2,5-DMP by strain DP-45 was not inhibited by the presence of these pyrazines in the culture.

Table 2 Degradation of different pyrazines by *R. erythropolis* strain DP-45 as a sole carbon and nitrogen source for growth, by resting cells (induced with 2,5-DMP) and by cometabolic conversion during growth with 2,5-DMP[a]

Compound[b]	Growth[c]	Degradation (μM day^{-1})[d]	Transformation with resting cells (μM min^{-1})[e]	Cometabolic conversion during growth with 2,5-DMP (μM day^{-1})[f]
AP	–	–	–	–
DM	–	–	6.3	360
DEP	–	–	3.6	158
2,3-DMP	+	33	2.1	72
2,5-DMP	+	750	25	ND
2,6-DMP	+	189	12.5	ND
EMP	+	422	12.5	ND
EP	+	84	12.5	416
IP	–	–	–	–
MP	+	40	6.2	380
P	–	–	0.2	33
TMP	+	94	2.1	250
TTMP	–	–	–	–

[a] Data are means from duplicate experiments, and the standard deviations were within 8% of the mean values (n = 2). +, growth; -, no activity or growth; ND, not determined.

[b] All the compounds were used at a final concentration of 0.5 mM.

[c] Cultivations of strain DP-45 in NF-M1 medium containing the compound as a sole substrate were carried out for 10 days. Cell growth was determined every day.

[d] Cultivations of strain DP-45 in NF-M1 medium containing the compound as a sole substrate were carried out for 10 days. The concentration of the substrates in the culture medium was determined every day. Maximum degradation rate of the compound during cultivation is shown.

[e] Resting cells experiments were carried out for 48 h. The optical density at 550 nm of the cells suspensions was 15 (corresponding to a cell density of approximately 3.2 mg dry weight ml^{-1}). The concentrations of the substrates in the cells suspensions were determined every 20 min up to 4 h.

[f] Cultivations of strain DP-45 in NF-M1 medium containing 0.5 mM 2,5-DMP and 0.5 mM of the compound as the substrate were carried out for 10 days. The concentration of the compounds in the culture medium was determined everyday. Maximum degradation rate of the compound during cultivation is shown.

Metabolite formation during degradation of 2,5-DMP

2,5-DMP had a maximum absorption at 275 nm. The accumulation of a metabolite showing absorption maxima at 223 and 319 nm was detected in the culture medium after 16 h of cultivation. Amounts of the metabolite reached a maximum after 24 h. Thereafter the metabolite decreased and disappeared after 28 h. No other metabolite showing absorption in the UV/Vis range was detected in the culture medium during the entire culture course (48 h). Controls without cells showed neither a decrease of 2,5-DMP nor formation of the metabolite or ammonium. In the absence of molecular oxygen no metabolite was formed.

By using GC/MS analysis only one new substance in the culture broth was detected. This metabolite had a molar mass that was 16 a.u higher than the molecular mass of 2,5-DMP. Therefore, the metabolite was regarded as the product of an enzymatic oxidation of 2,5-DMP. According to the mass spectrum of the acetylated metabolite, oxygenation of the nucleus had taken place. This was in agreement with the finding that the UV absorption maxima of the metabolite shifted to longer wavelengths, which suggested that the metabolite was hydroxylated at the ring site. To prove this hypothesis, HDMP was synthesized. The structure of the compound was confirmed by mass spectroscopy, ^{1}H-NMR, and ^{13}C-NMR analyses. The results obtained are shown in Table 1. The UV-absorption spectrum of the synthetic HDMP with absorption maxima at 223 and 319 nm was identical to the spectrum of the metabolite obtained during bioconversion of 2,5-DMP by strain DP-45. In addition, mass

spectrum and GC-retention time of the synthetic product and the metabolite were identical, proving the structure of the metabolite to be HDMP.

Degradation of HDMP by *R. erythropolis* strain DP-45

The time course of the degradation of HDMP by strain DP-45 was investigated. As shown in Fig. 2, under aerobic conditions HDMP was utilized immediately after starting the cultivation with a rate of 50 μM h^{-1} during the first 18 h. Thereafter the degradation rate increased up to 180 μM h^{-1}. All the HDMP was utilized after 30 h of cultivation. Growth with a doubling time of 5.4 h was observed after a lag phase of 8 h. A stationary phase was reached after 30 h of cultivation. During degradation of HDMP, ammonium was released into the medium. The rate of ammonium accumulation during the first 8 h was 20 μM h^{-1}. Subsequently, the rate increased to 240 μM h^{-1}. The amount of ammonium in the culture medium reached the maximum value of 5 mM after 30 h. Without molecular oxygen, strain DP-45 did not grow on HDMP and neither HDMP degradation nor ammonium accumulation was observed (Fig. 2). Nitrate could not replace molecular oxygen.

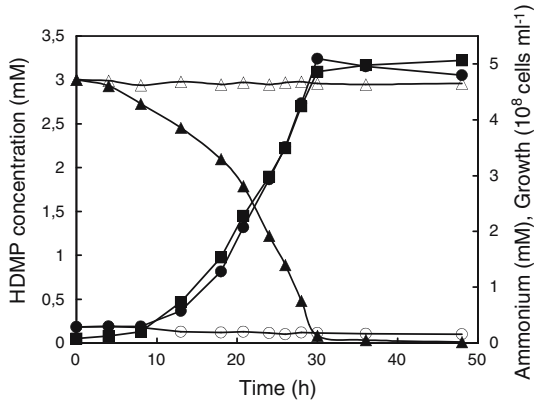

Fig. 2 Time course of utilization of HDMP by *R. erythropolis* strain DP-45 in liquid NF-M1 medium with 3 mM HDMP as the sole carbon and nitrogen source in the presence (closed symbols) and absence (open symbols) of molecular oxygen. Symbols: circles, growth; triangles, HDMP concentrations; squares, ammonium concentrations. Data are means of results of duplicate experiments, and the standard deviations were within 5% of the mean values (n = 2)

Regulation of 2,5-DMP and HDMP degrading enzymes in cells of *R. erythropolis* strain DP-45

As shown in Fig. 3A, 2,5-DMP-induced and HDMP-induced cells of strain DP-45 transformed 2,5-DMP quickly, while no transformation of 2,5-DMP was observed with non-induced cells. The 2,5-DMP-induced cells transformed 2,5-DMP with the initial rate of 28 μM min^{-1}. The rate of 2,5-DMP transformation by the HDMP-induced cells was 174 μM min^{-1}; about 6 times faster than by 2,5-DMP-induced cells.

Transformation of HDMP by 2,5-DMP-induced, HDMP-induced, and non-induced cells of strain DP-45 was also investigated. As shown in Fig. 3B, all cells transformed HDMP. The HDMP transformation rates by these cells were

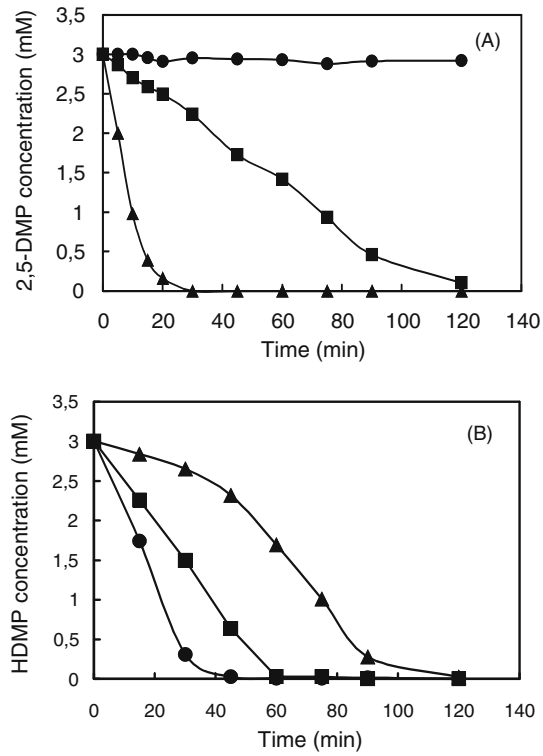

Fig. 3 Conversion of 2,5-DMP **(A)** and HDMP **(B)** by non-induced and induced resting cells of *R. erythropolis* strain DP-45 under aerobic conditions. Symbols: circles, non-induced cells; squares, 2,5-DMP-induced cells; triangles, HDMP-induced cells. Data are means of results from duplicate experiments, and the standard deviations were within 6% of the mean values (n = 2)

90 μM min^{-1} by non-induced, 50 μM min^{-1} by 2,5-DMP-induced, and 30 μM min^{-1} by HDMP-induced cells of strain DP-45.

Effect of inhibitors on 2,5-DMP and HDMP degradation

In order to determine the nature of the enzyme system involved in 2,5-DMP and HDMP degradation by strain DP-45, effects of various inhibitors on the degradation ability and growth of strain DP-45 were investigated. DPI, methimazole, and sodium tungstate were chosen as irreversible inactivator of flavoproteins (Shiemke et al. 2004), as competitive inhibitor of flavin-containing monooxygenase (Tomasi et al. 1995), and as specific antagonist of molybdate (Siegmund et al. 1990), respectively. In addition, PHZ and ABT were tested as cytochrome P450 inhibitors (Bhushan et al. 2003). As shown in Table 3, in the presence of 25 μM DPI, the 2,5-DMP and HDMP degradation activities and growth of strain DP-45 were inhibited. The same DPI concentration did not affect growth of strain DP-45 in LB medium. In resting cells the presence of 1 mM DPI completely inhibited the transformation of 3 mM 2,5-DMP. 3 mM HDMP was completely degraded by 2,5-DMP-grown resting cells within 2 h whereas in the presence of 1 mM DPI only 40% of HDMP was degraded after 20 h of incubation. The addition of 5 mM methimazole strongly inhibited 2,5-DMP degradation and growth of strain DP-45 during 24 h of cultivation while it did not inhibit HDMP degradation and growth of strain DP-45 on HDMP. Transformation of HDMP by resting cells of strain DP-45 was also not inhibited by 20 mM methimazol. The presence of 9 mM sodium tungstate did not inhibit the degradation of 2,5-DMP or growth of strain DP-45. The presence of 20 mM ABT or PHZ in the reaction mixtures completely inhibited both 2,5-DMP and HDMP transformation by resting cells of strain DP-45 (Table 3).

Discussion

In this paper, we describe a strain, which is capable of using 2,5-DMP as sole carbon and nitrogen source. This strain was classified as *R. erythropolis*. According to the US patent number 5284767, *R. erythropolis* strain DSM 6138 has been described as a microorganism useful for the production of hydroxylated heterocycles (Kiener et al. 1994). Strain DSM 6138 also utilizes 2,5-DMP as the sole substrate. However, the physiological data for species-identification of strain DP-45 and strain DSM 6138 are different. It was found that strain DP-45 could utilize D-turanose, L-proline, galactose, 4-aminobutyrate (α4B), benzoate, and 3-hydroxybenzoate while strain DSM 6138 could not. Strain DSM 6138 could utilize 4-aminobutyrate (o4B), pimelase, and gluconate but strain DP-45

Table 3 Effects of inhibitors on 2,5-DMP- and HDMP-degradation and on growth of *R. erythropolis* strain DP-45[a]

Inhibitor[b]	3 mM 2,5-DMP		3 mM HDMP	
	Degradation	Growth	Degradation	Growth
without inhibitor	+	+	+	+
25 μM DPI	–	–	–	–
5 mM methimazole	–	–	+	+
9 mM sodium tungstate	+	+	ND	ND
20 mM ABT	–	ND	–	ND
20 mM PHZ	–	ND	–	ND

[a] All inhibitor experiments (except for ABT and PHZ) were carried out in NF-M1 medium containing 3 mM 2,5-DMP or HDMP as the sole source of carbon, nitrogen and energy. Effect of ABT and PHZ were measured in resting cells assays. Data are results of duplicate experiments. – indicates no degradation activity or no growth was observed. + indicates degradation activity or growth was observed. ND means not determined.

[b] The concentration of inhibitors used (except for ABT and PHZ) did not inhibit growth of strain DP-45 when cultivated in LB medium.

could not. Moreover, strain DSM 6318 has mycolic acids with a chain length of C_{35}-C_{40} (Kiener et al. 1994) whereas strain DP-45 has C_{32}–C_{42}. These results indicate that *R. erythropolis* strain DP-45 and *R. erythropolis* strain DSM 6318 are not the same strain.

The new strain isolated here degraded a variety of pyrazines. In addition to the substrate used for its isolation, all isomers of methylpyrazine, dimethylpyrazine, trimethylpyrazine, and ethylpyrazine were growth substrates. With these properties, the strain uses a much broader substrate spectrum than the previously described strain DM-11 (Rappert et al. 2006). However, like strain DM-11 the new strain did not use unsubstituted pyrazine or pyrazines carrying other substituents like acetyl-groups or methoxy-groups. In addition, the fully methylated TTMP is not a substrate for both strains. The basic requirements for growth substrates for strain DP-45 seem to be pyrazines substituted with at least one methyl or ethyl group and with one free position at the ring.

The 2,5-DMP was transformed only when the enzyme for the degradation had been induced whereas the metabolite HDMP could be transformed by non-induced cells. These findings suggest that the enzyme for 2,5-DMP degradation is inducible but the enzyme for HDMP degradation is constitutively expressed. The synthesis of the first enzyme was also induced by HDMP. The induction of enzymes by a metabolite formed was also found in the degradation of aromatic hydrocarbons by *Mycobacterium* sp. strain 6PY1 (Krivobok et al. 2003) and in the degradation of DM by *Mycobacterium* sp. strain DM-11 (Rappert et al. 2006). Interestingly, it was found that the rate of 2,5-DMP transformation by HDMP-induced cells was about 6 times higher than that from 2,5-DMP-induced cells. Therefore, HDMP seems to be the better inducer for the enzyme. Although the enzyme responsible for HDMP degradation is constitutively expressed, different HDMP degradation rates were obtained among different types of resting cells. The HDMP transformation by non-induced cells was faster than by 2,5-DMP-induced and HDMP-induced cells. At the moment, we have no simple explanation for this phenomenon. One possible reason may be that the different growth substrates affected the membrane transport system for HDMP in strain DP-45. This phenomenon was found in *Saccharomyces cerevisiae* (Van Zyl et al. 1993). Alternatively, the presence of 2,5-DMP or HDMP in the growth medium could decrease the level of expression of the enzyme involved in HDMP degradation in strain DP-45. The constitutive enzyme expression level can be influenced by growth substrate as was found in *Lactobacillus collinoides* (Arthurs and Lloyd 1999). However, these hypotheses need further investigations.

Generally, the metabolism of N-heterocycles is initiated by a ring hydroxylation at the carbon adjacent to the N-heteroatom, followed by ring cleavage (Kaiser et al. 1996; de Wever et al. 1998; Fetzner 1998; Rappert et al. 2006). The hydroxylation of the N-heterocycles normally occurred under anaerobic conditions and the hydroxyl group originated from water and not from molecular oxygen (Kaiser et al. 1996; Tinschert et al. 1997; Fetzner 1998; Uchida et al. 2003). So far, in most cases molybdenum-containing hydroxylases are responsible for this reaction (Stephan et al. 1996; Fetzner 2000). However, not much is known about microbial degradation of pyrazines. Mattey and Harle (1976) proposed in the metabolism of hydroxypyrazine by *Pseudomonas* sp. that the first step is the dihydroxylation to 2,6-dihydroxypyrazine, followed by ring cleavage by an oxygenase. The regioselective monohydroxylation of derivatives of pyrazine-2-carboxylic acid at position 3 to form 3-hydroxypyrazine-2-carboxylic acid, 3-hydroxy-5-methylpyrazine-2-carboxylic acid, or 3-hydroxy-5-chloropyrazine-2-carboxylic acid by cells of *Ralstonia/Burkholderia* sp. strain DSM 6920 was reported (Tinschert et al. 2000). These reactions were catalyzed by 6-methylnicotinate-2-oxidoreductase (Tinschert et al. 1997 and 2000). Uchida et al. (2003) also reported that the quinolinate dehydrogenase from *Alcaligenes* sp. strain UK21 catalyzed the hydroxylation of pyrazine-2,3-dicarboxylic acid to 5-hydroxypyrazine-2,3-dicarboxylic acid. All these enzymes introduced a hydroxyl group containing oxygen derived from water and not from molecular oxygen (Tinschert et al. 1997 and 2000; Uchida et al. 2003). The growth of strains using

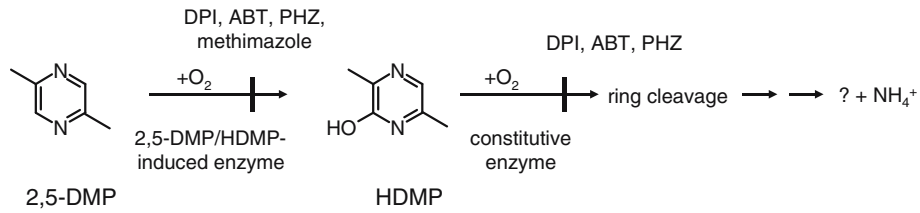

Fig. 4 Suggested initial degradation of 2,5-DMP by *R. erythropolis* strain DP-45. In this pathway, proposed sites of inhibition by DPI, ABT, PHZ, and/or methimazole are indicated by a vertical bar

molybdenum-containing enzymes is strictly inhibited by the presence of tungstate in the culture medium, because tungstate is a specific antagonist of molybdate (Siegmund et al. 1990). Growth and 2,5-DMP degradation of strain DP-45 were not inhibited by the presence of sodium tungstate in the culture medium. This clearly indicates that the enzyme involved in the degradation of 2,5-DMP is not belonging to the molybdenum containing enzyme family. Also 2,5-DMP degradation and HDMP formation were not observed in the absence of molecular oxygen, indicating that the transformation of 2,5-DMP to HDMP requires molecular oxygen. In morpholine degradation by *Mycobacterium* sp. strain RP1 and *Mycobacterium* sp. strain HE5, the formation of 2-hydroxymorpholine by a cytochrome P450-dependent monooxygenase was suggested as the initial step (Poupin et al. 1998; Schräder et al. 2000; Sielaff et al. 2001). The transformation of 2,5-DMP to HDMP was inhibited by the presence of DPI, an irreversibly inactivator of flavoproteins (Shiemke et al. 2004), methimazole, a competitive inhibitor of flavin-containing monooxygenases (Tomasi et al. 1995). This would suggest an involvement of a flavin containing enzyme. However, cytochrome P450 inhibitors (ABT and PHZ) (Bhushan et al. 2003) also inhibited transformation of 2,5-DMP. These data suggest that a cytochrome P450-dependent monooxygenase is involved. A final decision which type of enzyme is involved can only be made, when the enzyme is purified and characterized. This work is now in progress in our laboratory.

The conversion of HDMP, the first intermediate of the pathway, was accompanied by a complete disappearance of the UV absorption and the liberation of ammonium. These findings strongly indicate that ring fission occurs immediately after the initial hydroxylation like in the degradation of DM by strain DM-11 (Rappert et al. 2006). This step was also oxygen dependent indicating the involvement of an oxygenase in this step. The involvement of bacterial dioxygenases in ring cleaving of N-heterocycles has been reviewed comprehensively by Fetzner (2002). The transformation of HDMP was inhibited by the presence of DPI and cytochrome P450 inhibitors but not methimazole. These results suggest that the enzyme involved in this reaction belongs to the cytochrome P450 family, and is not a flavoprotein monooxygenase. However, the mechanism of ring fission and further catabolism of HDMP are still unclear and need further investigations, which are in progress in our laboratory. From the results obtained so far, an initial pathway for the microbial metabolism of 2,5-DMP by *R. erythropolis* strain DP-45 is proposed in Fig. 4.

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