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Full Length Research Paper

Isolation and identification of *Bacillus* strains with antimycobacterial activity

Souraya El Guendouzi[1], David Suzanna[2], Mohammed Hassi[1], Abdellatif Haggoud[1], Saad Ibn souda[1], Abdellah Houari[1] and Mohammed Iraqui[1]*

[1]Université Sidi Mohammed Ben Abdellah, Faculté des Sciences et Techniques de Fès. B.P: 2202, Fès, Maroc.
[2]Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA,IP), 1649-016, Lisbon, Portugal.

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Tuberculosis is the principal cause of death worldwide due to an infectious disease. The resurgence of tuberculosis, followed by the increase in prevalence of infections caused by nontuberculous mycobacteria (NTM), as well as the multi-drug resistance of mycobacteria to the majority of currently available antibiotics, have encouraged research for new antimycobacterial agents. Soil and water samples from different Moroccan biotopes, have led to the isolation of four bacterial strains (*M*, *R*, *G* and *S*), showing an inhibitory effect on mycobacterial growth. This effect was shown to be due to secreted substances in the growth medium. From subsequent analysis it was concluded that these strains produced different active substances. Sequencing of the 16S rRNA showed that these isolates belong to the genus *Bacillus*. The active substance from isolate *M*, showed the more important inhibitory effect on mycobacterial growth. It precipitated with ammonium sulfate and lost all activity when treated with Proteinase K, revealing its protein nature.

Key words: Tuberculosis, mycobacteria, antimycobacterial agents, *Bacillus*.

INTRODUCTION

Tuberculosis is a major public health problem (Musser, 1995; Berthet et al., 1999; Rossetti et al., 2002; Chen et al., 2011). Nevertheless, as of 1952, with the discovery of effective antituberculosis agents and the begining of chemotherapy, the implementation of BCG (Bacille Calmette Guérin) vaccination programs (Ann Ginsberg, 2002; Bonnaud, 1996), and the improvement of living conditions, the decline in tuberculosis was considerable in industrialized countries (the rate of infection dropped as much as from 200/100 000 inhabitants in 1900 to less than 10/100 000 inhabitants in 1980) (Rastogi et al., 2001). However, since 1986, there has been a worldwide resurgence of tuberculosis (Newton et al., 2000) to which the HIV epidemic has undeniably been a major contributor, as individuals with the immunological deficiency are more apt to develop the disease (Chin and Hopewell, 1996; Iredia et al., 2011). According to the World Health Organization (WHO) report on global tuberculosis control, the number of new cases was estimated at 9.2 million in 2006, or 139 cases per 100,000 inhabitants (WHO, 2009). The number of deaths from tuberculosis in 2006 was estimated at 1.7 million of which 0.2 million occurred in HIV infected individuals. Developing countries continue to be the most effected by tuberculosis with a mortality rate of nearly 40% as compared to 7% in industrialized nations (Emile, 1996; Dharmarajan et al., 2007).

Two resistant forms of tuberculosis include multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB). MDR-TB is caused by *Mycobacterium tuberculosis* strains resistant to at least the two major first-line TB drugs, isoniazid (INH) and rifampicin (RIF). XDR-TB is caused by an MDR-TB strain also resistance to at least one of the three second-line injectable TB drugs (capreomycin, kanamycin or amikacin) in addition to any one of the fluoroquinolones. In 2007, the worldwide estimate was of 500 000 cases of MDR-TB; 85% of these were from 27 countries (of which 15, from the European region). At the end of 2008, 55 countries and territories had reported at least one case of XDR-TB (WHO, 2009).

In Morocco, 500 to 1000 people die each year from tuberculosis. According to the last figures from the

*Corresponding author. E-mail: mhiraqui@yahoo.fr.

Ministry of Health, the Kingdom registered 25,500 new cases in 2007, or 82 per 100,000 inhabitants. Seventy percent of these patients were between 15 and 45 years of age (Ben Cheikh et al., 1996), mainly from the population residing in the most heavily populated urban areas. The sanitary services often receive patients in an advanced stage of disease. The delay in diagnosis not only endangers the patient but contributes to tuberculosis transmission as, without adequate treatment, an individual with active disease can infect on the average from 10 to 15 people in a one year time span. Also, inadequate treatment, either in dosage or duration, may result in acquired drug resistance to antituberculosis drugs.

In today's context, new drugs are a priority to better the control of mycobacterial infections and contribute to solution the problem of the emergence of strains resistant to antituberculosis agents. The purpose of this investigation consists in the search for novel substances with an antimycobacterial effect from the Moroccan microbial flora, which has not been extensively explored in this respect.

MATERIALS AND METHODS

Mycobacterial strains

All tests were carried out on non-pathogenic mycobacteria. These include: *Mycobacterium aurum* A^+: a rapid growing scotochromogenic species, with a generation time of 6 h, having a similar spectrum of drug susceptibility to *M. tuberculosis* (Chung et al., 1995).

Mycobacterium smegmatis MC^2 155: a rapid growing non-pigmented, thermophilic species with a generation time of 3 h.

Isolation of microorganisms secreting substances with antimycobacterial effect

Several samples (water, soil and water) were taken from different Moroccon ecological niches. The samples were kept under agitation for two hours. The supernatant was recovered after sedimentation of the heavier particles. Serial dilutions (of 10^{-1} to 10^{-5}) were carried out in sterile Luria Bertoni (LB) broth. An aliquot of 70 µl of each dilution was spread on LB-agar previously inoculated with *M. aurum* A^+ or *M. smegmatis* cultures having an optical density at 600 nm ($D.O_{600nm}$) of 0.3. After incubation at 37°C for 48 h, colonies surrounded by an inhibition zone were purified. The four isolates thus obtained were designated *M*, *R*, *G* and *S*.

To confirm that the antimycobacterial effect of these isolates was due to secreted substances their cultures were filtered and the filtrate was tested on the mycobacteria using the well or diffusion method. Briefly, the strains were cultures in 50 ml of LB broth, for 48 h at 37°C with agitation; the cultures were then centrifuged at 6000 rpm for 5 min to remove bacteria in suspension. The supernatant was recovered and filtered using 0.45 µm porosity filters. The filtrate obtained was tested by depositing 100 µl in wells cut out from solid media in plates previously inoculated with 100 µl of mycobacterial culture (*M. aurum* or *M. smegmatis*) at $D.O_{600\ nm}$ = 0.3. The plates were incubated at 37°C for 48 h. After incubation, the presence or absence of an inhibition zone was reported. The inhibition tests were repeated three times for each strain.

The control used corresponded to a culture filtrate from *Escherichia coli* prepared under the same conditions. 100 µl of this filtrate was deposited in the wells prepared in plates previously inoculated with mycobacterial culture (*M. aurum* or *M. smegmatis*) at $D.O_{600\ nm}$ = 0.3. The plates were incubated at 37°C for 48 h. After incubation, the presence or absence of an inhibition zone was reported.

Activity spectrum of the isolates

In order to get an idea on the spectrum of activity, the antimicrobial effect of the isolates under study was evaluated against the following bacteria: *M. aurum A^+*, *M. smegmatis* MC^2 155, *E. coli DH5α*, *Bacillus subtilis*, *Staphylococcus haemolyticus* (Hassi et al., 2007).

A few colonies from young cultures of each isolate were deposited at the center of a plate previously inoculated with 100 µl of microbial culture (for *E. coli, B. subtilis* and *S. haemolyticus* the $D.O_{600\ nm}$ was at 0.5, for *M. aurum* and *M. smegmatis* the $D.O_{600\ nm}$ was at 0.3). The plates are incubated at 37°C for 48 h. After incubation, the presence or absence of an inhibition zone is reported. The tests were repeated three times for each strain.

Confrontation test between the studied isolates

A confrontation test between the studied isolates was carried out in order to determine if these bacteria produced the same active substance or not. For this, A few colonies from young cultures of each isolate were deposited at the center of a plate previously innoculated with 100 µl of culture of another of the isolates under study at $D.O_{600\ nm}$ = 0.5. The plates were incubated at 37°C for 24 h. After incubation, the presence or absence of an inhibition zone was reported. The confrontation test was repeated three times for each strain.

Identification of the isolates

The molecular identification of the isolates was carried out by polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA gene. In order to classify a microorganism within a genus or as a species, a number of criteria must be respected. The molecular definition of genus states that 16S rRNA sequence similarity should be superior or equal to 97%. Similarity superior to 99% indicates identical species; on the other hand, no identification results if the similarity is inferior to 97% (Drancourt et al., 2000).

DNA for the molecular studies was extracted from a young LB broth culture of the isolates (24 h). A 1 ml aliquot of the culture was transferred to a 1.5 ml microtube (Eppendorf, XXX) and centrifuged at 6000 rpm for 5 min. The pellet was resuspended in 100 µl of sterile distilled water, frozen at -20°C for 30 min, then heated at 95°C for 3 min. This thermolysis procedure was repeated twice in order to burst a large number of cells. After centifugation at 7000 rpm for 10 min, 2 µl of the supernatant (containing the DNA) were used in the amplification reaction (Rodrigues Cavalcanti et al., 2007).

For, amplification, universal primers, fD1 (5' AGAGTTTGATCCTGGCT CAG 3') and Rs16 (5' TACGGCTACCTTGTTACGACTT 3'), used in the identification of bacteria by sequencing of 16S rDNA were selected (Weisberg et al., 1991). The reaction mix was prepared in a final reaction volume of 20 µl and contained: 4 µl of Taq buffer (5x), 1.2 µl of $MgCl_2$ (25 mM), 4 µl of dNTPs (1 mM), 2 µl of fD1 (10 µM), 2 µl of Rs16 (10 µM), 0.2 µl of Taq polymerase (5 U/µl), 4.6 µl of pure H_2O and 2 µl of the DNA. The of amplification conditions consisted in an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C

Table 1. Antimycobacterial effect of the filtrate from the isolated strains.

Filtrate from strain	Diameter of the inhibition zone (cm) [a]	
	Effect against MC[2 b]	Effect against A[+ c]
M	2.75 ± 0.15	3.22 ± 0.12
R	2.29 ± 0.21	2.71 ± 0.11
G	2.21 ± 0.22	2.56 ± 0.16
S	1.78 ± 0.17	2.22 ± 0.18
E. coli	0	0

[a] Tested on Luria Bertoni agar, [b] *MC*^{2}*: M. smegmatis*, [c] *A*$^+$*: M. aurum*.

for 30 s, annealing at 55°C for 45 s and extention at 72°C for 1 min 30 s; final extension was performed at 72°C for 10 min. The amplified fragment of approximately 1.5 kb was visualized by electrophoresis using a 1% agarose gel. Two amplification controls were used, a negative control (T-) corresponding to a no template control, and a positive control (T+) corresponding to the 16S rDNA gene of *B. subtilis.*

For sequencing, PCR products were purified using a PCR Product Purification Kit (JETquick, Genomed) as described by the manufacturer. Amplification was carried out using 2.7 µl of the DNA product. The sequencing reaction mixture contained 2 µl BigDye V 1.1 and 0.25 µl of the primer (forward or reverse), PCR grade water was added for a final reaction volume of 10 µl. Amplification conditions were the following: initial denaturation at 96°C (3 min), 35 cycles of denaturation at 96°C (20 s), annealing at 60°C (5 s) and extension at 60°C (4 min). All sequences were determined in an automated DNA capillary sequencer AbiPrism 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA) using BigDye™ Terminator Cycle Sequencing Ready reaction Kit (PE Biosystems, Applied Biosystems, Foster City, USA). Two sequencing reactions were performed using the two primers used for PCR amplification.

The sequences of were analyzed using the data base of the National Center for Biotechnology Information (NCBI) and the BLAST N program.

Other than sequencing of the 16S rRNA gene, the molecular identification of the isolates under study was complemented using conventional preliminary tests: macroscopic observation, Gram stain and growth at 50°C (Meyer et al., 2004).

Determination of the protein nature of the active substance of one of the isolates

Precipitation of the active substance of the isolate by ammonium sulfate

The precipitation protocol used was described by Shimei and collaborators for the purification and characterization of antimicrobial peptides (Subpeptin JM_4-A et JM_4-B), produced by *B. subtilis* JM_4 (Shimei et al., 2005). Briefly, 100 ml of LB broth was inocularted with the selected strain and incubated at 37°C under agitation for 48 h. After incubation, it was centrifuged at 6000 rpm, at 4°C, for 5 min. The supernatant was recovered and 52.3 g of ammonium sulfate were progressively added under agitation. The mixture was left overnight under agitation at 4°C. The precipitate was centrifuged at 10000 rpm, at 10°C, for 20 min. The pellet was re-suspended in 200 µl of buffer (potassium phosphate KH_2PO_4 50 mM, pH = 6).

The suspension obtained was dialyzed against the same buffer at 4°C for 12 h. The dialisate was filtered using a 0.45 µm de porosity filter. The effect of the dialisate was then tested (three times) against *M. aurum* and *M. smegmatis* using the well method.

The controls used in this experiment were the LB broth (with no inoculums) and the LB broth inoculated with *E. coli*. These controls were precipitated by ammonium sulfate, using the same protocol used for the precipitation of the active substance of the strain under study. The effect of the dialisate of these two controls was tested (three times) against *M. aurum* and *M. smegmatis* using the well method.

Sensitivity of the isolate's protein precipitate to Proteinase K

A 100 µl volume of the filtered dialisate was added to 40 µl of a Proteinase K solution (1 mg/ml). The mixture was homogenized and incubated at 37°C for 3 h. The effect of the Proteinase K treated dialisate, was tested (three times) against *M. aurum* and *M. smegmatis* using the well method. The control was a solution of Proteinase K at the same concentration.

RESULTS

Isolation of microorganisms secreting substances with antimycobacterial effect

Four bacterial strains (*M*, *R*, *G* and *S*) were isolated from the samples analyzed. They exerted growth inhibition against the indicator mycobacterial strains as seen by the formation of inhibition zones (Table 1). The inhibitory effect of the active substance from each strain was relatively more important against *M. aurum* than *M. smegmatis*. The *E. coli* filtrate, used as control, did not present any antimycobacterial activity.

Activity spectrum of the isolates

Results show that the isolates under study have an antimicrobial effect against Gram positive bacteria, Gram negative bacteria, and mycobacteria (Table 2).

Confrontation test between the studied isolates

In order to determine if the isolated strains produce the same active substances, confrontation tests were carried out between the various isolates. Results show that each isolate exerted an antimicrobial effect against the

Table 2. Activity spectrum of the isolates.

Strains	Inhibitory effect against [a,b]				
	M. aurum **(mycobacteria)**	***M. smegmatis*** **(mycobacteria)**	***E. coli*** **(Gram-)**	***B. subtilis*** **(Gram+)**	***S. haemolyticus*** **(Gram+)**
M	+	+	+	+	+
R	+	+	+	+	+
G	+	+	+	+	+
S	+	+	+	+	+

[a] Tested on Luria Bertoni agar. [b] +: presence of an inhibition zone.

Table 3. Confrontation between the isolates.

Strain deposited at the center of the plate	Strain spread over the plate [a]			
	M	**R**	**G**	**S**
M	-	+	+	+
R	+	-	+	+
G	+	+	-	+
S	+	+	+	-

[a] Tested on Luria Bertoni agar. +: presence of an inhibition zone. -: absence of an inhibition zone.

indicator strain as could be seen by the formation of an inhibition zone around the colonies deposited at the center of the plate (Table 3).

Identification of the isolates

According to the identification criteria described, results from the sequence analysis, indicated that the isolates under study belong to the genus *Bacillus* (Table 4). Concordantly, the isolates were Gram+ and capable of growth at 50°C.

Determination of the protein nature of the active substance of isolate M

Isolate M, which showed a more important activity than that of the other isolates, was selected to study the sensitivity of the isolate's protein precipitate to Proteinase K. The precipitate of the active substance of the isolate inhibited mycobacterial growth creating an inhibition zone around the wells. This activity was eliminated upon treatment by Proteinase K (Table 5). There were no inhibition zones around the wells, with the test controls (LB broth and LB broth inoculated with *E. coli*).

DISCUSSION

Numerous microorganisms are used in industry in the production of metabolites: enzymes, antibiotics, amino acids, vitamins, etc. The main microorganismes used by industry are Mycetes. However, some bacterial genus, such as *Streptomyces* and *Bacillus*, are also of industrial value (Xiaofeng et al., 2005). The production of anti-microbial compounds is known of an important number of bacteria. This phenomenon corresponds to a defensive mechanism of these bacteria, producing antibiotics, organic acids and lytic agents such as lysozymes. Furthermore, several types of protein exotoxins and bacteriocines are produced (Mota et al., 2004).

Since the 1950's, antibiotherapy is the major defensive means against microbial infections. Amongst industrial fermentations, the production of antibiotics is one of the most important sectors. Screening for strains producing natural antibiotics has led to the production of first generation antibiotics. The majority of antibiotics of microbial origin are produced by actinomycetes and Bacillus (Duval, 1989, Xiaofeng et al., 2005). Bacteria of the genus Bacillus produce a variety of antimicrobial agents, although none used as antituberculosis agents. These are mainly peptides, lipopeptides and phenolic derivatives (Mota et al., 2004). The large majority of peptides are antibiotics. Others are known as antifungal, antitumoral and cytotoxic drugs (Sachtowakyama Fumiy Ishikawa and Kuni, 1984). As examples we can mention the synthesis of iturin by *B. subtilis*, bacitracin by *Bacillus licheniformis*, tyrothricin by *Bacillus brevis*, and polymyxine by Bacillus polymyxa (Duval, 1989).

In this investigation, water and soil and water samples from Morocco led to the isolation of four bacterial strains (M, R, G and S) which inhibited growth of *M. smegmatis* and *M. aurum*. Their antimycobacterial activity was

Table 4. Identification of the isolates.

Isolates under study	Bacterial species showing a high degree of sequence similarity with the isolates under study	Size of the sequenced fragment using primer Rs16 [b] (pb)	% of similarity obtained using primer Rs16	Size of the sequenced fragment using primer fD1 [b] (bp)	% of similarity obtained using primer fD1
M	*Bacillus* sp. (FR773880.1)[a]	500	100	561	100
	Bacillus subtilis (HQ678671.1)[a]		100		100
	Bacillus amyloliquefaciens (HQ668178.1)[a]		100		100
R	*Bacillus* sp. (HQ728329.1)[a]	609	100	618	100
	Bacillus subtilis (HQ694434.1)[a]		100		100
	Bacillus amyloliquefaciens (HQ179100.1)[a]		100		100
G	*Bacillus* sp. (GU361655.1)[a]	478	100	563	100
	Bacillus subtilis (HQ323421.1)[a]		100		100
	Bacillus amyloliquefaciens (HM597236.1)[a]		100		100
S	*Bacillus* sp. (HQ603746.1)[a]	535	100	623	100
	Bacillus subtilis (HQ678662.1)[a]		100		100
	Bacillus amyloliquefaciens (HM992829.1)[a]		100		100

[a]Access number National Center for Biotechnology Information (NCBI) database. [b]bp: base pairs.

Table 5. Sensitivity of the active substance from isolate M to Proteinase K.

Protein precipitate	Diameter of the inhibition zone (cm) [a]			
	Precipitate not subject to Proteinase K treatment		Precipitate treated by Proteinase K	
	Effect on MC[2 b]	Effect on A[+ c]	Effect on MC[2]	Effect on A[+]
Bacillus (isolate M) [d]	2.8 ± 0.15	3.3 ± 0.21	0	0
E. coli	0	0	-	-
LB broth [e]	0	0	-	-

The control used was a solution of proteinase K. This control showed no antimycobacterial activity.- :Test not performed since the precipitate not subject to the Proteinase K treatment showed no effect.[a] Tested on Luria Bertoni agar.[b] MC[2]: *M. smegmatis*.[c] A[+]: *M. aurum*.[d] M: *Bacillus*. [e] LB: Luria Bertoni broth.

demonstrated by the occurrence of growth inhibition zones around the colonies, resulting from the diffusion in the LB agar growth medium of the bioactive substances produced by the isolate. The study of the activity spectrum of the isolates showed that these exert an antimicrobial

activity against Gram positive and Gram negative bacteria as well as mycobacteria.

Inhibition zones were also produced when using the isolate filtrates. The absence of mycobacterial growth (*M. smegmatis* and *M. aurum*) around the wells containing the filtrate, confirmed that the antimycobacterial effect observed was due to diffusion in the agar medium of secreted substances. The inhibitory effect of the active substance from each of the strains was comparatively more active against *M. aurum* than *M. smegmatis*. This important inhibition could be due to the fact that M. aurum has a longer generation time than *M. smegmatis*, which would permit a longer diffusion time for the inhibitor. As the filtrates from the studied isolates are active against *M. aurum*, it is highly probable that they will also be active against *M. tuberculosis* since these two mycobacteria present the same antibiotic susceptibility profiles (Chung et al., 1995).

Ammonium sulfate is widely used to precipitate water soluble proteins. It is one of the most common methods used to concentrate active substances of protein nature secreted by microorganisms in liquid medium. The active substance from the isolate showing the more important antimycobacterial activity was shown to precipitate by ammonium sulfate. The precipitate was also rendered inactive by Proteinase K. These results indicated the isolate's activity was due to a protein substance.

Sequencing of the 16S rRNA gene is presently the most commonly used molecular approach in bacterial phylogeny (Woese et al., 1990). It has led to the creation of large databases (Maidack et al., 1996; Van de Peer et al., 1999), whose use has allowed the identification of many environmental microorganisms as well as others that have been impossible to grow in culture (Relman et al., 1992; Strous et al., 1999). Using this methodology, the isolates were identified as Bacillus strains. These strains are Gram+ bacilli resistant to 50℃, confirming their classification in the genus Bacillus. The confrontation test showed that these isolates produced different active substances, which suggests that they are different strains although all from the genus Bacillus. The active substances could be antibiotics since most natural antimicrobial agents of bacterial origin are mainly synthesized by Bacillus and actinomycetes.

Since mycobacteria can infect macrophages, the effect of the active substances isolated will be studied ex vivo. The effect will also be studied against pathogenic bacteria, namely those responsible for nosocomial infections. Finally, the substances shown to be active *ex vivo* (macrophages infected by mycobacteria) will be purified and their structures determined.

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