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Isolation and identification of a *Staphylococcus warneri* strain with anti-mycobacterial activity

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Tuberculosis is the principal cause of death from infection in the world. The resurgence of tuberculosis and the increase in mycobacterial infections, as well as multidrug-resistance of mycobacteria to available antibiotics, has incentivized research on new antimycobacterial agents. Therefore, research based on water and soil samples from the Moroccan biotopes, has led to the isolation of a bacterial strain capable of inhibiting mycobacterial growth (*Mycobacterium smegmatis* and *Mycobacterium aurum A*$^{+}$). The effect was due to an active substance secreted into the culture medium. Sequencing of the 16S rRNA gene identified the strain as belonging to the species *Staphylococcus warneri*. The active substance precipitated using ammonium sulfate, maintained its inhibitory properties, which were lost when treated with proteinase K. These results indicated that the active substance was protein. Study of the activity of the metabolite revealed its effect on *M. smegmatis* cell wall, facilitating genomic DNA extraction.

Key words: Tuberculosis, mycobacteria, anti-mycobacterial agents, *Staphylococcus warneri*, DNA extraction.

INTRODUCTION

Tuberculosis, caused by *Mycobacterium tuberculosis*, undeniably remains a menacing social disease (Musser, 1995; Berthet et al., 1999; Rossetti et al., 2002). Nevertheless, as of 1952, the onset of effective chemotherapy, followed by widespread Bacille de Calmette et Guérin (BCG) vaccination (Ann Ginsberg, 2002; Bonnaud, 1996), the discovery of effective antituberculosis agents and the improvement of living conditions, have led to a considerable decline in tuberculosis in industrialized nations. In fact, the rate of infection dropped from 200/100.000 inhabitants in 1900 to less than 10/100.000 inhabitants in 1980 (Rastogi et al., 2001; Jost et al., 2001).

However, in the early 90's, population mobility, economic and political migrations, together with ineffective tuberculosis control programs, where adequate treatment failed to reach many patients, gave rise to an increase in the global incidence of tuberculosis (Newton et al., 2000). These conditions also favored the onset of problematic multidrug-resistant strains (Jost et al., 2001). Furthermore, immunocompromised patients, especially those infected by the human immunodeficiency virus (HIV),

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Abbreviations: BCG, Bacille de Calmette et Guérin; **HIV,** human immunodeficiency virus; **WHO,** World Health Organization; **MDRTB,** multidrug-resistant tuberculosis bacilli; **XDRTB,** extensively drug-resistant tuberculosis; **NCBI,** National Center for Biotechnology Information;**BLAST,** Basic Alignment Search Tool.

are particularly vulnerable (Mohle-Boetani et al., 2002; Chin and Hopewell, 1996). Developing countries remain the most affected by tuberculosis with mortality rates close to 40% compared to 7% in the industrialized nations (Emile, 1996).

In spite of scientific progress, this disease continues to take its toll mainly in the poorer regions of the globe (Dharmarajan et al., 2007). According to the World Health Organization (WHO) 2010 report, more than two billion people, close to one third of the world's population, are contaminated with the tuberculosis bacillus. One out of ten infected individuals will develop active disease in the course of his or her lifetime. The risk is considerably higher for people living with HIV. WHO has estimated that between 2000 and 2020, close to one billion people will be newly infected and that 200 million of these will develop the disease, of these 35 million will die of tuberculosis, if adequate measures are not taken to improve disease control (WHO, 2005).

Resistance to antituberculosis agents is due to insufficient treatment in either duration or dosage. There are two forms of resistance, multidrug-resistance and extensive drug-resistance. In 2007, the estimate was of 500 000 tuberculosis cases due to multidrug-resistant tuberculosis bacilli (MDRTB); 85% of these were from 27 countries (of which 15 were from the European region). By the end of 2008, 55 countries and territories had reported at least one case of extensively drug-resistant tuberculosis (XDRTB) (WHO, 2009). In Morocco, 500 to 1.000 people die every year from tuberculosis. According to the latest reports from the Ministry of Health, the kingdom registered 25.500 new cases in 2007, corresponding to 82 cases for every 100.000 inhabitants. Seventy percent of these patients were between 15 and 45 years of age (Ben cheikh et al., 1996), and those populations from urban populated areas remain the most vulnerable.

The WHO, the Global Fund to Fight AIDS, Tuberculosis and Malaria and the Stop TB Partnership have appealed to world leaders to reinforce their commitment and increase their contributions so as to reach the objective of one million people diagnosed and treated against MDRTB between 2011 and 2015, implicating the necessity of developing new and effective antimycobacterial agents (Newton et al., 2000). The production of antimicrobial compounds is a phenomenon observed amongst an important number of, mainly Gram positive, bacteria (Sosunov et al., 2007). This corresponds to a defensive mechanism of these bacteria, through the production of antibiotics, organic acids, and lytic agents such as lysozymes. Furthermore, several types of protein exotoxins, and bacteriocins are produced (Mota et al., 2004). The purpose of this investigation is to search for new antimycobacterial substances from the Moroccan bacterial flora, that has not been extensively explored, that could improve control of mycobacterial infections bringing a solution to the problem caused by the emergence of strains resistant to the available antituberculous drugs.

MATERIALS AND METHODS

Bacterial strains

The tests were performed on non-pathogenic mycobacteria. These include the following:

**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). Accordingly, this strain has been proposed as a surrogate for antimycobacterial drug discovery (Chung et al., 1995).

**Mycobacterium smegmatis MC^2 155*

A thermophilic, nonpigmented species (cultures may turn yellow with time). This is a rapidly growing species having a generation time of 3 h. This species has been used as a model in antituberculosis drug discovery (Mitscher et al., 1998).

Isolation of a bacterial strain secreting a metabolite with antimycobacterial effect

Several samples (water, soil and water) were taken from different Moroccan ecological niches. The samples were kept under agitation for 2 h. 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 (peptone: 10 g/l; yeast extract: 5 g/l; sodium chloride: 10 g/l). 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 (OD_{600nm}) of 0.3. After incubation at 37°C for 48 h, one colony surrounded by an inhibition zone was isolated.

To confirm that the antimycobacterial effect of this isolate was due to secreted substances, their cultures were filtered and the filtrate was tested on the mycobacteria using the well or diffusion method according to the following protocol: The bacterial strain was cultured in 50 ml of LB broth, for 48 h at 37°C with agitation; 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 OD_{600nm} = 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 was 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 OD_{600nm} = 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 isolate

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: *Mycobacterium aurum A*$^+$, *Mycobacterium smegmatis MC^2 155*, *Escherichia coli DH5α*, *Bacillus subtilis* (EL Guendouzi et al., 2011), *Staphylococcus haemolyticus* (Hassi et al., 2007). The test was repeated three times for each strain. A few colonies from young cultures of the isolate were deposited at the center of a plate previously inoculated with 100 µl of microbial cul-

ture (for *E. coli, B. subtilis* and *S. haemolyticus* the $D.O_{600nm}$ was at 0.5, for *M. aurum* and *M. smegmatis* the $D.O_{600nm}$ was at 0.3). The plates were incubated at 37°C for 48 h. The presence or absence of an inhibition zone was then reported.

Identification of the isolate

The identification of the isolate was based on rRNA gene sequencing. This is the molecular approach more commonly used in bacterial phylogeny (Woese et al., 1990). It has permitted the constitution of important databases (Maidack et al., 1996; Van de Peer et al., 1999), and its use has led to the identification of several microorganisms from the environment including when culture was not successful (Relman et al., 1992; Strous et al., 1999). A 1 ml aliquot from a young LB broth culture of the isolate (24 hours) was transferred to a 1.5 ml microtube. The tube was 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 (PCR) (Rodrigues Cavalcanti et al., 2007). For, amplification, universal primers, fD1 (5'AGAGTTTGATCCTGGCTCAG3') 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 (5 x), 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 amplification conditions consisted of an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension 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-) corresponds to a no template control, and a positive control (T+) corresponding to the 16S rDNA gene of *B. subtilis.*

Sequencing of the PCR products was carried out as previously described (EL Guendouzi et al., 2011). Sequences were compared to those from reference strains using the National Center for Biotechnology Information (NCBI) database and the Basic Alignment Search Tool (BLAST) N program. Similarity was determined according to sequence homology as previously described (EL Guendouzi et al., 2011; Hassi et al., 2012). 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).

Precipitation of the active substance of the isolate by ammonium sulfate

Ammonium sulfate is frequently used to precipitate water soluble proteins. It is one of the most commonly used methods for the concentration of the active protein substances secreted by microorganisms in liquid culture (EL Guendouzi et al., 2011; Hassi et al., 2012). The precipitation protocol used was described for the purification and characterization of antimicrobial peptides (Subpeptin JM_4-A et JM_4-B), produced by *Bacillus subtilis* JM_4 (Shimei et al., 2005). Briefly, 100 ml of LB broth was inoculated 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 potassium phosphate buffer (KH_2PO_4, 50 mM, pH = 6). The suspension obtained was dialyzed against the same buffer at 4°C for 12 h. The dialysate was filtered using a 0.45 µm de porosity filter. The effect of the dialysate was then tested (3 x) 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 same protocol for the precipitation of the active substance of the strain under study. The effect of the dialysate of these two controls was tested (three times) against *M. aurum* and *M. smegmatis* using the well method. The inhibition test was repeated three times.

Sensitivity of the isolate's protein precipitate to proteinase K

A 100 µl volume of the filtered dialysate 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 dialysate, was tested (three times) against *M. aurum* and *M. smegmatis* using the well method (Hassi et al., 2007; EL Guendouzi et al., 2011). The control was a solution of proteinase K at the same concentration. The test was repeated three times.

Effect of the bioactive metabolite from the study isolate on the mycobacterial cell wall

In order to determine if contact with the bioactive metabolite secreted by the isolate resulted in lysis of the mycobacterial cell wall, its effect on the extraction of genomic DNA of *M. smegmatis* was studied as follows: A 3 ml volume of an overnight culture of *M. smegmatis* was centrifuged at 5000 rpm for 5 min. The bacterial pellet was resuspended in 400 µl of the bioactive metabolite, prepared using ammonium sulfate as described above, and recovered after the precipitation step in sterile distilled water instead of potassium phosphate buffer. The bacterial suspension was incubated at 37°C for 3 h and centrifuged at 5000 rpm for 5 min. The supernatant was then treated twice with phenol-chloroform. The DNA was precipitated using ethanol absolute, washed with 70% ethanol then dried, and finally dissolved in 20 µl of Tris-EDTA (TE) buffer (pH = 8) for electrophoretic analysis using agarose gel (1%). This experiment was repeated three times. Two controls were used; the first corresponded to *M. smegmatis* treated with the protein precipitate of the non-inoculated LB broth, and the second to the use of classical mycobacterial DNA extraction procedure (Houssaini-Iraqui et al., 1991).

RESULTS

Isolation of a bacterial strain secreting a metabolite with antimycobacterial effect

One bacterial strain was isolated from the samples analyzed. It exerted a growth inhibitory effect against the indicator mycobacterial strains (Figure 1).

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

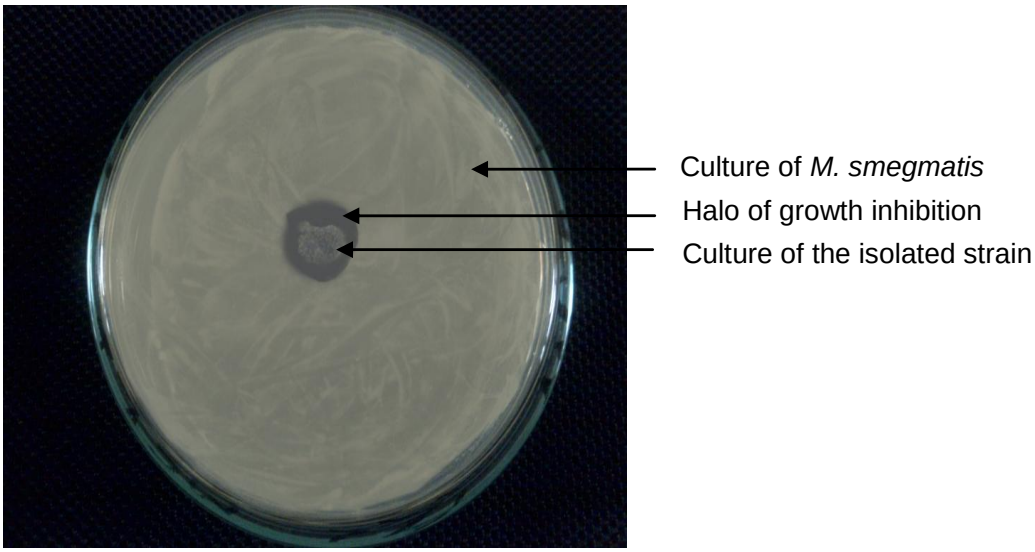

Figure 1. Antimycobacterial activity of the study isolate.

Table 1. Antimycobacterial effect of the filtrate from the study isolate.

Filtrate	Diameter of the inhibition zone (mm)	
	Effect against *MC²*	Effect against *A⁺*
Filtrate from the study isolate	23 ± 0,7	26 ± 0,5
Filtrate from *E. coli*	0	0

MC², *M. smegmatis* ; *A⁺*, *M. aurum*.

Table 2. Activity spectrum of the study isolate.

Bacterial species tested				
M. aurum **(mycobacteria)**	***M. smegmatis*** **(mycobacteria)**	***E. coli*** **(Gram⁻)**	***B. subtilis*** **(Gram⁺)**	***S. haemolyticus*** **(Gram⁺)**
+	+	+	+	+

+, Presence of an inhibition zone.

Activity spectrum of the isolate

Results of the activity spectrum of the isolate are shown in Table 2. These show that the isolate exerts an anti-microbial effect for all the bacterial strains tested.

Identification of the study isolate

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). Results of the sequences obtained using primers Rs16 and fD1, 762 bp and 551 bp respectively, were compared to those of the data base. According to the identification criteria described, results from the sequence analysis, indicated that the isolate corresponded to a strain of *Staphylococcus warneri* (Table 3). This result was also confirmed by the fact that the isolate was Gram+ and incapable of growth at 50°C.

Determination of the protein nature of the active substance of isolate *S. warneri*

Sensitivity of the active substance from S. warneri to proteinase K

The results obtained concerning the mycobacterial growth inhibitory effect of the protein extract from the study isolate are shown in Table 4. The protein extract from the study isolate exerted a growth inhibitory effect against mycobacteria showing an inhibition zone around the wells. This extract was inactivated upon treatment

Table 3. Identification of the study isolate.

Bacterial species showing a high degree of sequence similarity with the isolates under study	% of similarity obtained using primer Rs16	% of similarity obtained using primer fD1
Staphylococcus warneri (strain G72) (HQ407248)[a]	100%	100%
Staphylococcus warneri (strain LNP2) (GQ181035)[a]	100%	100%
Staphylococcus warneri (strain M-S-TSA 96) (JQ795875)[a]	100%	100%

[a], access number

Table 4. Sensitivity of the active substance from *S. warneri* to proteinase K.

Protein precipitate	Diameter of the inhibition zone (mm)			
	Precipitate not subject to Proteinase K treatment		Precipitate treated by Proteinase K	
	Effect against *MC²*	Effect against *A⁺*	Effect against *MC²*	Effect against *A⁺*
S. warneri	25 ± 1,4	28 ± 1,2	0	0
E. coli	0	0	-	-
LB broth	0	0	-	-

MC², *M. smegmatis*; *A⁺*, *M. aurum*; -, test not performed because the corresponding extract showed no biological effect.

with proteinase K (Table 4). A proteinase K solution, used as control, showed no antimycobacterial activity. No inhibition zones around the wells were observed for the study controls (LB broth and LB broth inoculated with *E. coli*).

Effect of the bioactive metabolite on the mycobacterial cell wall

The bioactive metabolite secreted by *S. warneri*, was tested for its capacity to lyse the cell wall of *M. smegmatis*. Results are shown in Figure 2. The protein extract from *S. warneri* allowed the extraction of DNA from *M. smegmatis* as efficiently as with the classic method. The control (non-inoculated LB broth culture protein precipitate) did not allow the extraction of *M. smegmatis* DNA.

DISCUSSION

The sample analyzed allowed the isolation of a bacterial strain of which the culture filtrate was capable of inhibiting mycobacterial growth due to the diffusion of an active substance produced on LB-agar. The inhibitory effect of the active substance of the study strain was relatively more accentuated against *M. aurum* than *M. smegmatis*. This difference could be due to the fact *M. aurum* has a longer generation time than *M. smegmatis*, which would allow for a more consequent diffusion of the inhibitory substance. Since the filtrate of this isolate was active against *M. aurum*, it is very probable that it would also be active against *M. tuberculosis* due to the fact that these two mycobacterial species have the same antibiotic susceptibility spectrum (Chung et al., 1995).

Results from the study of the activity spectrum of the isolate indicated that it exerted an antimicrobial effect against Gram positive bacteria (*B. subtilis*, *S. haemolyticus*), Gram negative bacteria (*E. coli*) and mycobacteria (*M. smegmatis*, *M. aurum*). Molecular analysis identified the isolate as a strain of *S. warneri*. This study shows for the first time that this species secretes an active substance against mycobacterial growth. Other studies have shown that the genus *Staphylococcus* includes several species (*S. haemolyticus, S. aureus*) known for their production of antibacterial substances (Frenette et al., 1984; Beaudet et al., 1982; Hassi et al., 2007). Verdon et al. (2008) isolated a strain of *S. warneri* that inhibited the growth of *Bacillus megaterium* and *Legionella pneumophila*.

This study reports for the first time a strain of *S. warneri* capable of inhibiting the proliferation of mycobacteria. The *S. warneri* extract was proteic as the ammonium sulfate preparation used is known to precipitate proteins (Shimei et al., 2005). The protein extract showed a growth inhibitory effect against mycobacteria; this activity is lost in the presence of proteinase K, which corroborates the protein nature of the active substance from *S. warneri*. Previous investigations have shown that *S. aureus* secretes a protein (Aureocin A53) that exerts a bactericidal effect against lactic bacteria, *Listeria monocytogenes* (Oliveira et al., 1998a), and several other strains distant from *S. aureus* responsible for bovine mastitis (Oliveira et al., 1998b). Hassi et al. (2007) also showed that *S. haemolyticus* produced a protein that inhibited mycobacterial growth. The protein extract from *S. warneri* was capable of extracting mycobacterial DNA

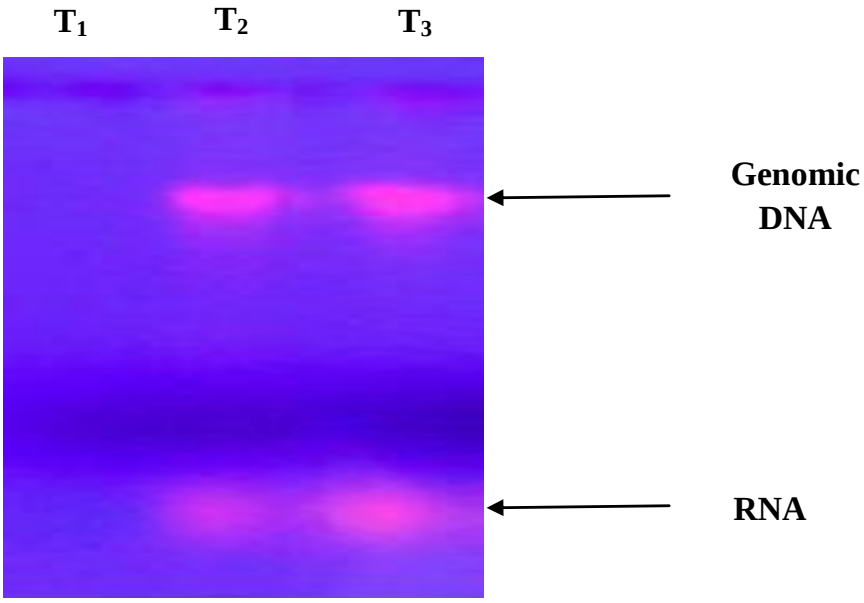

Figure 2. Extraction of genomic DNA from *M. smegmatis* by the bioactive metabolyte from *S. warneri*. T_1, Extract using the protein precipitate from non-inoculated LB broth; T_2, extract from *M. smegmatis* prepared using a conventional DNA extraction method (Houssaini-Iraqui et al., 1991); T_3, extract from *M. smegmatis* prepared using the *S. warneri* extract in replacement of the lysis agent in the conventional method.

and RNA, in the absence of conventional cell lysing agents (lysozyme, SDS, proteinase K). This shows that the active substance acts upon the cell wall of *M. smegmatis*. This result suggests that the growth inhibition of mycobacteria by this strain may be due to cell wall lysis.

Conclusion

This investigation focused on a bacterial strain isolated from a Moroccan biotope identified as *S. warneri*. This isolate secreted an active substance of protein nature that inhibited the growth of mycobacteria as well as Gram positive and negative bacteria. This active substance was capable of extracting DNA and RNA from *M. smegmatis* and therefore, may be useful in the extraction of the mycobacterial genome in molecular engineering. Since mycobacteria infect macrophages, the effect of the active substance may be tested *ex vivo*. Finally, this effect will also be tested against pathogenic bacteria, namely those responsible for nosocomial infections.

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