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ORIGINAL PAPER

Pseudomonas putida A ATCC 12633 oxidizes trimethylamine aerobically via two different pathways

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Abstract The present study examined the aerobic metabolism of trimethylamine in *Pseudomonas putida* A ATCC 12633 grown on tetradecyltrimethylammonium bromide or trimethylamine. In both conditions, the trimethylamine was used as a nitrogen source and also accumulated in the cell, slowing the bacterial growth. Decreased bacterial growth was counteracted by the addition of $AlCl_3$. Cell-free extracts prepared from cells grown aerobically on tetradecyltrimethylammonium bromide exhibited trimethylamine monooxygenase activity that produced trimethylamine *N*-oxide and trimethylamine *N*-oxide demethylase activity that produced dimethylamine. Cell-free extracts from cells grown on trimethylamine exhibited trimethylamine dehydrogenase activity that produced dimethylamine, which was oxidized to methanal and methylamine by dimethylamine dehydrogenase. These results show that this bacterial strain uses two enzymes to initiate the oxidation of trimethylamine in aerobic conditions. The apparent K_m for trimethylamine was 0.7 mM for trimethylamine monooxygenase and 4.0 mM for trimethylamine dehydrogenase, but both enzymes maintain similar catalytic efficiency (0.5 and 0.4, respectively). Trimethylamine dehydrogenase was inhibited by trimethylamine from 1 mM. Therefore, the accumulation of trimethylamine inside *Pseudomonas putida* A ATCC 12633 grown on tetradecyltrimethylammonium bromide or trimethylamine may be due to the low catalytic efficiency of trimethylamine monooxygenase and trimethylamine dehydrogenase.

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Introduction

Trimethylamine (TMA) is a dietary-derived tertiary amine that has the characteristic odor of rotting fish (Al-Waiz et al. 1984). The origin of TMA in fish has not been established, but there is evidence that it is produced by microbial activity on choline, betaine or trimethylamine *N*-oxide in marine fish (King 1984; Mouné et al. 1999; López-Caballero et al. 2001). TMA is known to inhibit the synthesis of macromolecules, such as DNA, RNA and proteins (Guest and Varma 1992). In addition, TMA is a malodorous pollutant frequently found in effluents from fish-meal manufacturing processes (Sandberg and Ahring 1992; Rappert and Muller 2005 and citations therein). Microorganisms can transform hazardous organic compounds into harmless organic products, if the environmental conditions are suitable and the initial compounds are not toxic to microorganisms. The biological degradation of some compounds, such as TMA, is important to the development of strategies for effective biological treatment technologies (King 1984; Kim et al. 2001, 2003). Therefore, the metabolism of TMA by microorganisms has attracted considerable attention.

Recently, we reported that *P. putida* A ATCC 12633 can be grown with the cationic surfactant tetradecyltrimethylammonium bromide as the sole carbon, nitrogen and energy source (Liffourrena et al. 2008). The tetradecyltrimethylammonium bromide degradation was initiated by N-dealkylation catalyzed by a monooxygenase activity resulting in the formation of tetradecylalkanal and TMA

(Liffourrena et al. 2008, 2009). The TMA produced in the initial step was used by *P. putida* A ATCC 12633 as a nitrogen source and also accumulated inside the cell, decreasing the bacterial growth. This effect was counteracted by the addition of $AlCl_3$. In the presence of 0.1 mM $AlCl_3$, the intracellular concentration of free TMA decreased by the formation of an Al^{3+}-TMA complex, and bacterial growth increased in conjunction with the disappearance of tetradecyltrimethylammonium bromide through the action of the monooxygenase activity, which was no longer inhibited by free TMA. Thus, the formation of an Al^{3+}-TMA complex is thought to enable the bacteria to overcome the damage caused by TMA accumulation inside the cell (Liffourrena et al. 2008).

The TMA degradation pathway by aerobic bacteria has been intensively studied (Colby and Zatman 1973; Boulton and Large 1977; Large and Haywood 1981; Urakami et al. 1990; Ohara et al. 1990; Rappert and Muller 2005). However, no information on the metabolism of TMA by *P. putida* is available. In *P. aminovorans* grown on TMA, two enzymes are involved in the oxidation of TMA, suggesting that two TMA oxidation pathways exist, one involving oxygenation to give TMA *N*-oxide catalyzed by a trimethylamine monooxygenase (TMA monooxygenase) and the other involving the direct dehydrogenation of TMA to formaldehyde and dimethylamine (DMA), catalyzed by a trimethylamine dehydrogenase (TMA dehydrogenase) (Boulton et al. 1974).

The present study examined the enzymatic activities implicated in the TMA catabolism of *P. putida* A ATCC 12633 grown on tetradecyltrimethylammonium bromide or TMA. Cell-free extracts prepared from cells grown aerobically on tetradecyltrimethylammonium bromide exhibited the activity of TMA monooxygenase, whereas a TMA dehydrogenase activity was detected in cell-free extracts prepared from cells grown aerobically on TMA. These results confirmed that *P. putida* A ATCC 12633 uses two enzymes to initialize the oxidation of TMA in aerobic metabolism. Activities of other enzymes implicated in TMA metabolism were also examined. Additionally, the relationship of each enzyme with TMA accumulation and its negative effect on bacterial growth is also discussed.

Materials and methods

Organisms and culture conditions

P. putida A ATCC 12633 were grown aerobically at 30°C, with shaking in a basal salt liquid medium (HPi-BSM) (Lucchesi et al. 1989) with 50 mg l^{-1} (0.15 mM) tetradecyltrimethylammonium bromide or 295 mg l^{-1} (5 mM) TMA as the carbon and nitrogen source. To prevent loss of tetradecyltrimethylammonium bromide by adsorption on glass surfaces, all glassware was conditioned by overnight treatment with an aqueous 5 mg l^{-1} tetradecyltrimethylammonium bromide solution (Fernández et al. 1996). Control bacterial cultures were grown in HPi-BSM with 20 mM glucose and 18.7 mM NH_4Cl. Growth was measured as attenuance at 660 nm (D_{660}) with a spectrophotometer (Beckman DU 640).

Preparation of crude enzyme extracts

Cells of *P. putida* A ATCC 12633 were grown on tetradecyltrimethylammonium bromide or TMA to a stationary phase (D_{660} = 0.4). Bacteria were harvested by centrifugation at 10,000*g* for 10 min at 4°C, washed with 10 mM phosphate buffer (pH 7.4) and resuspended in the same buffer. The cells were disrupted by sonication in an ice bath at 20,000 Hz using a Vibra cell ultrasonic processor 10 times for 10 s each time. After centrifuging at 20,000*g* for 30 min, the clear supernatant was removed and used as a crude enzyme extract.

Enzyme assays

TMA monooxygenase activity was assayed at 30°C in a 1 ml reaction mixture containing 0.5 mM TMA, 0.5 mM NADPH, 1 mM KCN, 14 mM phosphate buffer (pH 7.4) and 0.3 mg ml^{-1} protein (Colby and Zatman 1973). TMA dehydrogenase activity was assayed in the reaction mixture described above without KCN and NADPH. Trimethylamine *N*-oxide demethylase and dimethylamine dehydrogenase activities were assayed in the same reaction mixture without KCN, replacing TMA with TMA *N*-oxide (0.5 mM) or dimethylamine (0.5 mM) as substrate, respectively. After 30 min of incubation at 30°C, the reaction was stopped by addition of 0.5 ml of 37.5% TCA. After centrifugation at 12,000*g* for 10 min, aliquots of 2 µl of the supernatant were used to detect TMA or the product TMA *N*-oxide and DMA by GC–MS, as described below. Boiled cell extracts were used as a blank. Enzyme activity for TMA dehydrogenase was expressed as nmol of DMA min^{-1} mg $prot^{-1}$ and for TMA monooxygenase as nmol of TMA *N*-oxide min^{-1} mg $prot^{-1}$. Protein concentrations were measured by the Bradford (1976) method with bovine serum albumin (BSA, Sigma Chemical Co., SL, USA) used as a standard.

TMA, TMA *N*-Oxide and DMA determination by GC–MS

TMA and DMA were estimated by GC–MS with standards, as described previously (Liffourrena et al. 2008). The GC was equipped with an HP-1 column (crosslinked methyl silicone gum, 25 m × 0.32 mm × 0.17 µm film thickness).

The flow rate of the helium carrier gas was 30 ml min^{-1}. The detector and the injector temperatures were 280 and 250°C, respectively, the injection volume was 2 µl, and the column temperature was 50°C. For TMA *N*-oxide, the GC was equipped with a ZB-1 ms column (dimethylpolysiloxane phase, 30 m × 0.25 mm × 0.2 µm film thickness). The flow rate of the helium carrier gas was 30 ml min^{-1}. The detector and the injector temperatures were 280 and 250°C, respectively, and the injection volume was 2 µl. The column temperature was initially 40°C for 1 min; ramped at 6°C min^{-1} to 100°C for 10 min.

TMA concentration determination by fluorescence

The intracellular TMA concentration was determined by fluorescence using the fluorochrome 2′,3,4′,5,7-pentahydroxyflavone (morin reagent) with the addition of $AlCl_3$, as described previously (Liffourrena et al. 2008). The fluorescence of the Al^{3+}-morin complex was measured with a Fluoromax-3 spectrophotometer using an excitation wavelength of 440 nm and an emission wavelength of 494 nm. The concentration of TMA was calculated by calibration graphs constructed by plotting the fluorescence intensity of Al^{3+}-morin complex versus TMA concentration. The intracellular concentration of free TMA was calculated by taking the difference between the total TMA detected by GC–MS and TMA present in the complex Al^{3+}-TMA.

Cellular fraction volume

Cellular fraction volume was determined by the number of cells in the sample and the volume of one cell. Briefly, cultures were mixed to ensure a uniform suspension and used to count *P. putida* A ATCC 12633 in all growth phases. The cells were aseptically withdrawn and counted using a Neubauer's hemocytometric chamber. The volume of one cell, calculated as described by Roy and Packard (1998), was 0.8 μm^3 ($8 \cdot 10^{-10}$ µl).

Results and discussion

Previously, we showed that when *P. putida* A ATCC 12633 cells are grown on tetradecyltrimethylammonium bromide, TMA accumulated in the cell (0.56 mg l^{-1}; 1.5 mM based on cell volume); however, the addition of 0.1 mM $AlCl_3$ decreased the internal concentration of free intracellular TMA to approximately zero by the formation of an Al^{3+}-TMA complex. In addition, the tetradecyltrimethylammonium bromide was fully consumed without the accumulation of undesirable compounds (Liffourrena et al. 2008). In order to determine whether the accumulation of TMA and its negative effect on bacterial growth also occurs when the bacteria are grown on TMA, *P. putida* A ATCC 12633 was grown with 295 mg l^{-1} (5 mM) TMA as the sole carbon and nitrogen source. After 48 h, the cells entered a stationary phase (D_{660} = 0.37); at this point, the total consumption of TMA was 138 mg l^{-1} (47% of initial concentration), and the accumulation of intracellular TMA was 0.93 mg l^{-1} (2.5 mM based on cell volume). Considering that the average N content of proteins is about 15%, the ratio of TMA consumed (138 mg l^{-1}) and biomass produced (OD_{660} = 0.37; 6.75 mg protein l^{-1}) showed that the initial nitrogen content in the TMA consumed by *P. putida* A ATCC 12633 needed to reach the stationary phase was incorporated into the biomass and also accumulated inside the cells. When the culture was supplemented with 0.1 mM of $AlCl_3$, increased bacterial growth ($D_{660\ nm}$ = 0.72) and TMA consumption (63%; 194 mg l^{-1}) was observed, concomitant with the TMA intracellular concentration decreasing to 0.06 mg l^{-1} (0.16 mM based on cell volume). Based on these data, in concordance with that demonstrated when *P. putida* A ATCC 12633 are grown on tetradecyltrimethylammonium bromide (Liffourrena et al. 2008), the addition of Al_3Cl promotes the efficient utilization of TMA and the formation of an Al^{3+}-TMA complex, which is thought to enable the bacteria to overcome the damage caused by the TMA that has accumulated inside the cell.

Table 1 shows the enzymatic activities associated with the metabolism of TMA detected in cell-free extracts of *P. putida* A ATCC 12633 grown on tetradecyltrimethylammonium bromide or TMA as the only carbon and nitrogen source. Extracts from tetradecyltrimethylammonium bromide-grown cells had TMA monooxygenase activity that produces trimethylamine *N*-oxide. No TMA oxidation was observed in the absence of NADH or NADPH. The addition of NADH resulted in the highest TMA oxidation observed. In cells grown on tetradecyltrimethylammonium bromide, TMA is oxidized to dimethylamine by way of trimethylamine *N*-oxide, and a trimethylamine *N*-oxide demethylase activity was found. On the other hand, cell-free extracts from TMA-grown cells had TMA dehydrogenase activity. NADH or NADPH was not required for enzyme activity, and neither coenzyme stimulated the reaction. The resulting product, dimethylamine, is oxidized to methanal and methylamine by dimethylamine dehydrogenase (Table 1).

The enzymes catalyzing the first step of TMA oxidation metabolism detected in cells grown on tetradecyltrimethylammonium bromide or TMA were not detected in cells grown on glucose–NH_4Cl, suggesting that these enzymes were induced by their substrates. Furthermore, both enzyme activities, TMA monooxygenase and TMA dehydrogenase, were induced before growth began when glucose-grown *P. putida* were transferred to a

Table 1 Enzyme activities in cell-free extracts of *P. putida* A ATCC 12633 grown on trimethylamine and tetradecyltrimethylammonium bromide

Enzyme	Substrate		Enzyme activity in cells aerobically grown on	
			Tetradecyltrimethylammonium bromide	Trimethylamine
Trimethylamine monooxygenase[(a)]	Trimethylamine	+NADPH[(e)]	0.029 ± 0.0066	0
		−NADPH	0.004 ± 0.0009	0
Trimethylamine *N*-oxide demethylase[(c)]	Trimethylamine *N*-oxide	+NADPH	0.084 ± 0.009	0
		−NADPH	0.011 ± 0.005	0
Trimethylamine dehydrogenase[(b)]	Trimethylamine	+NADPH	0	0.031 ± 0.009
		−NADPH	0	0.033 ± 0.005
Dimethylamine dehydrogenase[(d)]	Dimethylamine	+NADPH	0	0.09 ± 0.01
		−NADPH	0	0.11 ± 0.01

Enzyme activities were determined with crude extracts as described in Materials and methods and expressed as: (a) nmol of TMA *N*-oxide min^{-1} mg $protein^{-1}$; (b) nmol of DMA min^{-1} mg $protein^{-1}$; (c) nmol of DMA min^{-1} mg $protein^{-1}$; (d) nmol of DMA min^{-1} mg $protein^{-1}$. (e) Indicates the presence (+) or the absence (−) of 0.5 mM NADPH

All experiments were repeated three times, and the standard deviations are shown

tetradecyltrimethylammonium bromide or TMA growth medium, suggesting that the enzymes are required to initiate growth on these substrates (not shown).

By varying the concentration of TMA in the standard assay, its apparent Km_{app} was 0.7 mM for TMA monooxygenase and 4.0 mM for TMA dehydrogenase. Both enzymes were not activated by $AlCl_3$, and only TMA dehydrogenase was inhibited by TMA from 1 mM (Fig. 1). The high TMA Km_{app} value detected for TMA dehydrogenase may be explained by taking into account that the enzyme fails to reach the V_{max} because it is inhibited by its substrate, which produces an inactive or less-effective enzyme. This inhibition may produce a shift in the Km_{app}, resulting in an increased value. However, the change in the affinity was accompanied by a change in the V_{max} that allowed the enzyme to maintain a similar catalytic efficiency (V_{max}/K_m) to that obtained for TMA monooxygenase (Table 2).

According to the above-mentioned results, *P. putida* A ATCC 12633 displayed different enzymes with different properties to initialize the oxidation of TMA depending on the substrate utilized for growth. In addition, we postulated that the low catalytic activities of these enzymes for TMA are responsible for TMA accumulation inside the cells when *P. putida* are grown on tetradecyltrimethylammonium bromide or TMA. Previously, we showed in cell-free extracts of *P. putida* A ATCC 12633 grown on tetradecyltrimethylammonium bromide that the TMA formation by the tetradecyltrimethylammonium monooxygenase activity was 4.9 ± 0.6 nmol min^{-1} mg $prot^{-1}$ ($n = 4$) (Liffourrena et al. 2008). With the same extracts, TMA was oxidized to dimethylamine by TMA monooxygenase, although at slower rate (0.029 ± 0.007 nmol min^{-1} mg $prot^{-1}$ ($n = 4$) (Table 1). Thus, the different catalytic activities for tetradecyltrimethylammonium monooxygenase and TMA monooxygenase

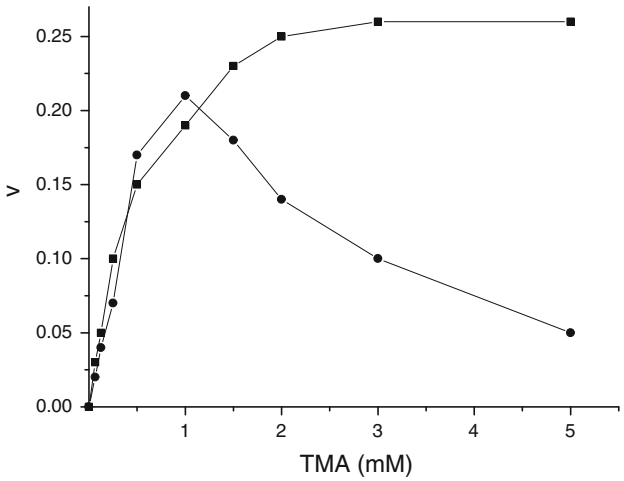

Fig. 1 Saturation curves of *P. putida* A ATCC 12633 TMA monooxygenase (*filled square*) and TMA dehydrogenase (*filled circle*) by TMA. The velocity (v) is expressed as: nmol of TMA *N*-oxide min^{-1} mg $protein^{-1}$ for TMA monooxygenase and nmol of DMA min^{-1} mg $protein^{-1}$ for TMA dehydrogenase. Data correspond to a representative experiment of three independently performed runs

Table 2 Kinetics constants of *P. putida* A ATCC 12633 TMA monooxygenase and TMA dehydrogenase

Enzyme	Km_{app} (mM)	V_{max}	V_{max}/Km_{app}
TMA monooxygenase	0.7	0.3[(a)]	0.5
TMA dehydrogenase	4.0	1.5[(b)]	0.4

The Km_{app} values were calculated from double-reciprocal plots from saturation curves by TMA. The V_{max} is expressed as: (a) nmol of TMA *N*-oxide min^{-1} mg $protein^{-1}$; (b) nmol of DMA min^{-1} mg $protein^{-1}$

are responsible for TMA accumulation. On the other hand, when *P. putida* A ATCC 12633 were grown on TMA as the sole carbon and nitrogen source, TMA was oxidized by a

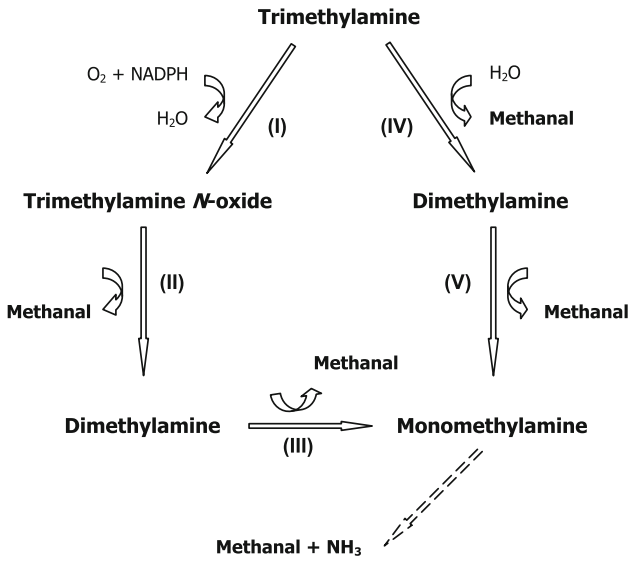

Fig. 2 Oxidation of trimethylamine by *P. putida* A ATCC 12633. The enzymes of the pathways are as follows: (I) trimethylamine monooxygenase; (II) trimethylamine *N*-oxide demethylase; (III) dimethylamine monooxygenase; (IV) trimethylamine dehydrogenase; (V) dimethylamine dehydrogenase

TMA dehydrogenase at a low rate (0.031 nmol min^{-1} mg $prot^{-1}$ ($n = 4$), and the enzyme was inhibited by its substrate (Fig. 1). Thus, the accumulation of TMA inside the cells may be explained by the low catalytic activities of this enzyme that was not sufficient to completely metabolize the TMA incorporated in the cell. Therefore, free intracellular TMA is accumulated (2.5 mM based in cell volume) and sufficient to inhibit the TMA dehydrogenase activity.

According to the above-mentioned results, *P. putida* A ATCC 12633 displayed different pathways to remove the N-trimethylated group of TMA depending on the initial substrate utilized for growth under aerobic conditions (Fig. 2). Two different enzymes can initiate the oxidation of TMA in aerobic metabolism, a TMA monooxygenase or a TMA dehydrogenase. This property could give this organism a selective advantage over others for the microbial removal of TMA from contaminated environments. In addition, we validated that the use of a Lewis acid such as $AlCl_3$ to sequester toxic TMA is a strategy to combat a stress situation that allows the bacteria to more efficiently utilize the N-trimethylated contaminant as a carbon, nitrogen and energy source.

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