

Isolation and molecular identification of biodegrading Mycobacteria from water supplies of Iranian hospitals

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ABSTRACT

Molecular isolation of biodegradable mycobacteria from water supplies of Iranian hospitals Background and objectives: Some microorganisms, mainly members of two genera including *Pseudomonas* and *Mycobacterium*, were found to be capable of transforming and degrading of polluting agents. We herein report the isolation of a few mycobacteria with the ability to biodegrade organic and inorganic compounds from water supplies of Iranian hospitals.

Materials and methods: The water samples were collected from hospital water supplies. Isolation processes were done according to standard methods. The colonies were subcultured on Löwenstein-Jensen medium to obtain a pure culture. The identification and characterization of the isolates were based on conventional and molecular methods including direct sequence analysis of almost full length of 16S rRNA gene.

Results: The almost complete 16S rRNA gene sequences of the studied strains revealed that the isolates WP16, AW18-1 and AW18-3 were identified as *M. fredriksbergense*, AW18-2 as *M. austroafricanum*, AW27-2 as *M. obuense* and AW27-6 as *M. phocaicum*. The relationship between our isolates and standard strains of *Mycobacterium* was supported by a phylogenetic tree of 16S rRNA gene.

Conclusion: In the current study we were able to isolate and characterize six mycobacteria with capability of transforming and degrading polluting agents from Iranian hospital environments. This is indeed the first report on isolation and characterization of mycobacteria with degrading capability of polluting agents from Iranian hospitals. It can be considered as a pioneer study to open up a new horizon in the study of microbial diversity in Iran with an objective-based and applied approach to tackle environmental challenges.

Keywords: Biodegradation, *Mycobacterium*, 16SrRNA gene, water supply

INTRODUCTION

Biodegradation is a natural process by which microorganisms break down organic chemicals to simpler substances (1). Non degradable compounds

are often present in environment from industries such as pesticides, oil refining, coal gasification, dye and petrochemicals, pharmaceutical and resin manufacturing plants. The ability of a number of microorganisms to degrade a vast array of pollutants that have previously been considered non degradable has therefore been studied to provide efficient strains for biodegradation processes (2). Many isolated bacterial and fungal species, mainly members of two genera including *Pseudomonas* and *Mycobacterium* have been reported to be capable of transforming and biodegrading effectively polluting agents (3, 4).

Biodegradation requires specified microorganisms

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and operation conditions to accelerate the natural biodegradation rates by overcoming limiting factors. For example, the biodegradation of polluted cold fields requires cold-adapted microorganisms (5, 6). Degradation of organic compounds in the natural environments is often the result of a community-interacting microbial population. Therefore, a synergistic effect is expected in the biodegradation by simultaneous use of mixed biodegradable bacteria (7).

Although non degradable compound has been shown to be biodegradable, there is minimal information about the organisms capable of this degradation and little is understood of the mechanisms or biochemistry involved. The focus of this study was to isolate mycobacteria with the ability to biodegrade organic and inorganic compounds from water supplies of Iranian hospitals.

MATERIALS AND METHODS

Sampling. The water samples were collected between September 2011 and December 2012 as part of a more comprehensive project to analyze microbial diversity of hospital environments. The samples were processed according to the described methods (8). In short, each 1,000-mL sample was transported at 4°C to the laboratory and processed within 24 hours. They were first decontaminated with cetylpyridinium chloride (0.005%, 20 min) and filtered by vacuum through cellulose nitrate filters (0.45 µm, Sartorius AG, Gottingen, Germany). The filters were rinsed and macerated in 3 mL sterile distilled water. Aliquots (0.1 mL) transferred in duplicate to Löwenstein-Jensen (LJ) and Sauton's media, and incubated at "25°C, 32°C, 37°C and 42°C" in an atmosphere of 5% CO₂.

Microbiological analysis. The Iranian isolates coded " WP16, AW18-1, AW18-2, AW18-3, AW27-2 and AW27-6" were subjected to identification by conventional phenotypic tests including Ziehl-Neelsen (ZN) staining and standard biochemical assays, i.e., growth rate, growth at 25°C, 32°C, 37°C and 42°C on LJ medium, pigment production, semi-quantitative and heat-stable (68°C) catalase production, nitrate reduction, tellurite reduction, urease activity, tween opacity, pyrazinamidase, niacin accumulation, and tolerance to 5% NaCl, tests (9).

Molecular identification. Chromosomal DNA

was extracted using a modified method of Pitcher *et al.* (10). In brief, after thermal inactivation, the bacterial cells were lysed with lysozyme and digested with proteinase K in the presence of sodium dodecyl sulfate (SDS) and treated with guanidine-sarcosyl solution. The DNA was purified by phenol chloroform-isoamyl alcohol and precipitated with isopropanol. The precipitate was washed in 70% ethanol, dehydrated and dissolved in 100 µl of sterile double-distilled water.

The *Mycobacterium*-specific PCR amplification protocol targeting a 228-bp region of the 65-kDa heat shock protein (*hsp*) gene carried out according to optimal PCR amplification conditions recommended by Khan and Yadav (11).

Sequencing based identification. The amplification and direct sequence analysis of almost full length of 16S rRNA gene was carried out as described previously (12). Sequencing was performed by Bioneer Company (South Korea) with an ABI 3100 genetic analyzer. The obtained sequences were aligned manually with all existing sequences of the rapidly growing mycobacteria retrieved from GenBank™ database and compared with the relevant sequences and analysed using the jPhydit program (13).

Nucleotide sequence accession numbers. The GenBank accession number for the 16S rRNA gene of *M. frederiksbergense* isolates, namely, WP16, and AW18-1, *M. austroafricanum* isolate AW18-3, *M. obuense* isolate AW27-2 and *M. phocaicum* isolate AW27-6 determined in this work are JX566890.1, KF019696, KF019697, KF028777 and KF019699, respectively.

RESULTS

The isolates WP16, AW18-1, AW18-2, AW27-3, and AW27-6 were recovered from water samples collected from different hospitals. The strain WP16 was isolated from the drinking water dispenser in the emergency department of a general hospital in suburb, Isfahan. The strains AW18-1, AW18-3 were isolated from tap water of a general hospital in Isfahan and the strain AW27-2, AW27-6 were isolated from water running of the shower in a university hospital in Isfahan.

The recorded temperature for the water samples

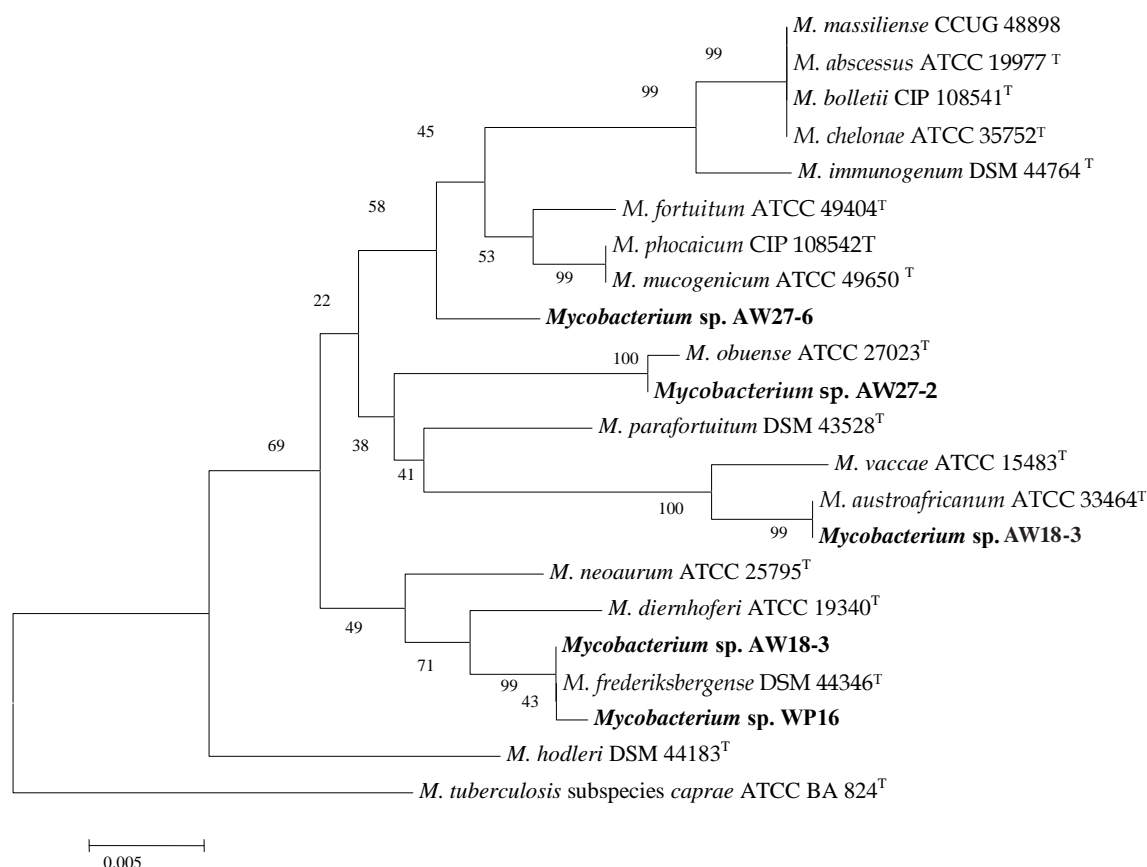


Fig. 1. 16S rRNA sequence based phylogenetic tree for Iranian isolates of biodegrading mycobacteria and closely related reference species of rapidly growing mycobacteria by using the neighbor-joining method. The figure at each node represent bootstrapping values. The tree was rooted with *M. tuberculosis*.

from which the strains, i.e., WP16, AW18-1, AW18-2, AW18-3, AW27-2, AW27-6 were isolated recorded as 15°C, 28°C, 14°C, 13°C, 16°C and 17°C. The corresponding pH of the water samples were 7, 6.6, 6.8, 6.9, pH: 7.9 and 8.1, respectively.

Based on phenotypic characteristics including growth rate and pigmentation all Iranian isolates recovered from water samples categorized as the rapidly growing Runyon Group IV mycobacteria. The phenotypic and biochemical features of Iranian isolates are detailed in Table 1.

The genus specific PCR amplification recommended by Khan and Yadav reliably produced a 228-bp amplicon of the *hsp 65* gene that confirmed belonging of the isolates to the genus *Mycobacterium* (11). Moreover, the presence of the short helix 18 in the 16S rRNA gene of strains represented the typical molecular signature of rapidly growing mycobacteria (14).

The almost complete 16S rRNA gene sequences of the studied strains WP16, AW18-1 and AW18-3 showed 99.4%, similarities with the type strain of *M.*

frederiksbergense 44346^T. The strain AW18-2 showed 99.9% similarities with *M. austroafricanum* ATCC 33464^T. The strain AW27-2 showed 99.6% similarities with *M. obuense* ATCC 27023^T and the strain AW27-6 showed 99.4% similarities with *M. phocaicum* CIP 108542^T. The relationship between our isolates and standard strains of mycobacteria was supported by a phylogenetic tree of 16S rRNA gene (Fig. 1).

DISCUSSION

The increasing irregular release of organic pollutants to environment by industries and other chemical materials causes many health-related problems (15, 16). It is now realized that microbial metabolites can provide a safer, more efficient, and less expensive alternative method rather than physicochemical methods for pollution abatement (17). Biodegradation is a cost-effective method that can be used for degradation of toxic compounds into innocuous products.

Actinomycetes, constitutes a phylogenetically coherent group such as the genera *Corynebacterium*, *Rhodococcus*, *Nocardia*, *Gordonia*, and *Mycobacterium* (18, 19). These organisms harbor many degraders of environmental pollutants: members of *Rhodococcus* are able to degrade hydrocarbons, chlorophenols, polychlorinated biphenyls and sulfonated azo dyes (20). Mycobacteria degrade diverse polycyclic aromatic hydrocarbons (PAH) and polychlorophenols (21-23), while *Gordoniae* metabolize alkanes (24). Although most species of the above genera are rapidly growers, mycobacteria are usually considered slow growers. As a result, they are excellent survivors of unfavorable conditions since they can compete successfully with fast growing strains, e.g. *Pseudomonas* and related bacteria, which are also known to metabolize pollutants such as aromatic compounds in the environment (25).

It is clear that chemotaxis is a selective advantage to the degradative mycobacteria for guiding them to sense and locate pollutants that are present in the environment (20, 26).

In the current study we report on isolation and molecular characterization of a few biodegrading mycobacteria from Iranian hospital water supplies. They are potentially capable of successful removal of many dangerous compounds from the soil, water and environment by implantation of them in the bioremediation process and source of contamination.

Consistent with the phenotypic features, the molecular tests used in the current study provided evidences that the isolates WP16, AW18-1, AW18-3, AW18-2, AW27-2 and AW27-6 belong to mycobacteria that is, having *Mycobacterium* genus specific genetic marker, representing the short helix signature of rapidly growing mycobacteria, and showing the highest similarities in terms of 16S RNA sequence with those of type species of related mycobacteria.

The isolates WP16, AW18-1 and AW18-3 were identified as *M. frederiksbergense*, *M. frederiksbergense* is a PAHs bacterium which was first isolated and characterized in 2001 from coal tar contaminated soil in Denmark (27). The species is capable to degrade phenanthrene, fluoranthene and pyrene.

The isolate AW18-2 was identified as *M. austroafricanum*. This species has shown an extended capacity for the degradation of various hydrocarbons

Table 1. Biochemical characterizations of biodegradable mycobacteria isolated from Iranian hospital water systems.

| Strain | Growth rate | Pigmentation | Runyon group | Growth at (°C) | | | | Semi-quantitative catalase production* | Heat-stable (68°C) catalase | Nitrate reduction | Tellurite reduction | Urease activity | Tween opacity | Pyrazinamidase | Niacin accumulation | Tolerance to 5% NaCl |
|--------|-------------|----------------|--------------|----------------|-----|-----|-----|--|-----------------------------|-------------------|---------------------|-----------------|---------------|----------------|---------------------|----------------------|
| | | | | 25° | 32° | 37° | 42° | | | | | | | | | |
| WP16 | <7 days | Scotochromogen | IV | + | + | + | - | + | - | + | - | + | - | - | + | |
| AW18-1 | <7 days | Scotochromogen | IV | + | + | + | - | + | + | - | + | + | - | - | + | |
| AW18-2 | <7 days | Scotochromogen | IV | + | + | + | - | + | - | - | - | - | - | - | + | |
| AW18-3 | <7 days | Scotochromogen | IV | + | + | + | - | + | + | - | + | + | - | - | + | |
| AW27-2 | <7 days | Scotochromogen | IV | + | + | + | - | + | - | + | - | + | - | - | + | |
| AW27-6 | <7 days | Nonchromogen | IV | + | + | + | - | + | - | + | - | + | + | + | - | |

* : < 45 mm indicates as negative and >45 mm as positive.
C: Celsius

classes including n-alkanes and other isoalkanes, as well cyclic alkanes and monoaromatic hydrocarbons. For instance, a new *M. austroafricanum* strain, IFP 2015 was reported to grow on methyl tert-butyl ether (MTBE) as a sole carbon source and was isolated from an MTBE-degrading microcosm inoculated with drain water of an MTBE-supplemented gasoline storage tank (28).

The isolate AW27-2 was identified as *M. obuense*. This organism is a rapidly growing scotochromogenic *Mycobacterium* with capacity to degrade Methoxychlor ethane which is an organochlorine insecticide (29, 30).

The isolate AW27-6 was identified as *M. phocaicum*. This organism was firstly isolated and characterized in 2006 from bronchial aspirate of a patient with chronic pneumonia (31). In a recent study the capability of a fluoroglycofen ethyl degrading strain of *M. phocaicum*, namely, MBWY-1, was reported. This strain was isolated from the soil of an herbicide factory (32).

There is currently substantial interest in the application of microbial biotechnology to degrade organic pollutants in the environment. However, one major factor determining the success of bioremediation is the availability of microorganisms which produce the catabolic enzymes required to degrade the chemicals of interest.

In conclusion our study dealing with isolation and characterization of six rare and difficult to isolate and cultivate *Mycobacterium* species from Iranian hospital environment with biodegrading features indicates that mycobacteria isolated from unfavorable environments, such as those that may be encountered in hospitals' water and soil possess biochemical and ecological capacity to degrade organic pollutants and to decrease the risk associated with chemicals. This affirms the idea that despite being abundant in environment, mycobacteria have been simply unnoticed for such significant usage. Indeed, there is an untapped potential with regard to biodegrading actinomycetes and in particular mycobacteria that has yet to be discovered and exploited in bioremediation of hazardous chemicals.

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