

A 60 kb Plasmid in *mycobacterium* (pip^+) Involved in Degradation of Antihelminthic Drug (Piperazine)

Giti Emtiazi*, Mohammad Reza Norri Dalooi and Mona Hosseini

Dept. of Biology, Isfahan University, Isfahan, Iran.

ABSTRACT

The biodegradation of secondary amines is particularly important due to their propensity for conversion either chemically or microbiologically to N-nitrosamines which are potent carcinogens. In this research, a weak Gram-positive organism was isolated from river and identified as *Mycobacterium*. This *Mycobacterium* grows slowly and effectively utilizes piperazine as the sole source of organic, carbon, nitrogen and energy. Also, it has one 60-kb plasmid on gel agarose which involves in piperazine degradation; however the mutant strain which were obtained by subculturing in nutrient broth (pip^-) has lost this plasmid. This might suggest that the ability to degrade piperazine are plasmid encoded. *Iran. Biomed. J. 5 (4): 113-116, 2001*

Keywords: *Mycobacterium*, Plasmid, Piperazine

INTRODUCTION

The biodegradation of some amines such as morpholine has been focused more than the other amines. Previous research on morpholine has not answered yet the question of why this structurally simple compound is relatively difficult to degrade and why it is in general only degraded by a very limited range of bacteria. Piperazine and morpholine are two similar carcinogens. Although the conversion of piperazine microorganism is of interest, it has attracted little attention [1].

Piperazine alternatively known as hexahydropyrazine is a cyclic secondary amine. There has been very little research on the biodegradation of the piperazine; however Dmitrenko *et al.* [1] and Emtiazi *et al.* [2] showed the biodegradation of this compound by *Arthrobacter* and *Mycobacterium*. The high resistance of piperazine to biodegradation is thought to be related to the presence of two imino groups in its molecule [3]. Whaterhouse *et al.* [4] showed that *Mycobacterium* mor G. had 4 plasmids and found evidence that the unstable morpholine degrading phenotype is plasmid encoded. In the present work, we show that piperazine degrading phenotype may be plasmid encoded. This plasmid helps degradation of carcinogenic amine residues in colon and environment.

MATERIALS AND METHODS

Isolation media. *Mycobacterium* isolated from growth media contained mineral salts of the following concentrations (g^{-1}). Iron and Magnesium salts were made up as 10% w/v $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1% w/v FeCl_3 . After autoclaving separately, $6\text{H}_2\text{O}$ was added to basal medium. Enrichment media contained 10 mM piperazine plus mineral salts, pH 7.0 in distilled water.

Die away test. Piperazine was assayed by modified method of Stevens and Skov [5]. This method involves the coupling of secondary amines to sodium 1:2 naphtho-quinone 4-sulphonate to produce an orange colour. This colour can be assayed spectrophotometrically by measuring its absorbance at 480 nm against a reagent blank. The sample containing amines (0.1-1 mM) was made up of 10 ml total volume in buffer (KH_2PO_4 , 2 g/L^{-1} pH 7.0). To this was added 0.04 ml of sodium 1 (3% w/v), 2-naphtho quinone-4 sulphonic acid solution, immediately followed by 0.1 ml of 1 M NaOH. The sample was mixed thoroughly by vortexing. After 20 minutes, the absorbance was read at 480 nm with a spectrophotometer against a similarly treated reagent blank. The growth rate of piperazine degrading bacterium was determined turbidimetrically by measurement of the change rate in piperazine concentration. Generation times were

*Corresponding Author; Tel. (98-311) 668 3480, E-mail: emtiazi@sci.ui.ac.ir.

calculated from semi-logarithm plots of turbidity or change of piperazine concentration against time.

Ammonia assay. Ammonia was estimated using Nessler's reagent [1], the resulting yellow colour was measured similarly treated at 410 nm against a reagent blank. This method involves the coupling of ammonium to Nessler's reagent to produce a yellow colour. This colour can be assayed spectrophotometrically by measuring its absorbance at 410 nm against a reagent blank. The supernatant was added to 10 ml distilled water. To this, 0.5 ml of Nessler's reagent was added. The sample was mixed thoroughly by vortexing. After 10 minutes, the absorbance was read at 410 nm with a spectrophotometer against a similarly treated reagent blank [1].

Method for detection of spontaneous loss of piperazine degradative ability in pure cultures.

The organism under test was grown in liquid piperazine medium to late exponential phase (starter culture). This culture (0.1 or 1.0 ml) was used to inoculate 100 ml of nutrient broth + Tween 80 (culture A). Then, it was incubated at 27°C with shaking (100 rpm).

The starter culture was diluted (10-fold series) in phosphate buffer; the first dilution was sonicated to disperse clumped growth. Appropriate dilutions were then used to inoculate nutrient agar in triplicate. Plates were incubated at 27°C until the colonies were visible. The colonies were then counted and 100 colonies were picked up and patched out firstly onto piperazine agar and then onto nutrient agar. After incubation at 27°C, the plates were examined. The strains that grew on the nutrient agar but failed to grow on the piperazine agar were counted, subcultured on nutrient agar and piperazine mineral salt agar and then tested in liquid piperazine medium to confirm that they were non-degraders.

When culture A reached stationary phase it was subcultured (0.1 or 1.0 ml) into nutrient broth + Tween 80 (culture B). The percentage of the cells in the culture (A) that was no longer capable of piperazine degradation was determined as above. This was repeated over several subcultures.

Reversion experiment method. Pip⁻ variants (can not use piperazine) of wild bacterial strains (were formerly able to degrade piperazine) were tested for reversion to pip⁺ phenotype (able to use piperazine), by serially subculturing in piperazine. Before

subculturing, a sample of culture was centrifuged, washed in sterile phosphate buffer, diluted and used to inoculate piperazine mineral salts agar. After the incubation at 27°C for at least 14 days, the piperazine mineral salt plates were examined for any colonies showing good growth. These possible revertants were subcultured onto piperazine mineral salts agar and nutrient agar and then subcultured into liquid piperazine to test for piperazine degradation and confirm reversion.

Plasmid extraction. Mycobacterial cell walls are not normally lysed by lysozyme and sodium dodecyl sulphate and therefore cultures must receive special treatment to make them susceptible. Cultures of *Mycobacterium* pip⁺ were made susceptible to lysis by the addition of glycine (1.2% w/v final concentration) to mid exponential phase cultures (glycine solution was sterilized by filtration). Cultures were then incubated for a further 40 h until stationary phase and harvested by minifuge. The plasmids DNA were isolated by the method of Bennett *et al.* [6]. Preparations of plasmids DNA were analysed by submerged, horizontal agarose gel electrophoresis.

RESULTS AND DISCUSSION

A pure isolate that grew on piperazine as a sole source of carbon, nitrogen and energy was obtained. The isolate was motile, acid fast rod with catalase and arylsulfatase positive and had positive growth on Tween 80 as the only source of energy. This isolate was identified as *Mycobacterium* and grew well on 10 mM piperazine mineral salts. The isolate could use piperidine and pyrrolidine but not morpholine for growth.

The biodegradation of piperazine was confirmed by die away test. Isolated *mycobacterium* grew on 10 mM piperazine with lag of 50 h. Piperazine degradation and production of ammonium are shown in Figure 1.

Experiments to study the spontaneous loose of the piperazine degradative phenotype were carried out on pip⁺. The results of these experiments are shown in Figure 2. After two subcultures, 15% of pip⁻ isolates were obtained for *Mycobacterium*. To make sure that the mutants obtained are piperazine negative (and not simply auxotrophs, which are incapable of growth on mineral salts media), pip⁻ isolate was subcultured onto glucose mineral salt agar and all had good growth on this medium. Pip⁻

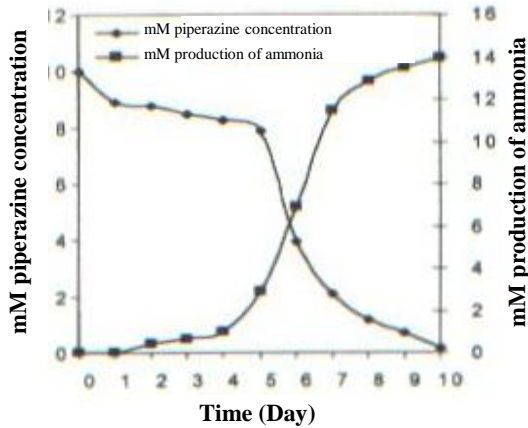


Fig. 1. Biodegradation of piperazine and production of ammonia by the isolate. Piperazine has two amine groups. From 10 mM piperazine 14 mM ammonia is released.

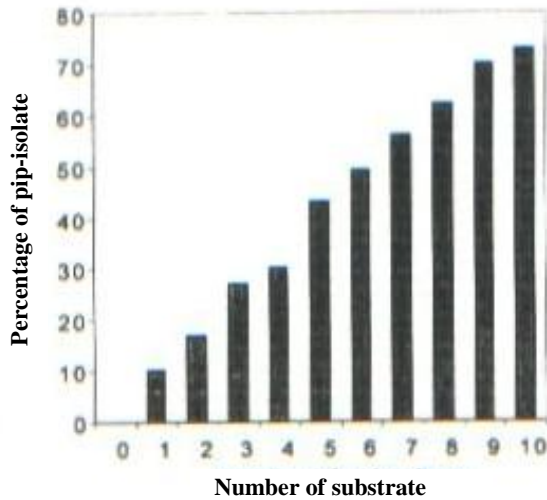


Fig. 2. Rate of loss of piperazine degradation ability during growth of the isolate in nutrient-broth. The height of each bar represents the percentage of the mutant isolates. The mutant was obtained after subculturing the wild on nutrient-broth. These mutants lost the ability to degrade piperazine.

tested to see if any revertants could be detected after serial subcultural in nutrient broth. They had not reverted to the parent piperazine degrading phenotype.

The isolated *Mycobacterium* was cultured at 27°C in nutrient broth containing glycine. The cells were harvested by centrifugation and disrupted chemically to give plasmid extracts which were then examined by agarose gel electrophoresis Figure 3. The pip⁺ had one 60-kb plasmid while none of pip⁻ was shown to have any plasmid. Plasmid was first isolated from *Mycobacterium* by

Crawford and Bates in 1979 [7]. Antibiotic resistance plasmid was observed among clinical *Mycobacterium* isolates [8]. Waterhouse [5] found 4 plasmids in morpholine degrader *Mycobacterium* which could not degrade piperazine. In this report, we found one 60-kb plasmid in *Mycobacterium* degrading piperazine. However, the pip⁻ phenotype did not have any plasmid and could not degrade piperazine. Danofloxacin is also a new synthetic fluoroquinolone antibacterial agent with piperazine ring, under development for exclusive use in veterinary medicine [9]. Such use could lead to deposition of low levels of danofloxacin residues in environment in manure from treated livestock. The photochemical degradation of quinolones and danofloxacin gives more stable compounds [10]; therefore, the degradation of piperazine and related carcinogenic compounds are very important. *Mycobacterium* that degrade the residues of this compound is slow growing microorganism. However, this 60-kb plasmid that is involved in degradation of antihelminthic drug could lead to clean up of low levels of piperazine residues in colon and environment.

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