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Full Length Research Paper

In vitro and intracellular antimycobacterial activity of a *Bacillus pumilus* strain

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Despite the declaration of tuberculosis (TB) as a global emergency by the world health organization (WHO) about 20 years ago, the worldwide problem of this disease has worsened due to increased drug resistance of tuberculosis bacilli and acquired immune deficiency syndrome (AIDS) pandemic. Consequently, fight against multidrug and extensively drug-resistant TB is a high priority for public health and research. The present work describes the isolation of a *Bacillus pumilus* strain secreting a metabolite of protein nature capable of inhibiting mycobacterial growth (*Mycobacterium smegmatis*, *Mycobacterium aurum* and *Mycobacterium bovis* BCG). This metabolite is not toxic, accumulates within the macrophage and inactivates the bacilli with a comparable efficiency to that of the pure commercial antimycobacterial substance Amikacin.

Key words: Drug resistance of tuberculosis bacilli, mycobacteria, antimycobacterial agents, *Bacillus pumilus*.

INTRODUCTION

At the beginning of the twenty first century, tuberculosis remains a disease of the present. It is the primary cause of infectious mortality in the world (Billy and Perronne, 2004). In 2007, the number of new tuberculosis cases was estimated at about 9.7 million and the number of deaths at 1.8 million (WHO, 2009). Although the disease is ubiquitous, 80% of the worlds' tuberculosis cases have been registered in Asia (55%) and Africa (31%) (WHO, 2009). The causes of the failure to control tuberculosis are multi-factorial and principally due to the advent of AIDS (acquired ammuno deficiency syndrome), the relative efficiency of the bacillus calmette-guerin (BCG) vaccine (Billy and Lévy-Bruhl, 2007) as well as the expansion of *Mycobacterium tuberculosis* multi and extensive drug resistant strains (Danilchanka et al., 2008; Gruber et al., 2008). Acquired drug resistance has been favored by the length of therapeutic management and the inappropriate use of antibiotics. Mono-resistance to antituberculosis drugs is not particularly a threat whereas multidrug resistance to drugs used in standard management schemes is of major concern (Migliori et al., 2002).

Consequently, along with synthetic drugs, the search for natural antibiotics from novel sources, such as micro-organisms from extreme biological niches, is primordial in the fight against this dreadful disease (Dharmarajan et al., 2006).

The antibiotics used today are derived principally from a restricted group of microorganisms of the genera *Penicillium*, *Streptomyces*, *Cephalosporium*, *Micromonospora* and *Bacillus*. In the pharmaceutical industry, several highly important antimicrobial peptides, such as Polymyxin, Gramicidin, Tyrocidine, Subtilin, Bacilysin etc., are produced by species from the genus *Bacillus* (Katz and Demain, 1977; Mendo et al., 2004).

The majority of these antibiotics are peptides of low molecular weight, produced via a nonribosomal biosynthetic pathway that depends on specific enzymes

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called peptide synthetases. These peptides possess a large range of biological activity (Nicolas and Mor, 1995). They are mainly active against Gram positive bacteria; nevertheless, compounds such as Polymixin, Colistin and Circulin inhibit Gram negative organisms, while bacillomycin, mycobacillin, and fungistatin are active against yeasts and fungi (Katz and Demain, 1977).

The present investigation describes the isolation of a strain of *Bacillus pumilus* that secretes a metabolite of protein nature showing antimycobacterial activity. This noncytotoxic metabolite is also capable of accumulating inside macrophages, resisting the hostile conditions within the phagocytes and specifically inactivating the bacilli with efficiency comparable to that of Amikacin.

MATERIALS AND METHODS

Bacteria and culture conditions

The mycobacteria used include:

1. *Mycobacterium aurum* A^+: A nonpathogenic mycobacteria with a generation time of approximately 6 h. *M. aurum* is considered a surrogate model that can be used to evaluate the effect of substances the on growth of *M. tuberculosis* (Chung et al., 1995);
2. *Mycobacterium smegmatis* MC^2 155: Atypical nonpathogenic mycobacteria with a generation time of approximately 3 h;
3. *Mycobacterium bovis* BCG IPP: The vaccine strain;
4. *Escherichia coli* DH5α.

The mycobacteria were grown on Löwenstein Jensen (LJ) and Sauton media, at 37°C (Allen, 1998; Papa et al., 1987).

The *E. coli* strain was cultivated on Luria Bertani (LB) medium (peptone (Biokarps diagnostics, Beauvais, France), 10 g/l; yeast extract (Biokarps diagnostics, Beauvais, France), 5 g/l; Sodium Chloride (Riedel- de Haën, Seelze, Germany), 10 g/l).

Screening for bacterial strains with antimycobacterial activity

Soil samples were collected from selected Moroccan biotopes. The samples were collected, after the removal of the first centimeters of soil, to a depth of 5 to 10 cm. These were placed, with the aid of a sterile spatula, over a sterile aluminum sheet for removal of gross debris (stones etc.) after which they were poured into a sterile vial for transport to the laboratory where they were treated independently for bacterial isolation as follows: Four grams of soil were mixed into 36 ml of sterile saline (NaCl 9 g/l) and vortexed for 2 h. A series of decimal dilutions of this suspension (from 10^{-1} to 10^{-7}) were prepared in LB broth. An aliquot of 100 µL of each dilution was spread over LB-agar previously inoculated with *M. aurum* A+ or *M. smegmatis* cultures of approximately 10^6 CFU/ml. After incubition at 37°C for 48 h, bacterial clones surrounded by an inhibition zone were subcultured. One of the isolated clones having shown important activity was selected for the following study.

Identification of the antimycobacterial producing strain

The identification of the isolate showing antimycobacterial activity was carried out using polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA gene. For this, genomic DNA was extracted using standard methodology (Marmur, 1961). Universal primers were used to amplify the 16S rDNA, fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP2 (5'TACGGCTACCTTGTTACGACTT 3') (Weisberg et al., 1991). These primers can be used to amplify the eubacterial 16S rRNA gene (Weisberg et al., 1991), including from the genus *Bacillus* (Weisberg et al., 1991; Drancourt et al., 2000). For PCR amplification the following program was used as follows : 94°C for 5 min, 35 cycles of (94°C for 30 s, 55°C for 45 s, 72°C for 1min 30 s), 72°C for 10 min. The PCR products were then sequenced using the ABI PRISM sequencer (ABI Prism 310 genetic analyser, applied biosyste). The 16S rRNA sequences obtained were compared to those in the Gen Bank, EMBL, DDJB and PDB databases using the BLAST N 2.2.11 program of the national center for biotechnology information (NCBI) website.

Extraction of the antimycobacterial substances by organic solvents

In order to show that the antimycobacterial effect of the bacterial isolate was due to secreted substances, an extraction procedure using two organic solvents (Ethyl Acetate and Ether) was used according to the following protocol:

The isolate was cultured under agitation in 100 ml of LB broth for 24 h at 37°C. After centrifugation at 6000 rpm for 10 min, the supernatant was recovered then filtered using a filter of 0.22 µm of porosity. The filtrate was added to an equal volume of organic solvent and kept under agitation at ambient temperature for 1 h. The extract obtained was evaporated under vacuum at 37°C using a rotary evaporator. The dried residue was dissolved in 1 ml of sterile distilled water, filtered and used to measure the antimycobacterial activity using the "well method" as follows. Aliquots of 100 µl from mycobacterial cultures (*M. aurum* A+, *M. smegmatis*), containing approximately 10^6 CFU/ml, were spread on LB-agar where wells, 6 mm in diameter, had previously been cut. 100 µl of the prepared organic extract was deposited in the wells. The plates were incubated at 37°C. The diameters of the inhibition zones were measured after 48 h of incubation at 37°C. The test was repeated three times.

Experimental controls included LB-agar plates inoculated with the same mycobacterial strains but when the wells were filled with 100 µl of an *E. coli* culture extract prepared in an identical manner as described for the bioactive isolate.

Characterisation of the protein nature of the antimycobacterial metabolite

Purification of the antimycobacterial metabolite was carried out according to the method described by Wu et al. (2005). The isolate was cultured under agitation in 100 ml of LB broth for 24 h at 37°C. After centrifugation of the culture at 6000 rpm for 10 min, the supernatant was recovered and filtered. Precipitation using a saturated solution of Ammonium Sulfate (80% ammonium sulfate) was carried out overnight under agitation at 4°C. After centrifugation at 10000 rpm for 30 min, the pellet was suspended in 2 ml of Potassium Phosphate buffer (pH 6; 50 mM) and dialysed against the same buffer at 4°C for 24 h. The buffer was substituted several times to improve dialysis efficiency. The antimycobacterial activity of the dialysate was tested against *M. aurum* A+ using the diffusion method.

The precipitated metabolite was tested regarding its sensitivity to degradation by proteinase K at a final concentration of 1 mg/ml (Wu et al., 2005). After addition of proteinase K, the sample was incubated at 37ºC for 3 h. Experimental controls included: samples prepared under the same conditions except for using 100 ml of LB broth either without inoculums or inoculated with *E. coli*, the extract

without enzyme treatment and the enzymatic solution alone. The antimycobacterial effect of the different preparations was examined using the well method against *M. aurum* A+. The tests were repeated three times.

Study of the inhibitory activity of the antimycobacterial metabolite against *M. smegmatis* hosted by macrophages

Murin peritoneal macrophages isolation

Murin peritoneal macrophages were isolated according to the protocol described by Sosunov et al. (2007). After the anesthesia of the mouse, 20 ml of cold Hanks balanced salt solution was injected into abdomen. After 15 min, a short abdominal incision was performed and the cellular suspension recovered by aspiration using a syringe. After a washing step using the Hanks' balanced salt solution, the cells were suspended in RPMI-1640 medium supplemented with 2% fetal calf serum. The cellular suspension at a density of 5.10^5 cells per well was added to 96-well microtitration plates and incubated for 2 h at 37°C in 5% CO_2 atmosphere for cellular adhesion. Macrophage viability, determined using the trypan bleu exclusion method, was 92%.

Cytotoxicity assay

Cytotoxicity of the antimycobacterial metabolite was evaluated by neutral red uptake by the murine peritoneal macrophages (Repetto et al., 2008). Only live cells are able to fix neutral red. The macrophages were seeded into 96-well culture plates containing RPMI-1640 medium at a density of 5.10^5 cells per well in the presence of the antimycobacterial metabolite (crude protein extract obtained by Ammonium Sulfate precipitation), 0.25% sodium dodecyl sulphate (SDS) (used as a cytotoxic control) or the protein extract from the LB broth without inoculums. After 72 h incubation, the wells were washed with Hanks balanced salt solution, and then filled with a 0.075% neutral red solution in RPMI-1640 medium. After 2 h of incubation, the wells were again washed until the all traces of the dye were eliminated and the absorbance at 540 nm of each well was read on a plate reader.

Infection of macrophages by *M. smegmatis*

Adherent macrophages in the wells of the 96-well microtitration plates were infected by 7.10^5 bacilli/well. *M. smegmatis* phagocytosis was favored by incubation of the macrophages for 4 h at 37°C in 5% CO_2 atmosphere (Rastogi et al., 1987). After phagocytosis, the culture medium was removed from the wells, and the cells were treated with 10 µg/ml amikacin for 1 h. This treatment was followed by several washings, with Hanks balanced salt solution, to eliminate extracellular bacilli (Sharbati-Tehrani et al., 2005; Rastogi et al., 1991; 1987).

Protein extracts activity against *M. smegmatis* hosted by macrophages

Crude protein extract, obtained by ammonium sulfate precipitation, in solution in RPMI-1640 medium was added to the wells containing the *M. smegmatis* infected macrophages. Intercellular persistence was determined by counting mycobacterial colony forming units (CFU) at 4, 12, 24, 48, 72 and 96 h after the adding of the antimycobacterial metabolite. For this, intracellular mycobacteria were recovered after lysis of the macrophages using 0.25% SDS solution. After centrifugation, the SDS was eliminated and the bacilli suspension plated on LB-agar. CFU counts initiated after 72 h of incubation at 37°C. As described by Rastogi et al. (1987), and confirmed by our work, the SDS did not show any effect on mycobacterial viability. Results were expressed as the proportion of bacilli surviving treatment by the bioactive metabolite. These results were compared to those obtained using untreated macrophages or macrophages treated with amikacin at 200 ug/mL (Sharbati-Tehrani et al., 2005).

RESULTS AND DISCUSSION

Isolation of a bacterial strain secreting a metabolite with antimycobacterial effect

The bacterial isolate produced an inhibition zone in cultures of *M. aurum* A+ and *M. smegmatis* on LB agar. The filtrate, from a subculture of this clone, confirmed this result showing that the isolate secrets an active substance capable of inhibiting mycobacterial growth (Table 1). Results in Table 1, also show that the organic solvents used (Ethyl Acetate and Ether) were able to extract and concentrate the active substance from the filtrate. The extracts were also active against *M. bovis* BCG.

Identification of the isolate

The identification of the isolate, showing the observed activity, was carried out by 16S rDNA sequencing. This molecular approach is the most currently used in bacterial phylogeny (Woo et al., 2008; Mignard and Flandrois, 2006; Woese et al., 1990). It has led to the creation of large databases (Van de Peer et al., 1999; Maidack et al., 1996) and its application has been used in the identification of environmental bacteria as well as “virtual” bacteria impossible to grow in culture (Tortoli, 2003; Relman et al., 1992; Strous et al., 1999). This identification assigned the isolate to the genus *Bacillus* showing 100% similarity with *B. pumilus* (Accession number: HQ625388).

Bacillus is highly ubiquitous in soil, and is comprised of several species synthesizing a considerable number of antibiotics with different chemical structures (Wu et al., 2005). Amongst these species, we mention *Bacillus subtilis* (more than 66 different peptides used for their antibacterial activity), *Bacillus brevis* (approximately 23 molecules), *Bacillus licheniformis*, *Bacillus polymyxa*, *B. pumilus,* and *Bacillus circulans* (Katz and Demain, 1977).

Characterization of the protein nature of the active substance from the *B. pumilus* isolate

The antimycobacterial metabolite, purified by ammonium sulfate precipitation, was capable of inhibiting *M. aurum A+* growth (Table 2). This activity is lost in the presence

Table 1. Antimycobacterial activity of the bacterial isolate (measured in mm).

Microorganism	Filtrate	Ethyl acetate extract	Ether extract
M. smegmatis	9 ± 0	28 ± 0.5	22 ± 1
M. aurum A+	10.5 ± 0.7	29.5 ± 0.7	25 ± 0

Data are average values of triplicate ± standard deviation.

Table 2. Characterization of the protein nature of the antimycobacterial metabolite.

Variables	Inhibition diameters against *M. aurum* A+ (mm)				
	Without treatment with proteinase K	After treatment with proteinase K	Controls		
			T1	T2	T3
ASP	31 ± 1.4	0	0	0	0
EAE	29.5 ± 0.7	0	0	0	0
EE	25 ± 0.0	0	0	0	0

ASP: Ammonium sulfate precipitate; EAE: Ethyl Acetate extract; EE: Ether extract; T1: Ammonim sulphate precipitate of sterile LB medium; T2: Ammonim sulphate precipitate of LB medium inoculate with *E. coli*; T3: proteinase K solution. Data are average values of triplicate ± standard deviation.

of proteinase K. These results were also obtained with the Ethyl Acetate and Ether extracts (Table 2), showing that the active substance, responsible for the observed activity, is of protein nature. This is in agreement with bibliographical data showing that *Bacillus* produces antimicrobial substances of protein nature (Wu et al., 2005). These molecules are essentially active against Gram-positive bacteria, nevertheless compounds such as polymixin and colistin inhibit Gram-negative forms (Bottone and Peluso, 2003), whereas Bacillomycin, Mycobacillin, and Fungistatin are active against yeasts and fungi (Katz and Demain, 1977). Bottone and Peluso (2003) have reported the inhibition of species from the genera *Mucoraceae* and *Aspergillus* by an antifungal compound produced by *B. pumilus*. This species also showed antibacterial effects inhibiting *Staphylococcus aureus* and d'*Enterococcus faecalis* resistant strains through a Bacteriocin Pumilicin 4 (Aunpad and Na-Bangchang, 2007). This species is also known for the elaboration of other antimicrobial peptides such as Micrococcin P, Pumilin and Tetain (Katz and Demain, 1977). Our work described for the first time was the antimycobacterial activity of a strain of *B. pumilus* isolated from Moroccan soil. The activity was shown to be due to a metabolite of protein nature for which the structure will be determined in subsequent research.

Cytotoxicity assay

Amongst the hundreds of natural antibiotics, only few are sufficiently nontoxic for clinical use. For this reason, the cytotoxicity of the antimycobacterial agent was evaluated as well as its effect on mycobacteria within macrophages. As shown in Figure 1, peritoneal macrophages treated with the antimycobacterial substance or the protein extract from LB medium (crude protein extract obtained by ammonium sulfate precipitation) fixed neutral red in the same manner as untreated macrophages. However, macrophages treated with SDS were almost all lysed and did not fix neutral red. These results (Figure 1) indicate that the active substance of the isolated strain did not exert cytotoxic activity against the peritoneal macrophages.

Study of the activity of the antimycobacterial metabolite against *M. smegmatis* hosted by macrophages

Figure 2 shows results of the effect of the metabolite against *M. smegmatis* infecting macrophages. In the absence of this metabolite, the elimination of intracellular bacilli requires more than 4 days. In the presence of amikacin or of the active substance produced by the *B. pumilus* strain, the elimination of *M. smegmatis* is much more rapid. Indeed, the majority of the bacilli are inactivated within 24 h in the presence of the metabolite. These results (Figures 1 and 2; Tables 1 and 2) show that the active substance is effective not only *in vitro* but also in the intracellular environment.

Research for new antimicrobial agents remains the most promising approach to the problem of emerging mycobacterial infections evermore resistant to available antibiotics. In this study we have isolated a strain of *B. pumilus* capable of inhibiting mycobacterial growth (*M. smegmatis*, *M. aurum* A+ and *M. bovis* BCG) through the action of a protein metabolite. This metabolite is nontoxic, capable of accumulating within the macrophage, resisting the intracellular environment of the phagocytes and

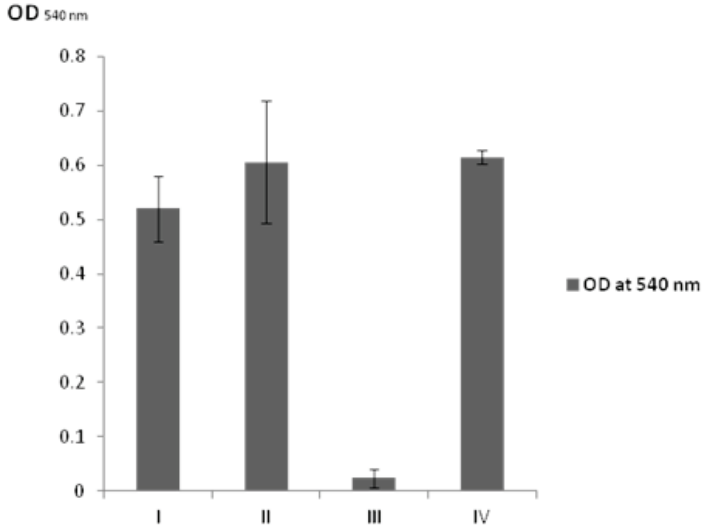

Figure 1. The cytotoxicity assay
I: peritoneal macrophages treated with the antimycobacterial substance.
II: peritoneal macrophages treated with LB medium protein extract.
III: peritoneal macrophages treated with SDS.
IV: Untreated peritoneal macrophages.
The Student t test revealed significant difference between groups (I, II, IV) and the group III.

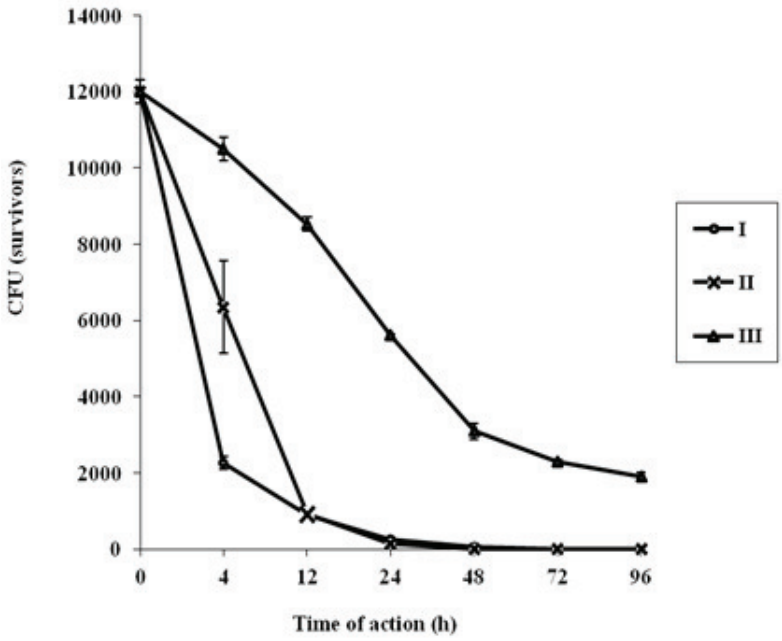

Figure 2. Action of the antimycobacterial substance from *B. pumilus* against *M. smegmatis* hosted by peritoneal macrophages
I: CFU of *M. smegmatis* surviving inside macrophages after treatment with *B. pumilus* metabolite.
II: CFU of *M. smegmatis* surviving inside macrophages after treatment with Amikacin.
III: CFU of *M. smegmatis* surviving inside untreated macrophages.
The Student test revealed significant difference between groups (I, II) and the group III.

inactivating the bacilli with efficiency comparable to the pure commercial antimycobacterial substance Amikacin. The structure and mode of action of the metabolite from *B. pumilus* will be the subject of further studies.

REFERENCES

Allen BW (1998). Mycobacteria: general culture methodology and safety considerations. Methods. Mol. Biol., 101: 15-30.

Aunpad R, Na-Bangchang K (2007). Pumilicin 4, A Novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* Strain WAPB4. Curr. Microbiol., 55(4): 308–313.

Billy C, Lévy-Bruhl D (2007). BCG vaccine and place of tuberculin skin test in 2006. Rev. Med. Interne., 28 (3): 151-160.

Billy C, Perronne C (2004). Tuberculosis in children and adults: clinical features and treatment. EMC - Maladies Infectieuses, 1(2): 81-98.

Bottone EJ, Peluso RW (2003). Production by *Bacillus pumilus* (MSH) of an antifungal compound that is active against *Mucoraceae* and *Aspergillus* species: preliminary report. J. Med. Microbiol., 52(Pt1): 69–74.

Chung GA, Aktar Z, Jackson S, Duncan K (1995). High throughput screen for detecting antimycobacterial agents. Antimicrob. Agents. Chemother., 39(10): 2235-2238.

Danilchanka O, Mailaender C, Niederweis M (2008). Identification of a novel multidrug efflux pump of *Mycobacterium tuberculosis*. Antimicrob Agents. Chemother., 52(7): 2503–2511.

Dharmarajan S, Perumal Y, Ruth VD (2006). Synthesis, *in vitro* and *in vivo* antimycobacterial activities of diclofenac acid hydrazones and amides. Bioorg. Med. Chem., 14(9): 3113–3118.

Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D (2000). 16S Ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates J. Clin. Microbiol., 38(10): 3623-3630.

Gruber A, Durham AM, Huynh C, del Portillo, HA (2008). Bioinformatics in tropical disease research: a practical and case-study approach. Editors Bethesda (MD): National Library of Medicine (US), NCBI.

Katz E, Demain AL (1977). The peptide antibiotics of *Bacillus*: chemistry, biogenesis and possible functions. Bacteriol. Rev., 41(2): 449–474.

Maidack BL, Olsen GJ, Larsen N, Overbeek R, McCaughey MJ, Woese CR (1996). The ribosomal database project (RDP). Nucleic. Acids. Res., 24(1): 82–85.

Marmur J (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol., 3(2): 208-218.

Mendo S, Faustino NA, Sarmento AC, Amado F, Moir AJ (2004). Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus licheniformis* strain. Biotechnol. Lett., 26(2): 115-9.

Migliori GB, Fattorini L, Vaccarino P, Besozzi G, Saltini C, Orefici G, Iona E, Matteelli A, Fiorentini F, Codecasa LR, Casali L, Cassone A (2002). SMIRA (Italian Multicentre Study on resistance to Anti-tuberculosis drugs) Study Group. Prevalence of resistance to anti-tuberculosis drugs: results of the 1998/99 national survey in Italy. Int. J. Tuberc. Lung. Dis., 6(1): 32–8.

Mignard S, Flandrois JP (2006). 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. J. Microbiol. Methods, 67(3): 574-581.

Nicolas P, Mor A (1995). Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. 49: 277– 304.

Papa F, Rivière M, Fournié JJ, Puzo G, David H (1987). Specificity of a *Mycobacterium kansasii* phenolic glycolipid (Mycoside A) immunoserum. J. Clin. Microbiol., 25(12): 2270-2273.Rastogi N, Labrousse V, Goh KS, De Sousa JP (1991). Antimycobacterial spectrum of sparfloxacin and its activities alone and in association with other drugs against *Mycobacterium avium* complex growing extracellularly and intracellularly in murine and human macrophages. Antimicrob. Agents. Chemother., 35(12): 2473-2480.

Rastogi N, Potar MC, David HL (1987). Intracellular growth of pathogenic mycobacteria in the continuous murine macrophage cell line J-774: Ultrastructure and drug-susceptibility studies. Curr. Microbiol., 16(2): 79-92.

Relman DA, Schmidt TM, MacDermott RP, Falkow S (1992). Identification of the uncultured *Bacillus* of Whipple's disease. N. Engl. J. Med., 327(5): 293–301.

Repetto G, del Peso A, Zurita JL (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat. Protoc. 3(7): 1125-1131.

Sharbati-Tehrani S, Stephan J, Holland G, Appel B, Niederweis M, Lewin A (2005). Porins limit the intracellular persistence of *Mycobacterium smegmatis*. Microbiology, 151(Pt7): 2403-2410.

Sosunov V, Mischenko V, Eruslanov B, Svetoch E, Shakina Y, Stern N, Majorov K, Sorokoumova G, Selishcheva A, Apt A (2007). Antimycobacterial activity of bacteriocins and their complexes with liposomes. J. Antimicrob. Chemother., 59(5): 919–925.

Strous M, Fuerst JA, Kramer EH, Logemann S, Muyzer G, Van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MS (1999). Missing lithotroph identified as new planctomycete. Nature, 400(6743): 446-449.

Tortoli E (2003). Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin. Microbiol. Rev., 16(2): 319-354.

Van de Peer Y, Robbrecht E, de Hoog S, Caers A, De Rijk P, De Wachter R (1999). Database on the structure of small subunit RNA. Nucleic. Acids. Res., 27(1): 179-183.

Weisberg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol., 173(2): 679-703.

WHO (2009). Report 2009 - Global tuberculosis control. Epidemiology, strategy, financing. www.who.int/tb/publications/global report/2009

Woese CR, Kandler O, Wheelis ML (1990). Towards a natural system of organisms: proposal for the domains Archae, Bacteria and Eukarya. Proc. Natl. Acad. Sci. USA, 87(12): 4576-4579.

Woo PC, Lau SK, Teng JL, Tse H, Yuen KY (2008). Then and now: Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin. Microbiol. Infect., 14(10): 908-934.

Wu S, Jia S, Sun D, Chen M, Chen X, Zhong J, Huan L (2005). Purification and characterization of two novel antimicrobial peptides Subpeptin JM4-A and Subpeptin JM4-B produced by Bacillus subtilis JM4. Curr. Microbiol., 51(5): 292-296.