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The correlation of Mycolic acid production by a toluene degrading *Mycobacterium* in the presence of cholesterol

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Abstract

Introduction: Cell wall mycolic acids (MA) from *Mycobacterium tuberculosis* are presented as antigens that can be used to detect antibodies as surrogate markers of active Tuberculosis (TB) disease, even in HIV coinfected patients. The use of the complex mixtures of natural MA is complicated by apparent antibody cross-reactivity with cholesterol. Mycolic acid is not only related to diagnosis of TB disease but also similar structure of mycolic acid in saprophyte strains are excellent candidate for drug delivery especially for nasal spray. The aim of this work was to investigate the effect of cholesterol on mycolic acid synthesis and potential of saprophyte mycolic acid to drug delivery.

Materials and methods: Toluene enrichment medium was used for isolation of mycolic acid producing bacteria. A fast growing, acid fast bacterium was identified by PCR reaction and the related sequence of 16S rRNA gene was deposited in the NCBI Genbank with accession number jn64433. Production of mycolic acid was investigated by high-performance liquid chromatography (HPLC) in different media.

Results: Here the effect of cholesterol on biosynthesis of mycolic acids by saprophyte *Mycobacterium* isolated from marine water is was reported. HPLC analyses showed the mycolic acid extracted from saprophyte. *Mycobacterium* had one, early, cluster of peaks, like *Mycobacterium tuberculosis* and *Mobacterium bovis.*This strain is a fast growing bacterium and cholesterol might promote longer fatty acid production.

Discussion and conclusion: The similarity between mycolic acid from isolated and patogenic mycobacteria offers that mycolic acid obtained from saprophyte *Mycobacterium* can be useful as a drug carrier. Addition of egg yolk to media induced longer fatty acid production by isolating that is suitable for delivery of drugs into the macrophage.

Key words: *Mycobacterium*, Toluene, Mycolic acid, Cholesterol

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Introduction

Mycolic acids are found in species of *Corynebacterium*, *Nocardia*, *Rhodococcus*, and *Mycobacterium*. These fatty acids have been isolated and used for *Mycobacterium tuberculosis* identification (1). Their common structure was published in 1950 and shown to be formed of a hydroxy and alkyl branched chain. The species of *Mycobacterium tuberculosis* and *M.leprae* are the most known species of *Mycobacterium* genus which are the causative agents tuberculosis and leprosy. Nevertheless most of other Mycobacteria, found in the environment, are non pathogenic and fast growing strains (2).

Mycolic acids present in the outer cell membrane of the *Mycobacteria* represent up to %40 of cell wall dry mass. Hydrophobic compounds architecture of the mycolic acid plays an important role in cell structure and is essential for Mycobacterial growth.

Mycolic acid covalently bonded to the arabinogalactan. Arabinogalactan-mycolate linked with the other component in the cell wall to form a thick layer with an extremely low fluidity. This barrier protects the Mycobacteria from hydrophilic agents (3). Mycolic acid is a key component for the survival of *M.tuberculosis* for example low permeability of MA doesn't allow to hydrophobic drugs such as general antibiotics to inter the cell. *M. tuberculosis* grows inside the macrophages, so this bacterium is responsible for substantial health problems by effect on immune system (4, 5). Each species of Mycobacteria has a specific MA as a mixture of structurally related molecules. Although biosynthesis pathway of MA was shown to be similar in different Mycobacterial species, the proximal and distal position of meromycolic carboxyl group is different in each species (3, 4). Precursors of mycolic acid have long fatty acids with different functional groups, such as double bonds, keto, ester, epoxy, methoxy, and cyclopropane ring. All the sub-classes of this fatty acid do not exist in all species but methoxy and keto are less than alpha in most organisms. During the exponential growth phase the production of keto mycolic acid is more than exponential phases (5, 6). The genera which has mycolic acid, posses a broad family of over 500 species, that several remarkable differences such as the length of fatty acid and the number of carbon atoms are important for their identification. In *Mycobacterium* species the most prominent fatty acid, posses 60-90 carbon atoms that provide a crucial barrier for the cell. However, other genera such as *Nocardia* and *Corynehbacterium* contain no cardiomycolic and corynemycolic acids with carbon atoms between 22-60 units. Nocardiomycolic acid has 44-60 and corynemycolic acid has 22-36 carbon atoms (4).

Acetyl-CoA and malonyl-CoA are initial substrates for biosynthesis of mycolic acid, and FAS-I and FAS-II systems are two systems for MA biosynthesis. FAS-I system elongates acetyl-CoA by two carbon units. The yield of first reaction is butyryl-CoA, and then C-16 and C-18 derived. Generally these fatty acids are used for synthesis of membrane phospholipids, but in *M. tuberculosis*, continued elongation of C-16 and C-18 by FAS-I system, leads to produce C-20 and C-26 products. The C-20 fatty acid is introduced to FAS-II system (7).

Mycobacteria are evolved in biodegradation of some xenobiotic substances. Toluene and xylene degradation have seen in *M.tuberculosis*, also two toluene-degrading strains, *Mycobacterium aurum* and *Mycobacterium komossense*, were isolated from rock surface in a freshwater stream contaminated with toluene. The strains exhibit different capacities for degradation of toluene and other aromatic compounds and have characteristics of the genus *Mycobacterium*. They have mainly straight-chain saturated

and mono unsaturated fatty acids with 10 to 20 carbon atoms and large amounts of tuberculostearic acid that are typical of Mycobacteria. Ecological studies reveal that toluene contamination has enriched for toluene-degrading bacteria in the epilithic microbial community. Biodegradation of phenanthrene, fluorene, fluoranthene, pyrene, vinyl chloride and morpholine by *Mycobaterium* have been reported previously (8- 11), however usually this xenobiotic does not affect on fatty acid syntheses.

Today this fatty acid is a good candidate for drug delivery. Nano-encapsulation of anti-TB drugs was successfully encapsulated four first line anti-TB drugs: Isoniazid (INH), Rifampicin (RIF), Ethambutol (ETB) and Pyrazinamide (PZA), as well as two second line TB drugs (MDR –TB drugs), namely: Capreomycin and Kanamycin, in particles of 250-400 nm, using a novel multiple emulsion spray-drying technique (11). These drugs penetrate better to damaged tissue. Natural polymers like chitosan, alginate, and PLGA alternative system has been used to the current anti-tuberculosis nano-drug delivery. The current anti-tuberculosis nano-drug delivery has been used mycolonic acid as fatty acid drug carrier. This study describes the isolation and identification of toluene-degrading strain of *Mycobacterium* species from Persian Gulf and characterization of cell wall mycolic acid and its similarity to others for possible drug delivery.

Materials and Methods

Sampling

For screening and isolation of toluene degrading bacteria, samples were collected from Persian Gulf water and transferred to lab in ice box.

Isolation and selection of toluene degrading bacteria

A solid mineral medium (MM) was used for the isolation of toluene degrading bacteria. This medium contained (gl^{-1}): KH_2PO_4, 4; Na_2HPO_4, 4; NH_4Cl, 2; $MgSO_4$, 0.2; $CaCl_2$, 0.001 and $FeCl_3$, 0.001 in 1000 mL. distillated water and pH was 6.8. Toluene was added in sterile MM media by 1% (v/v). About 10 mL seawater was centrifuged and 1 mL. of pellet was spread onto the toluene agar (mineral medium with toluene as the only sources of carbon and energy). Plates were incubated at 28°C. After 2 weeks of enrichment, cream colony was isolated on nutrient agar. This isolate must be salt and toluene tolerant bacterium. The sample did not dilute since there were few microorganism that tolerated the solvent toluene.

Identification of the isolates

Identification of the isolated strain was performed based on colony morphology, microscopic observation of the cell cycle, Gram stain, acid-fast stain, the catalase test, the oxidase test, oxygen requirement, motility and the ability to grow on different carbon sources according to the standards of microbial identification (17)

DNA was extracted for PCR reaction from culture by boiling. The samples were centrifuged at 10,000 rpm for 15 min. Cells are washed triple by water. The pellet was twice freezed-thaw and resuspended in 1 mL. of molecular biology-grade water and boiling at 100°C in a water bath for 15 min, centrifuged at 10,000 rpm for 5 min, and the supernatant was used for PCR. The 16S rRNA gene for identification of the toluene utilizing strain was amplified with DG74-AGGAGGTGATCCAACCGCA as a forward primer and RW01-AACTGGAGGAAGGTGGGGAT as a backward primer (12). The amplified PCR product is approximately 370 bp in length. Amplification reactions were done in a 25 µl reaction volume. Reaction tubes contained 1 µl of each primer-pair (0.3 µg μl^{-1}), 0.5 µl dNTPs (0.2 mmol l^{-1}), 2.5 µl X10 PCR buffer, 0.75 µl $MgCl_2$ (25 mmol l^{-1}), 17 µl PCR H_2O, 2 µl template DNA extracted from bacterium and 0.25 µl Taq

polymerase (5U μl^{-1}). The PCR reaction was performed in an Eppendorf Thermal Cycler using appropriate programs optimized for this primer. After denaturation of DNA through heating for 2 min at 94°C, The PCR program involved 30 cycles, each cycle consisted of: denaturation at 94°C for 2 min, annealing at 55°C for 1 min and extension at 72°C for 1 minute. This was followed by a final elongation step for 2 min at 72°C. The PCR products were separated on a 1.7% agarose gel containing ethidium bromide in 1 × TBE buffer, run at 100 V for 1.5 h and the gel was visualized on a UV Transilluminator. The purified PCR product was sequenced in both directions using an automated sequencer by Macrogen (Seoul, Korea). The sequences were edited using Finch TV V.1.4.0., and the BLASTN program was used for homology searches with the standard program default.

Growth in nutrient broth and egg media?

Isolated *Mycobacterium* was inoculated in nutrient broth (Sigma) and liquid Lowenstein-Jensen (Merk) medium (L.J) contained (g/1600 mL): potato starch, 30; asparagines, 3.6; KH_2PO_4, 2.4; magnesium citrate, 0.6; $MgSO_4. 7H_2O$, 0.24; 10 mL; glycerol, 12 mL; Homogenized whole egg, in 600 mL distillated water. Cultures were grown at 25°C with continuous shaking, for 10 days (3). After this time, samples were checked for purify.

Extraction and analysis of mycolic acid

Lipids were extracted from cells using the method described by Rafidinarivo et.al (3). The cells were harvested by centrifugingt at 4000 rpm for 15 min. Mycolic acids were extracted from 20 gr of wet cells with 10mL chloroform/methanol (1:2, v/v) for 17 h at room temperature with continuous stirring. The residues were re-extracted for 23 h with 10 mL chloroform/ methanol (1:1, v/v) were added residues, for 17 h under the same conditions. After mixing and centrifuging at 6000rpm for 10min, organic phase of nutrient broth and bottom phase of L.J medium were pooled and were analyzed with HPLC. For the chromatographic separation, an HPLC instrument equipped with a reverse phase C18 Ultrasphere XL analytical cartridge column and with a detector set at 254-260 nm. The gradient of solvents, methanol (M) and methylene chloride (MC), is changed from the initial conditions (98% M:2% MC) to 80% M:20% MC.

Results

The isolated *Mycobacterium* from marine water was enriched with toluene and isolated on nutrient agar. The isolated bacterium had cream colony, growed fast in compare with other Mycobacteria and visible colonies were observed after 4 days incubation at 28°C on nutrient agar. In compare with other *Mycobacterium* it is a rapid grower strain. It was catalase positive, non motile, rod shaped acid fast bacilli with metachromatic granules (Fig. 1), using universal primer was identified as *Mycobacterium* sp. and has been submitted to NCBI with accesses number of jn64433. The cells were grown on nutrient broth and egg liquid media for 10 days. Then the grown cells were harvested and in several steps the mycolic acid was extracted and identified by HPLC (Fig. 2 A and B). *Mycobacterium bovis* (13) had one cluster of peaks and isolated toluene degradator *Mycobacterium* had one early cluster of peak according to figure 2B. The interface of *Mycobacterium bovis* and *Mycobacterium tuberculosis* to target cells are through similar mycolic acid which is the same as the isolated one, however the other toluene degrading *Mycobacterium* had different pattern of mycolic acid. It was interesting that the only difference between the additions of egg yolk was the highest peak at 3 min whereas in nutrient broth grown on the highest peaks, was at 1.5 min.

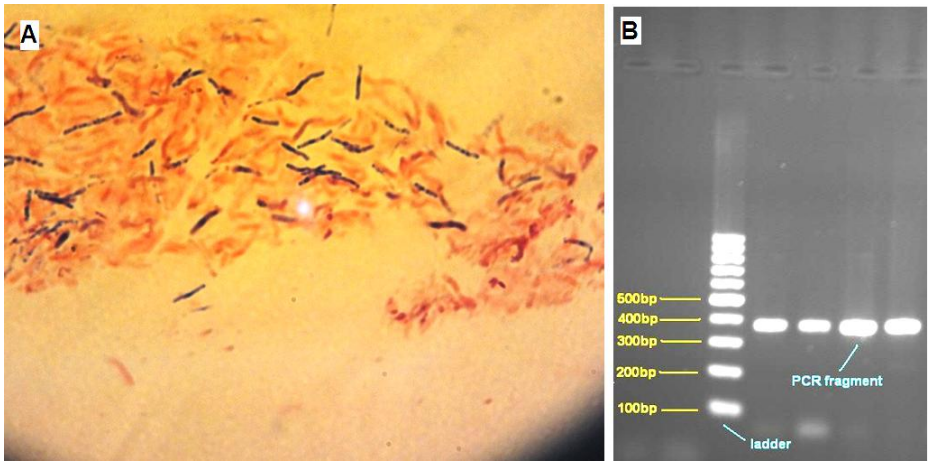

Fig. 1- A- Gram stain of isolated fast growing *Mycobacterium* and B- The PCR product of 16S rRNA gene.

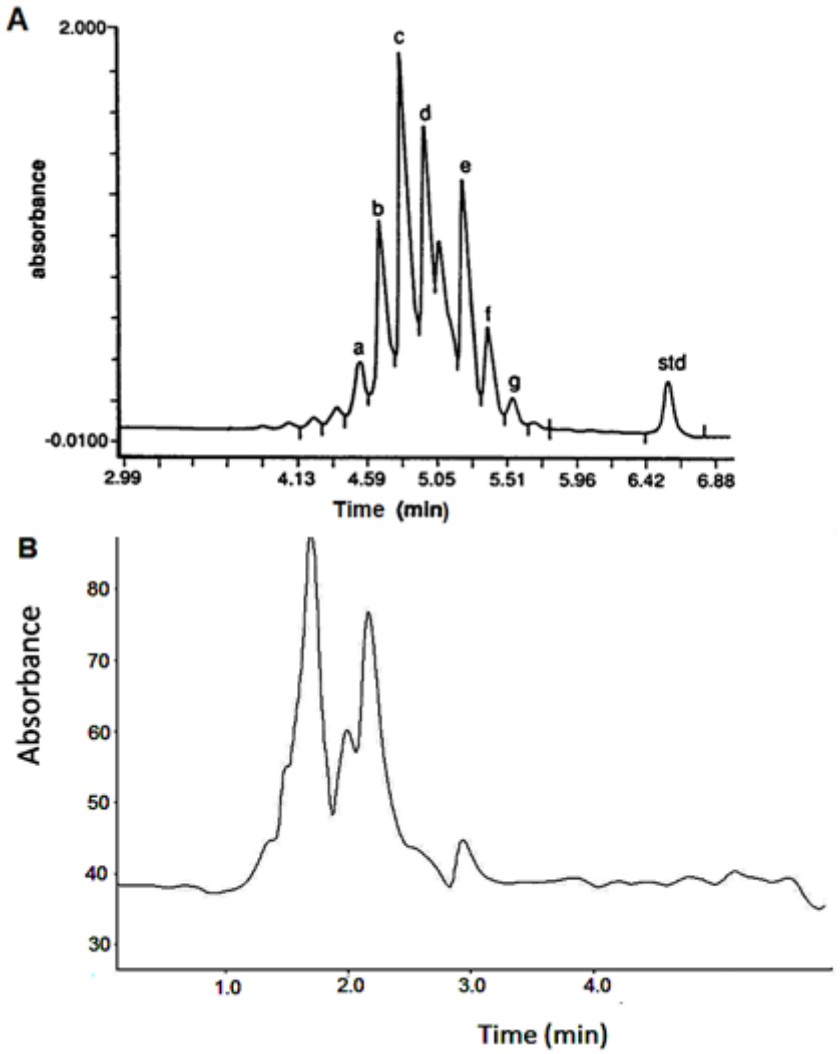

Fig. 2- A- The early first cluster of peaks of *Mycobacterium bovis* showen by Floyd et.al. in 1992, (13). B- Similar fatty acid pattern analyzed by HPLC are shown for isolated *Mycobacterium*

The result of present study offers that the production of different secondary metabolites can be induced by changing in culture ingredients to produce longer mycolic acid chains.

Discussion and conclusion

Mycolic acid is an essential component for survival of *M.tuberculosis.* For example, low permeability of this compound doesn't allow to hydrophobic drugs such as general antibiotics to inter the cell, or make them resistant to acid and solvents although acid and solvents may change the structure of this fatty acid. The drug delivers are liposomes, polyhydroxy butyrate or heavy chain of mycolic acids (14). Therefore production of mycolic acid from a non pathogen Mycobacterial is useful for introducing ways in identification of *Mycobacteria* (4), promoting second metabolite synthesis, drug deliveries (14) and inhibition of fatty acid synthesis (7). Here synthesis of long fatty acids in presence of cholesterol by a salt and toluene tolerate *Mycobacterium* is reported.

One of the challenges of Tuberculosis treatment is non-specific localization of the drugs into the body leads to reduction of their effectiveness due to decrease of drug concentration in target site of infection. Recent advances in intelligent drug delivery techniques cause effective cure for more infections. *M. tuberculosis* entry into host macrophages are adapted to survive in these cells (15).

Paradoxically, macrophages are the primary effector cells of the innate immune response. Nowadays, there are many drugs that inhibit specific enzymes which are involved in the biosynthesis of Mycobacterial cell wall (15). Mycolic acid can be used as drug delivery and can mimic antigenic structure of tuberculin too. This lipid is not soluble in water, Therefore, to overcome the poor solubility of MA in water, the bio-lipid has been incorporated into liposome as vehicles for drug delivery (16, 17). MA derivatives from saprophytic Mycobacteria, which are capable to resist the environmental condition (salt and solvents), are more stable. These fatty acids are suitable for delivery of drugs into the body and target them to the macrophage.

MA can insert to the peritoneal cavity or to the alveolar macrophage, similar to entering of *M tuberculosiss* observed in tuberculosis granulomas (4). The previous studies observed uptake of long-chain fatty acids from the culture medium by Mycobacteria and incorporation into triacylglycerol. C16 and C18 fatty acids in *M. tuberculosis*, could be used for the mycolic acids synthesis by entrance to the FAS-I pathway (7).

Also using of mycolic acid as capsule for drug can inhibit mycolic acid synthesis in pathogenic *Mycobacterium* which can efficiently inactivate pathogenic Mycobacteria. Our result indicated that changes in the culture medium of saprophytic *Mycobacterium* isolated from marine environment, had an influence on the mycolic acid component (Fig. 3), probably due to the effect of culture ingredient on activity of FAS-I and FAS-II systems (7).

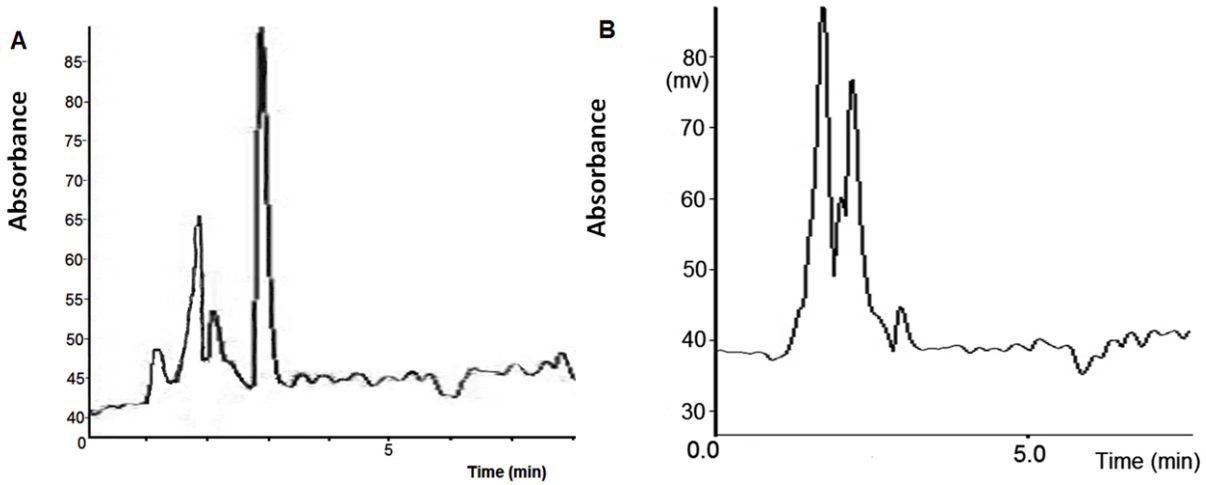

Fig. 3- The HPLC of mycolic acid obtained from *Mycobacterium* grown on egg yolk media (A) and cholesterol free media (B)

In the present study, we suggest that MA from a saprophytic toluene tolerate *Mycobacterium* has long fatty acid chain in presence of cholesterol and could be useful for treatment of Mycobacterial infection. According to the results, this isolated *Mycobacterium* are resistant to salt, toluene and pattern of cell wall mycolic acids were similar to pathogenic *Mycobacterium.*

Also Mycolic acid-containing bacteria can influence the biosynthesis of complex natural products in *Streptomyces* species (16). Each mycolic acid-containing strain induces different changes in secondary metabolism production (5). So, mycolic acid from non pathogen strain may promote *Streptomyces* for new drug syntheses.

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الگوی مایکولیک اسید در مایکوباکتریم تجزیه کننده تولوئن در حضور کلسترول

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چکیده

مقدمه: مایکولیک اسیدی که در دیواره‌ی سلولی مایکوباکتریوم توبرکلوزیس حضور دارد، داری عوامل آنتی‌ژنی است که می‌توانند به عنوان نشانگرهایی برای ردیابی آنتی‌بادی‌های بیماری فعال توبرکلوزیس استفاده شوند. ردیابی آنتی‌بادی توسط آنتی‌ژن‌های مایکولیک اسید حتی در عفونت‌های همراه با بیماری ایدز می‌تواند به‌کار گرفته شود. استفاده از مجموعه‌ای از مایکولیک اسیدها ممکن است باعث واکنش متقاطع آنتی‌بادی با کلسترول شود و از این‌رو مخاطره انگیز باشد. علاوه بر این‌که مایکولیک اسید می‌تواند در ارتباط با شناسائی بیماری توبرکلوزیس استفاده شود، مایکولیک اسید سویه‌های غیربیماری‌زا می‌توانند گزینه‌ی بسیار خوبی برای انتقال دارو به حساب بیایند. این ماده به‌خصوص برای اسپری دارو و انتقال آن از طریق بینی می‌تواند کارآمد باشد. هدف از این تحقیق، بررسی تاثیر کلسترول بر سنتز مایکولیک اسید و پتانسیل آن برای انتقال دارو است.

مواد و روش‌ها: به منظور غربال‌گری باکتری‌های تولیدکننده مایکولیک اسید از محیط غنی از تولوئن استفاده شد. یک باکتری اسیدفاست با رشد سریع جداسازی و با استفاده از روش PCR شناسائی شد. توالی مربوط به ژن 16S *rRNA* این باکتری با شماره دستیابی jn64433 در بانک ژنی NCBI ثبت و تولید مایکولیک اسید در محیط‌های مختلف با استفاده از کروماتوگرافی مایع با کارایی بالا(HPLC) بررسی شد.

نتایج: در این مطالعه، تاثیر کلسترول بر بیوسنتز مایکولیک اسید توسط یک سویه غیربیماری‌زا که از آب‌های دریایی جداسازی شده است بررسی شد. مطالعات HPLC نشان می‌دهد مایکولیک اسید سویه جداسازی شده یک دسته پیک اولیه دارد که این ویژگی در مایکولیک اسیدهای باکتری‌های مایکوباکتریوم توبرکلوزیس و مایکوباکتریوم بویس نیز دیده می‌شود. سویه‌ی جداسازی شده رشد سریعی دارد و کلسترول می‌تواند محرک تولید اسیدهای چرب طویل زنجیره در آن باشد.

بحث و نتیجه‌گیری: شباهت بین مایکولیک اسید سویه غیربیماری‌زای جداسازی شده با مایکوباکتریوم‌های بیماری‌زا این پیشنهاد را می‌دهد که مایکولیک اسیدی که از سویه‌ی غیربیماری‌زا جداسازی شده است می‌تواند به عنوان ناقل دارو استفاده شود. اضافه کردن زرده‌ی تخم مرغ به محیط کشت، باعث القای تولید اسیدهای چرب با طول بلند می‌شود که این حالت می‌تواند برای انتقال دارو به ماکروفاژها مناسب باشد.

واژه‌های کلیدی: مایکوباکتریوم، تولوئن، مایکولیک اسید، کلسترول

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