NOTE

Presence of an Inducible Semicarbazide-Sensitive Amine Oxidase in *Mycobacterium* sp. Strain JC1 DSM 3803 Grown on Benzylamine

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Mycobacterium **sp. strain JC1 was capable of growth on benzylamine as a sole source of carbon and energy. The primary deamination of benzylamine was mediated by an inducible amine oxidase, which can also oxidize tyramine, histamine, and dopamine. Inhibitor study identified this enzyme as a copper-containing amine oxidase sensitive to semicarbazide.**

Keywords: benzylamine oxidase, copper-containing, *Mycobacterium* sp. JC1, semicarbazide-sensitive

Many bacteria have been shown to convert primary amines via an oxidative deamination step into products that can be utilized either as a source of carbon and/or energy, as a source of nitrogen, or both (Levering *et al.*, 1981; Hacisalihoglu *et al.*, 1997). Two types of enzymes, amine dehydrogenase (ADH) and amine oxidase (AO), are normally implicated in this form of oxidation. ADHs catalyze the oxidative deamination of primary amines to their corresponding aldehyde and ammonia, but AOs generate hydrogen peroxide (H_2O_2) in addition to aldehyde and ammonia. Although many bacteria harbor the enzymes for primary amine deamination, the growth of bacteria with some primary amines as a sole source of carbon and energy has not been shown to be successful in general, due to the toxicity of the aldehydes generated during the oxidation processes.

Mycobacterium sp. strain JC1 DSM 3803 is a facultatively chemolithotrophic bacterium having versatile metabolic activities, including a unique ability to utilize toxic compounds, such as carbon monoxide and methanol (Ro *et al.*, 1997 and 2000; Park *et al.*, 2003). Recently, we observed that this bacterium could be enriched on benzylamine. When *Mycobacterium* sp. strain JC1 grown on 0.5% (v/v) methanol (t_d = 7.0 h) was inoculated into a standard mineral base (SMB) medium (Kim and Hegeman, 1981) supple-

mented with benzylamine (0.5%, w/v) as a sole source of carbon and energy, no growth was observed for the first week. The bacterium, however, then began to grow and after being subjected to several consecutive cultures in benzylamine-containing medium, it grew quite rapidly (t_d = 7.7 h) (Fig. 1A). The observed retardation at the beginning of growth on benzylamine may be attributable to an adaptation period, during which either benzylamine-oxidizing enzyme(s) and/or resistance to the toxic compounds (e.g. benzylamine and benzaldehyde) is induced, as the bacterium exhibited a similar adaptation period when it was initially subjected to growth medium containing toxic compounds such as CO and methanol (Cho *et al.*, 1985; Ro *et al.*, 1997).

In order to characterize the natures of the key enzyme(s) relevant to benzylamine oxidation in *Mycobacterium* sp. strain JC1, the activities of ADH and AO were assayed using cell-free extracts prepared from cells grown on benzylamine. Cells growing on benzylamine were harvested at the time points indicated in Fig. 1A (depicted as H1, H2, and H3), washed once with 50 mM potassium phosphate buffer (pH 7.0, standard buffer), and disrupted by sonication. The suspensions were then centrifuged for 10 min at 16,100 × g, and the resulting supernatants were employed as cell-free extracts (H1-, H2, and H3-extracts, respectively). No ADH activity was detected in the cell-free extracts upon the performance of an enzyme activity assay using a phenazine methosulfate/2,6-dichlorophenolindophenol coupled system (Eady and

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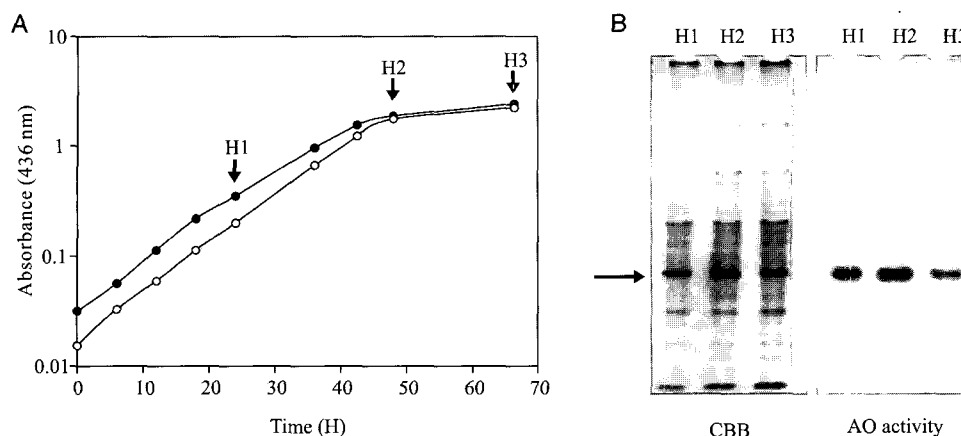(E-mail) ytaero@kku.ac.kr

Fig. 1. (A) Growth curves of *Mycobacterium* sp. JC1 growing on benzylamine and methanol. Cells were grown on 0.5% (w/v, -●-) benzylamine or 0.5% (v/v, -o-) methanol at 37°C and harvested at the indicated time points (H1, H2, and H3) for the preparation of the cell-free extracts. (B) CBB and amine oxidase (AO) activity stainings. After extracts (40 μg each) prepared from cells grown in SMB-benzylamine medium were subjected to non-denaturing PAGE (7.5% acrylamide), the proteins were stained by CBB or by AO activity using benzylamine as a substrate, as described in the text.

Large, 1968). The activity of AO was spectrophoto-metrically assayed at 25°C via the measuring of benzylamine-dependent benzaldehyde production (ϵ_{250} = 12,800 $M^{-1}cm^{-1}$) in a standard buffer at 250 nm, as previously described (Newton-Vinson *et al.*, 2000). One unit (U) of AO activity was defined as the quantity of enzyme required to oxidize 1 μmol benzylamine per minute. The H1-, H2-, and H3-extracts were determined to exhibit 0.048, 0.080, and 0.038 U of AO activities per mg of protein, respectively. AO activity staining was performed using a peroxidase-coupled system after electrophoresis on native polyacrylamide gel, as previously described (Lee *et al.*, 2002). Briefly, the gel after native PAGE was equilibrated twice for 20 min in potassium phosphate buffer (pH 7.5). Next, the gel was submerged and shaken for 5 min in a substrate solution [50 ml of potassium phosphate buffer (pH 7.5) containing 20 mg of benzylamine and 10 mg of 3-amino-9-ethylcarbazole]. Then, 200 μl of horseradish peroxidase (2 mg/ml) was added and the gel was gently shaken in darkness for 5-20 min, depending on band intensity. Comparisons between the two gels stained by Coomassie brilliant blue R-250 (CBB) and AO activity, after electrophoresis using Laemmli method (Laemmli, 1970) with 40 μg each of the proteins from the cell-free extracts, revealed that a major protein band apparent on CBB staining was highly specific for BAO activity (Fig. 1B). The enzyme activity was detected only in the benzylamine-grown cells, but not in the methanol- or methylamine-grown cells (data not shown).

In order to verify that *Mycobacterium* sp. strain JC1 harbors an inducible BAO, we conducted a growth substrate-switch experiment (Table 1). Cells

Table 1. Enzyme activities in cell-free extracts prepared from *Mycobacterium* sp. strain JC1 grown in substrate-switching media

Source of extracts	Sp. act[a]		
	BAO[b]	Catalase[c]	Peroxidase[d]
B cells[e]	0.089 (5.6)[f]	233.0 (3.2)	1.33 (7.8)
B-to-M cells[g]	0.016 (1.0)	72.5 (1.0)	0.17 (1.0)
M-to-B cells[h]	0.10 (6.3)	204.3 (2.8)	1.02 (6.0)

[a] Mean of three tests.
[b] Micromole of benzaldehyde produced per mg of protein per min.
[c] Micromole of H_2O_2 disappeared per mg of protein per min.
[d] Micromole of *o*-dianisidine oxidized per mg of protein per min.
[e] Cells harvested at the late exponential phase during growth in SMB medium supplemented with benzylamine (0.5%, w/v).
[f] Number in parenthesis means relative ratio of the enzyme activity when the activity in cell extracts from B-to-M cells was set as 1.0.
[g] Cells harvested at the late exponential phase during growth on methanol (0.5%, v/v). The origin of the inoculum was the B cells.
[h] Cells harvested at the late exponential phase during growth on benzylamine (0.5%, w/v). The origin of the inoculum was the B-to-M cells.

grown on benzylamine (B cells) were inoculated into a SMB-methanol medium, and allowed to grow into late exponential phase. The methanol-grown cells (B-to-M cells) were then transferred to a SMB-benzylamine medium, and these cells were also grown to late exponential phase (M-to-B cells). Each of these groups of cells was then harvested at the late exponential phase and their cell-free extracts were prepared as described above. It was determined that the cellular extracts prepared from the B-to-M cells exhibited 0.016 U of BAO activity, which was much

lower than that of the B cells (0.089 U) employed as an inoculum. The BAO activity was, however, shown to have recovered to original levels (0.1 U) in the M-to-B cells (Table 1). The BAO activity detected in the B-to M cells may represent the residual activity present in the B cells, since the cells that had been fully adapted in methanol-containing media exhibited no BAO activity (data not shown).

Next, we determined catalase and peroxidase activities in the cell-free extracts of *Mycobacterium* sp. strain JC1 via the measurements of the rates of H_2O_2 decomposition ($\varepsilon_{240} = 43.6$ $M^{-1}cm^{-1}$) and oxidation of o-dianisidine ($\varepsilon_{460} = 11.3$ $mM^{-1}cm^{-1}$) in the presence of 12.5 mM H_2O_2, respectively (Hochman and Goldberg. 1991; Ro *et al.*, 2003), because catalase activity in *Escherichia coli* K12 strain harboring an AO was significantly increased when it was grown on 2-phenylethylamine (PEA) (Cooper *et al.*, 1992) and this increase in catalase activity was presumed to ensure the removal of H_2O_2, which is a product from the exclusive result of an inducible AO activity in the bacterium (Parrott *et al.*, 1987). Our results revealed that catalase and peroxidase activities in benzylamine-grown cells (B cells and M-to-B cells) were determined to be 3- and 7-fold higher, on average, than those in B-to-M cells, respectively (Table 1). All of these results appear to bolster the notion that *Mycobacterium* sp. strain JC1 does express an inducible BAO, which is involved in the utilization of benzylamine as a sole source of carbon and energy.

In an effort to determine the substrate and inhibitor specificities of *Mycobacterium* sp. strain JC1 BAO, the cell-free extract (40 μg protein) from B cells was separated on 7.5% native polyacrylamide gels, and the gel-strips were subjected to AO activity staining in the presence of different substrates (20 mg of each substrate in 50 ml of the substrate solution) and inhibitors (2 mM of each inhibitor in the substrate solution). The results of AO activity staining showed that benzylamine (BA) and tyramine (TR) were the optimal substrates for *Mycobacterium* sp. strain JC1 BAO (Fig. 2A). Relatively low enzyme activities were recorded with dopamine (DP) and histamine (HT), but not with norepinephrine (NE) and methylamine (MA) (Fig. 2A). When the effects of semicarbazide (SC) and cuprizone (CZ) were tested in the activity staining as a carbonyl group reagent and a copper-chelating reagent, respectively, both reagents exerted profound inhibitory effects on the BAO activity (Fig. 2B). The BAO activity was also affected to a slight degree by o-phenylenediamine (PD) and 1,4-diamono-2-butanone (DB). The slight discrepancy of the migration rate of the BAO activity-stained protein shown in Fig. 2 (DP, NE, and MA lanes for substrate specificity; SC, CZ, and PD lanes for inhibitor specificity) was attributable to the utilization of two different sets of gels that were separated individually. The results showed that the *Mycobacterium* sp. strain JC1 BAO belongs to a class of copper-containing amine oxidases sensitive to semicarbazide (EC 1.4.3.6).

Many bacteria have been shown to utilize aromatic amines as sole sources of carbon and energy, and have also been demonstrated to employ different types of enzymes. *Pseudomonas aeruginosa* PAO1 was able to utilize PEA, tyramine, dopamine, octopamine, and norepinephrine, and the initial catabolism was shown to be mediated by a membrane-bound tyramine dehydrogenase (Cuskey *et al.*, 1987). *Alcaligenes*
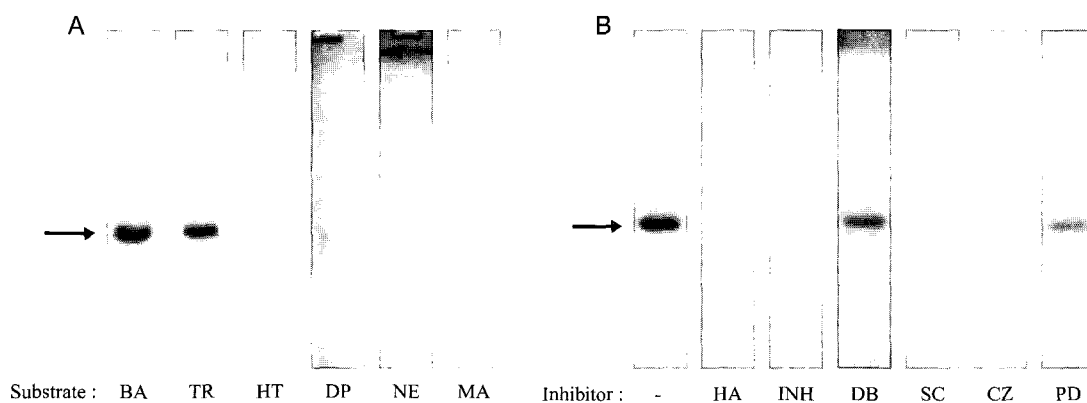

A   B

Substrate: BA   TR   HT   DP   NE   MA   Inhibitor: -   HA   INH   DB   SC   CZ   PD

Fig. 2. AO activity staining. (A) The substrate specificity of *Mycobacterium* sp. strain JC1 BAO was tested via AO activity staining with gel strips prepared after the non-denaturing PAGE (7.5% acrylamide) of extracts (40 μg each) from benzylamine-grown cells, as described in the text. The tested substrates (20 mg each per 50 ml substrate solution) were as follows: benzylamine (BA), tyramine (TR), histamine (HT), dopamine (DP), norepinephrine (NE), and methylamine (MA). (B) The effects of inhibitors on BAO activity. The inhibitor specificity of BAO was assayed via AO activity staining with gel strips prepared as described above in the presence of benzylamine (20 mg in 50 ml substrate solution) as a substrate, and different inhibitor candidates (2 mM each) including hydroxylamine (HA), isoniazid (INH), 1,4-diamono-2-butanone (DB), semicarbazide (SC), cuprizone (CZ), and o-phenylenediamine (PD).

faecalis and A. xylosoxidans grown on a PEA medium harbored an aromatic ADH with a quinone cofactor (Chistoserdov, 2001; Kondo et al., 2004). Klebsiella oxytoca, which possesses a soluble copper-topaquinone (TPQ) amine oxidase sensitive to semicarbazide, was capable of utilizing PEA and tyramine (Hacisalihoglu et al., 1997). Similarly, Escherichia coli K-12 and Arthrobacter golbiformis, when grown on PEA, were shown to have copper-containing amine oxidases (EC 1.4.3.6) (Cooper et al., 1992; Freeman et al., 1996). Flavin-containing amine oxidases (EC 1.4.3.4) were detected in several bacteria grown on or induced by tyramine, including Klebsiella aerogenes (Okamura et al., 1976), Salmonella typhimurium (Murooka et al., 1979), and Micrococcus luteus (Roh et al., 2000).

There have been only two reports, thus far, on the utilization of benzylamine by bacteria as a sole carbon and energy source, thereby suggesting that the benzylamine-utilizing bacteria had adopted some dehydrogenase-type enzymes. Pseudomonas putida (Durham and Perry, 1978) and Paracoccus denitrificans IFO 12442 (Takagi et al., 1999), when grown on benzylamine, were found to have a heme-containing, semicarbazide-sensitive ADH and a quinohemoprotein ADH, respectively.

In conclusion, the findings of this study appear to indicate that the primary deamination of benzylamine in Mycobacterium sp. JC1 DSM 3803 is catalyzed by a novel inducible copper-containing AO sensitive to semicarbazide. This study, to the best of our knowledge, is the first report regarding the AO-mediated bacterial utilization of benzylamine as a growth substrate.

Acknowledgment

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