AIX-MARSEILLE UNIVERSITÉ
FACULTÉ DE MÉDECINE – LA TIMONE
ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

THÈSE DE DOCTORAT
Présentée par

Monsieur **Otmane Lamrabet**

En vue de l'obtention du grade de Docteur d'Aix-Marseille Université en Pathologies Humaines
Mention Pathologies Humaines
Spécialité : Maladies Transmissibles et Pathologies Tropicales

Modifications génétiques de *Mycobacterium tuberculosis* : interactions avec les organismes hôtes.

Soutenue le 25 Septembre 2012

COMPOSITION DU JURY

Pr. Jean-Louis Mège	**Président de Jury**
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Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes;
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AVANT PROPOS

Le format de présentation de cette Thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier Raoult

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RÉSUMÉ

Les mycobactéries sont classées parmi les bactéries contenant des acides mycoliques dans leur paroi et un haut GC% dans leur génome. Elles peuvent être isolées à partir du sol ou d'environnement d'eau douce où vivent aussi les protozoaires libres. Plusieurs études ont montré une possibilité de co-isolement des mycobactéries et des amibes à partir de ces sources environnementales. Il a été montré également que la plupart des mycobactéries de l'environnement ont la capacité à survivre dans les trophozoites et les kystes d'amibes et dans certaines cellules eucaryotes, y compris les macrophages. Les manipulations génétiques des mycobactéries en général et des mycobactéries du complexe *Mycobacterium tuberculosis* en particulier sont compliquées et aucune étude de modification génétique des mycobactéries (pathogènes ou non pathogènes) n'avait été réalisée dans notre laboratoire avant notre travail de thèse.

Dans notre travail de thèse, nous avons montré que les amibes ou d'autres organismes phagocytaires peuvent servir comme sources et lieu de transfert des gènes chez les mycobactéries. Ce transfert des gènes peut avoir contribué à l'adaptation des mycobactéries à un mode de vie intracellulaire. Nous avons développé ensuite deux systèmes de coculture: *Mycobacterium smegmatis-Acanthamoeba polyphaga* et *Mycobacterium gilvum-A. polyphaga* et nous avons clarifié le spectre des interactions des mycobactéries à croissance rapide avec les amibes. Ce modèle d'interaction mycobactéries-amibes a été utilisé pour tester l'hypothèse contraire au paradigme dominant que l'addition

des gènes réduit la virulence des bactéries. Pour la première fois dans notre laboratoire, nous avons modifié deux espèces du complexe *M. tuberculosis*, *M. tuberculosis* H37Rv et *Mycobacterium bovis* BCG pour observer l'effet de ces changements sur leur pathogenicité et leur survie. De façon intéressante, nous avons observé que l'expression du gène *mspA* de *M. smegmatis* dans *M. tuberculosis* H37Rv et *M. bovis* BCG permet une augmentation significative de leur temps de croissance dans un milieu axénique, mais une diminution significative de leur survie dans les amibes et les macrophages, et une diminution de leur virulence dans un modèle souris.

En conclusion, l'addition des gènes plutôt que leur suppression peut être une autre façon de modifier le comportement des mycobactéries pathogènes. Ceci pourrait être utilisé comme une base pour un vaccin vivant atténué contre la tuberculose.

Mots clés: Mycobactéries, *Mycobacterium tuberculosis, Mycobacterium smegmatis, Mycobacterium gilvum*, amibes, cellules eucaryotes, transfert des gènes, modifications génétiques, porine MspA.

SUMMARY

Mycobacteria are mycolic-acid containing, high GC% bacterial organisms which can be recovered from soil and fresh water environments where free-living protozoa also live. Co-isolation of mycobacteria and amoeba collected from such environmental sources has been reported. Several experiments further demonstrated the ability of most environmental mycobacteria to survive in the amoebal trophozoites and cysts and in some eukaryotic cells including macrophages. Genetic modification of mycobacteria in general and mycobacteria belonging to *Mycobacterium tuberculosis* complex in particular are complicated and no studies using genetic modification of mycobacteria (pathogenic or non-pathogenic) had been performed in our laboratory prior to our work.

In our thesis work, we showed that amoebae or other phagocytic organisms can serve as sources and places for gene transfers in mycobacteria. Gene transfers may have contributed to the adaptation of mycobacteria to an intracellular lifestyle. In addition, we developed two co-culture systems: *Mycobacterium smegmatis-Acanthamoeba polyphaga* and *Mycobacterium gilvum-A. polyphaga* and we clarified the spectrum of rapid-growing mycobacteria and amoeba interactions. This model of mycobacteria-amoeba interactions was then used to test another hypothesis according to which unlike the prevailing paradigm, the addition of genes does not reduce the virulence of bacteria. For the first time in our laboratory we modified two species of the *M. tuberculosis* complex, *M. tuberculosis* H37Rv and *Mycobacterium*

bovis BCG to observe the effect of these changes on their pathogenicity and survival. Very interestingly we found that the expression of *M. smegmatis mspA* gene in *M. tuberculosis* H37Rv and *M. bovis* BCG increased significantly their time of growth in an axenic medium but decreased significantly their survival in amoeba and macrophages and their virulence in a mouse model.

In conclusion, gene addition rather than gene suppression may be another way to change the behaviour of mycobacteria in general and especially pathogenic species. The concept of using genetic engineering to add genes in the genome of *M. tuberculosis* could be used as a basis for attenuated vaccine against tuberculosis.

Keywords: Mycobacteria, *Mycobacterium tuberculosis, Mycobacterium smegmatis, Mycobacterium gilvum*, amoeba, eukaryotic cells, gene transfer, genetic modification, MspA porin.

INTRODUCTION ET OBJECTIFS

Introduction et objectifs

Les mycobactéries sont des bactéries du phylum des Actinomycètes, phylogénétiquement classées parmi les bactéries Gram-positive avec un haut GC%. Ce genre bactérien polymorphe comporte des mycobactéries à croissance rapide (moins de 7 jours pour produire des colonies visibles en sous-culture) et des mycobactéries à croissance lente (7-60 jours pour produire des colonies visibles en sous-culture dont *Mycobacterium tuberculosis* qui fait l'objet de notre travail) (Embley *et al.,* 1994). Ces bactéries comportent des espèces environnementales (sols, sources d'eau) saprophytes et des espèces associées à l'homme et aux animaux commensales ou pathogènes (Thomas *et al.,* 2006; Thomas *et al.,* 2008; Narang *et al.,* 2009). Les mycobactéries à croissance rapide comportent essentiellement des mycobactéries de pathogénicité inconnue ou pathogènes opportunistes, alors que les mycobactéries à croissance lente comportent des mycobactéries pathogènes pour l'homme, telles que les mycobactéries du complexe *Mycobacterium tuberculosis* responsables de la tuberculose, *Mycobacterium leprae* responsable de la lèpre et *Mycobacterium ulcerans* responsable de l'ulcère de Buruli (Taylor *et al.,* 2003; Mba Medie *et al.,* 2011).

Plusieurs études ont montré que les mycobactéries peuvent être isolées à partir d'environnement d'eau douce où vivent aussi les amibes libres (trophozoites unicellulaires) (Greub *et al.,* 2004; Thomas *et al.,* 2007). Egalement, plusieurs études expérimentales ont

montré la capacité des mycobactéries à survivre dans les trophozoites et dans les kystes d'amibes (Adekambi *et al.,* 2006; Ben Salah *et al.,* 2009; Mba Medie *et al.,* 2011).

Les manipulations génétiques des mycobactéries en général et des mycobactéries du complexe *M. tuberculosis* en particulier sont compliquées par leurs temps de génération supérieur à 15 heures et la présence d'une pellicule dense « capsule » qui pourrait empêcher l'introduction efficace de l'ADN à l'intérieur de la mycobactérie (Hatfull GF, 1993; Clark-Curtiss et Haydel, 2003 ; Daffe et Draper, 1998 ; Daffe et Etienne, 1999). Aucune étude de modification génétique des mycobactéries (pathogènes ou non pathogènes) n'avait été réalisée dans notre laboratoire avant notre travail de thèse.

Ce travail de thèse a comporté cinq parties et a consisté à étudier principalement les interactions entre les mycobactéries et les différents organismes hôtes. Dans un premier temps, nous avons effectué une revue de la littérature scientifique afin de résumer les techniques récentes qui ont été utilisées pour modifier et transformer les mycobactéries. Nous avons comparé ces techniques entre elles et nous avons discuté sur le rôle de ces outils génétiques dans des futures applications. (Lamrabet et Drancourt, Tuberculosis 2012, in-press).

En effet, les mycobactéries peuvent échanger naturellement des gènes par transfert horizontal à partir des eucaryotes mais aussi à partir des alpha- et gamma-Protéobactéries et d'autres Actinomycètes (Kinsella *et al.,* 2003; Marri *et al.,* 2006; Becq *et al.,* 2007). Néanmoins, les sources de ces échanges restaient inconnues.

Différents travaux avaient montré que les amibes étaient des lieux d'échanges génétiques entre microorganismes (Ogata *et al*., 2006; Saisongkorh *et al.,* 2010; Moliner *et al*., 2010; Thomas et Greub, 2010; Raoult et Boyer, 2010). Sur ces bases, nous avons analysé les génomes de 15 espèces de mycobactéries pour détecter des transferts horizontaux avec d'autres micro-organismes sympatriques ou avec leur propre hôte. Nos analyses phylogénétiques vérifiées expérimentalement ont montré que les mycobactéries ont échangé des gènes avec d'autres micro-organismes et que les protistes en général ont été les sources de ces transferts (Lamrabet *et al.,* PLoS ONE 2012a).

Plusieurs études avaient montré les interactions entre les mycobactéries à croissance lente avec les amibes (Ben Salah et Drancourt, 2010; Felix *et al.*, 2011) mais les interactions des mycobactéries à croissance rapide avec les amibes sont restées mal comprises (Krishna Prasad et Gupta, 1978; Cirillo *et al.,* 1997; Tenant et Bermudez, 2006). Par exemple, des résultats contradictoires ont été publiés concernant *Mycobacterium smegmatis*, allant de sa survie dans les amibes (Krishna Prasad et Gupta, 1978; Tenant et Bermudez, 2006) jusqu'à sa destruction par les amibes (Cirillo *et al.,* 1997; Sharbati-Tehrani *et al.,* 2005). Aucune étude n'avait été publiée sur l'interaction entre *Mycobacterium gilvum* et les amibes libres. Nous avons utilisé ces deux espèces de mycobactéries à croissance rapide comme des espèces modèles pour évaluer les interactions entre les mycobactéries à croissance rapide et les amibes. Nous avons

développé deux systèmes de coculture (*M. smegmatis-Acanthamoeba polyphaga* et *M. gilvum-A. polyphaga*) qui nous ont permis d'éclaircir le spectre des interactions entre les mycobactéries à croissance rapide et les amibes libres. Nous avons constaté que *M. smegmatis* pénètre et survit dans les amibes plus de cinq jours de coculture, entraînant la lyse des amibes et la libération des mycobactéries viables. En plus, nous avons remarqué que le culot ou le surnagent des amibes lysés permettent une augmentation significative de la croissance de cette mycobactérie (Lamrabet *et al.*, PLoS ONE 2012b). Dans un deuxième travail, nous avons constaté que *M. gilvum* pénètre et survit, mais ne se multiplie pas dans les amibes contrairement à *M. smegmatis.* Nous avons remarqué qu'il existe une corrélation significative entre la taille des mycobactéries à croissance rapide et leur capacité à tuer les amibes. Les mycobactéries à croissance rapide avec une taille de plus de 2 µm telles que *M. smegmatis* détruisent les amibes tandis que celles d'une taille inférieure à 2 µm telles que *M. gilvum* ne tuent pas les amibes (Lamrabet et Drancourt, soumis à Applied Environmental Microbiology).

Ce modèle d'interaction amibes-mycobactéries a été ensuite utilisé pour tester une autre hypothèse suivant laquelle l'ajout des gènes diminuerait la virulence des bactéries, contrairement au paradigme dominant. Pour tester cette hypothèse, nous avons modifié, pour la première fois dans notre laboratoire, deux espèces du complexe *M. tuberculosis, M. tuberculosis* H37Rv et *Mycobacterium bovis* BCG. Nous avons exprimé le gène *mspA* qui code pour une

porine spécifique de *M. smegmatis* (Niederweis M, 2003) dans *M. tuberculosis* H37Rv et *M. bovis* BCG et nous avons examiné l'effet de cette modification génétique sur leur croissance en milieu axénique, leur persistance intracellulaire dans différentes cellules phagocytaires (macrophages humains et souris, amibes *A. polyphaga*) et chez la souris. Toutes ces expériences ont été réalisées dans un laboratoire de Type 3. De façon très intéressante nous avons constaté que l'expression du gène *mspA,* dans *M. tuberculosis* H37Rv et *M. bovis* BCG entraine une augmentation significative ($p \leq 0.05$) de leur temps de croissance dans un milieu axénique mais une diminution significative ($p \leq 0.05$) de leur survie dans les amibes et les macrophages (souris et humains) ainsi qu'une diminution significative ($p \leq 0.05$) de la virulence dans un modèle souris. Cette dernière partie a fait l'objet d'un brevet (N°H52 888 cas 12 FR) et d'un article en cours de rédaction.

En conclusion, l'addition des gènes plutôt que leur suppression peut être un autre moyen de modifier le comportement des mycobactéries pathogènes. Ceci peut conduire à la contribution de nouveaux vaccins.

Chapitre I

REVUE DE LITTERATURE

Genetic engineering of *Mycobacterium tuberculosis*: a review

Otmane Lamrabet et Michel Drancourt

Tuberculosis 2012. En impression

Chapitre I – Avant propos

Les techniques de génie génétique ont été utilisées pendant des décennies afin de muter et de supprimer des gènes dans le génome de *Mycobacterium tuberculosis* dans le but de comprendre l'effet de ces mutations sur leur sensibilité aux antibiotiques antituberculeux. Le développement des plasmides et des mycobacteriophages, qui peuvent transférer l'ADN dans le chromosome de *M. tuberculosis,* a efficacement surmonté les difficultés retrouvées lors des manipulations génétiques de ce pathogène, du faite de sa croissance lente, sa capsule et sa paroi d'acides mycoliques qui limitent l'absorption d'ADN. L'utilisation des techniques de génie génétique a permis d'éclaircir de nombreux aspects sur les mécanismes de la pathogenicité de cette bactérie, ainsi que d'autres mécanismes liés à la croissance cellulaire, la biosynthèse des acides mycoliques, le métabolisme, la résistance aux médicaments et la virulence. Toutefois, une telle recherche n'a pas eu un impact concret sur le développement de nouveaux vaccins ou de nouveaux médicaments pour la pratique clinique de routine. En effet, l'utilisation des outils de génie génétique est basée sur le concept sous-jacent où la modification ou la réduction du génome de *M. tuberculosis* pourrait en diminuer la virulence. Cependant, des analyses post-génomiques récentes ont indiqué que la réduction des génomes bactériens est souvent associée à une augmentation de la virulence. Par conséquent, au lieu de déléter ou de muter des gènes dans *M. tuberculosis*, l'utilisation des techniques de

génie génétique pour ajouter des gènes dans le génome de *M. tuberculosis* pourrait conduire à atténuer la virulence de *M. tuberculosis* pour la production de nouveaux vaccins contre la tuberculose.

Genetic engineering of *Mycobacterium tuberculosis*: a review

Running title: Genetic engineering of *Mycobacterium tuberculosis*

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SUMMARY

Genetic engineering has been used for decades to mutate and delete genes in the *Mycobacterium tuberculosis* genome with the translational goal of producing attenuated mutants with conserved susceptibility to antituberculous antibiotics. The development of plasmids and mycobacteriophages that can transfer DNA into the *M. tuberculosis* chromosome has effectively overcome *M. tuberculosis* slow growth rate and the capsule and mycolic acid wall, which limit DNA uptake. The use of genetic engineering techniques has shed light on many aspects of pathogenesis mechanisms, including cellular growth, mycolic acid biosynthesis, metabolism, drug resistance and virulence. Moreover, such research gave clues to the development of new vaccines or new drugs for routine clinical practice. The use of genetic engineering tools is mainly based on the underlying concept that altering or reducing the *M. tuberculosis* genome could decrease its virulence. A contrario, recent post-genomic analyses indicated that reduced bacterial genomes are often associated with increased bacterial virulence and that *M. tuberculosis* acquired genes by lateral genetic exchange during its evolution. Therefore, ancestors utilizing genetic engineering to add genes to the *M. tuberculosis* genome may lead to new vaccines and the availability of *M. tuberculosis* isolates with increased susceptibility to antituberculous antibiotics.

Keywords: *Mycobacterium tuberculosis***;** genetic engineering; vaccine; antibiotics; pathogenicity; future developments.

Introduction

Mycobacterium tuberculosis, the causative agent of human tuberculosis, infects one-third of the world's population and is responsible for 9 million new cases and 1.5 million deaths each year.[1] The HIV/AIDS epidemic coincided with the reemergence of tuberculosis in developed countries[1] and was accompanied by an alarming emergence of drug-resistant strains.[2] Accordingly, the molecular biology of *M. tuberculosis* has been the subject of extensive research in many laboratories worldwide.[3-5] Complete genome sequencing of *M. tuberculosis* H37Rv, CDC1551, H37Ra, F11, KZN1435, KZN4207, CCDC5180, CCDC5079 and CTRI-2 isolates revealed a previously underestimated diversity within *M. tuberculosis* isolates (which are now grouped into families).[6] This diversity was found to encompass cellular growth and metabolism,[3,7,8] the repertoire of virulence genes[9-12] and antibiotic resistance.[5,13,14] This information has led to the development of multivalent vaccines.[15-19] Moreover, the scheduled sequencing of more than 95 additional *M. tuberculosis* genomes (http://genomesonline.org) (Figure 1) will further refine this knowledge.

M. tuberculosis genomics confirmed lack of plasmid and disclosed horizontal genetic exchanges between unidentified *Eukarya*, environmental alpha- and gamma-*Proteobacteria* and *Actinobacteria* that may have occurred before the emergence of *M. tuberculosis* sensu stricto.[20-23] Recently, we showed that amoebae and other phagocytic organisms may have been the places in which such ancestral mycobacteria exchanged genes with other bacteria.[24] Limitations to genetic exchanges include the presence of a thick cell wall comprising of the cell membrane, mycolic acid wall and a mixture of polysaccharide, protein and lipids called "capsule".[25,26] These components cause the mycobacteria to grow as a dense pellicle and prevents efficient DNA uptake.[5] In addition, manipulation of *M. tuberculosis* exposes laboratory personnel to a risk of contamination with this deadly pathogen, which is classified as a NSB3 organism.[27] Thus, the construction of DNA vectors for *M. tuberculosis* genetic engineering has been an important issue for the last two decades. The classical genetic approach for the study of mycobacteriology has been superseded by molecular techniques, which greatly enhanced the possibilities currently available for experimental design.

We herein summarize the recent developments regarding the techniques used to modify and to transform *M. tuberculosis* by critically reviewing the following: (i) the various methods used for DNA modification, (ii) the use of selectable genetic markers and (iii) the available mycobacterial cloning vectors. In addition, we compared all techniques between them and we discuss the future applications of these genetic tools.

Methods for literature review and technical assessment

The PubMed and Medline databases were queried using the following keywords: *M. tuberculosis*, genetic modification, genetic markers, mutagenesis, short and long linear DNA substrates, UV-irradiated DNA, phagemids, vectors systems, recombineering, genes addition, amoeba, vaccines, antituberculosis, treatment, pathogenesis, animal models and cell models. References from previous reviews[4,28,29] and references from selected papers were also reviewed. In this study we used 10 criteria to compare different techniques used to modify and to transform *M. tuberculosis*: efficiency and productivity, duration of manipulator formation, duration of experiment, materials and laboratory equipments, screening methods, inoculum, versatility, steps of experiment, publications and applications. Using published data, we indicated each variable for each technique with a note varying from -1, 0 or 1. The comparison results are presented in a chip-like form with clustering of the closest techniques using Mev.4.6.2 (http://www.softpedia.com/get/Science-CAD/MeV.shtml).

The *M. tuberculosis* genome

The *M. tuberculosis* genome consists of a single circular chromosome of 4.3-4.4 kbp with an average G+C content of 65%, 4,019-4,300 open reading frames, 48-104 intergenic spacers and no plasmids. Two prophage-like elements, φRv1 and φRv2, have been annotated in the genome of *M. tuberculosis* H37Rv and CDC1551,[30,31] and we have also detected these prophage-like elements in other *M. tuberculosis* genomes. The 10,892-bp φRv1 element, which is present at two different chromosomal locations in *M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551, is a presumably mobile element that is too small to encode infectious phages.[31] The 10-kbp φRv2 element exhibits an organization similar to that of φ Rv1 and encodes several proteins with sequence similarity to the φRv1 proteins.[32] Both elements encode a fully functional integration/excision system that utilizes multiple attachment sites in slow-growing mycobacteria to move from one chromosomal position to another.[30,31] In addition, mycobacteria can be infected by both temperate and virulent mycobacteriophages. The *M. tuberculosis* genome also includes 3 CRISPR-Cas (Clustered Regularly Short Palindromic Repeats Interspaced-Cluster associated) systems, which are characterized by a series of direct, short 21- to 37-bp repeats and regularly spaced 20-40-bp sequences that are generally unique.[33] This CRISPR-Cas system protects mycobacteria against mobile genetic elements, functioning in a manner similar to the eukaryotic RNAi system. This system is regarded as a form of a adaptive and heritable immune system because it is transmitted to daughter cells and allows mycobacteria to compete with the rapid changes induced by phages

and plasmids.[33,34,35] In addition, the *M. tuberculosis* genome encodes proteins such as the acyl carriers Rv0033 and Rv1344, which play central roles in cell wall mycolic acid synthesis,[36] proteins that protect mycobacteria against reactive oxygen and nitrogen intermediates such as Sod (superoxide dismutase), AhpC (alkylhydroperoxide reductase), KatG (catalase peroxidase) and NoxR (nitric oxide reductase)[37-41] and proteins involved in various stages of host–pathogen interactions and pathogenicity such as the ESAT-63, PE and PPE family members.[42,43] Indeed, this facultative intracellular pathogen successfully survives and multiplies inside its host despite the antimicrobial effectors functions of the host immune system. In addition, the *M. tuberculosis* genome contains a large number of genes encoding proteins that are attractive targets for new anti-tubercular drugs, such as fatty acid metabolism enzymes, two-component systems, cell wall biosynthesis-related enzymes and transcription factors,[42] Mce proteins and antigens belonging to the Antigen 85 complex.[44-46]

Genetic tools for modifying the *M. tuberculosis* genome

Legitimate site-specific mutation of *M. tuberculosis* had not been possible before the 1996 report of allelic exchange mutagenesis in a specific gene using linear DNA substrates of up to 50 kb.[3] Further research yielded more efficient and less cumbersome methodologies for the genetic manipulation of *M. tuberculosis*, including mutagenesis techniques and allelic exchange techniques. Stable genomic modification has been achieved by transposon mutagenesis,[3,11,12,14,47-52] signature-tagged mutagenesis[53-56] and site-directed mutagenesis.[36,39,58-65] Allelic exchange can be performed using long and short linear DNA substrates,[3,38,45,66-68] UV-irradiated double-stranded DNA,[69-72] phagemid systems,[10,70,73-78] the screening marker lacZ,[71,72,79-82] the counter-selectable marker sacB,[7,8,51,72,73,79-91] the counter-selectable marker galK,[92] the counter-selectable marker rpsL+[28,40,93,94] and various recombineering systems.[29,95-99] In addition, techniques aimed at mutagenesis and allelic exchange can be performed using mycobacterial extrachromosomal or integrating plasmid vectors (See below).

1. Mutagenesis

1.a Transposon mutagenesis

This technique allows genes to be transferred into the *M. tuberculosis* chromosome and aims to interrupt or modify the function of a specific gene via the introduction of a single stable mutation (Figure 2A). Using transposons derived from the *Mycobacterium smegmatis* insertion sequence (IS) IS1096, transposon mutant libraries have been constructed in *M.*

tuberculosis MT103,[53] Erdman,[50,74] H37Rv[11,14,48] and CDC1551.[12] A large number of mutants, which exceeded the number theoretically required to obtain at least one insertion in every nonessential gene, were obtained, including 10^6 for MT103,[51] 8 x 10^3 [74] and 10^4 [50] for Erdman, more than 10^4,[11] 7 x 10^3 [14] and 5 x 10^3 [48] for H37Rv and 8 x 10^3 for CDC1551.[12] The *M. tuberculosis* H37Rv library that was generated with the Tn5370 transposon is currently the most complete and detailed library. In total, 1,474 insertion sites were mapped; unique insertion sites (1,329 in total) were identified, 116 insertions were detected in the same position twice, 13 were identified in the same position three times and one was found in the same position four times. Altogether, these insertions correspond to the disruption of 351 genes.[11] Other transposition approaches previously used include the simple and efficient transposon system Tn552, which was modified from *Staphylococcus aureus* (100) to carry a phoA translational fusion (Tn5520phoA),[46] the mariner-derived transposon Himar1[100-102] and the MycoMarT7 transposon system (derived from the Mycomar phage),[52,103] which was used to easily identify insertion mutations in targeted genes.[49] These transposon libraries generated thousands of mutations that required effective screening methods to analyze. In theory, studying thousands of independent, randomly selected mutants would require thousands of animal or macrophage infections. This sort of large-scale screening is obviously unrealistic and would very long and difficult to perform. Recently, the transposon site hybridation (TraSH) a simple and more quickly method was used to screen and monitor the fitness of mutants in mixed population under different conditions.[104] Latter microarray was used in different studies to identify essential gene for mice infection,[105] survival in macrophage[52] and growth of mycobacteria in different medium.[106] Also, a new technique using deep sequencing was used to characterize transposon libraries.[107] The identification and selection of particular mutants of interest or the generation of signature-tagged or site-directed mutants would therefore be most applicable for *in vivo* screening.

1.b Signature-Tagged Mutagenesis

Signature-Tagged Mutagenesis (STM), a genetic method used to study gene function that was first described by Hensel et al. in 1995 for *Salmonella enterica* Typhimurium, enables the screening of large pools of mutants that exhibit an attenuated phenotype.[108] Briefly, each mutant is tagged with a different DNA sequence such that all of the tags can be co-amplified from the DNA of mixed populations of mutants in a single round of PCR (Figure 2B). These tags can also be simultaneously labeled to provide specific probes for the detection of mutants subjected to selection.[109] Therefore, the sequence tags act as a molecular barcodes to monitor

for the presence of each mutant in a mixed population.[109,110] STM has been applied to the *M. tuberculosis* MT103,[53,57] Erdman,[54-56,111] CDC1551[101] and H37Rv[56] isolates, and three different transposons were used for these studies: Tn5367, which was derived from IS1096 and delivered by a temperature-sensitive plasmid;[53] Tn5370, which was delivered by the temperature-sensitive mycobacteriophage phAE87[54-56,111] and mini-Tn5, which was delivered by a temperature-sensitive plasmid.[57] Screening of the mutants relied on the use of mouse models and the human macrophage THP1 cell line.[57]

1.c Site-directed mutagenesis

Site-directed mutagenesis, which is also referred to as site-specific mutagenesis, creates a mutation at a defined site within the *M. tuberculosis* chromosome (Figure 2C). In general, this form of mutagenesis requires knowledge of the wild-type gene sequence. The basic procedure also requires the synthesis of a short DNA primer that contains the desired base change. This synthetic primer needs to hybridize with single-stranded DNA containing the gene of interest. The single-strand fragment is then extended via a reaction with DNA polymerase. The resulting double-stranded molecule is then introduced into an expression vector, transformed into *Escherichia coli* for propagation and screened for the presence of the mutation within the vector[60] (Figure 2C). Mutant *M. tuberculosis* proteins have also been expressed in the *E. coli* BL21, UM255, UM262 and XL-1 strains[36,39,59,61-65] and *Mycobacterium vaccae*.[58]

2. Allelic exchange substrates

2.a Short and long linear DNA substrates

Several studies reported successful *M. tuberculosis* gene disruption using short[38,45,66-68,112] and long linear DNA fragments[3] as allelic exchange substrates for homologous recombination (Figure 3A). These linear DNA substrates harboring the target gene as well as kanamycin and/or hygromycin resistance markers were transformed into *M. tuberculosis* by electroporation. Generating allelic mutants in *M. tuberculosis* H37Rv,[45,67,112] Erdman[38,68] and CSU93[68] was most widely accomplished using a short linear substrate (< 5 kb), although one study used a 40-50 kb linear substrate to create a leucine auxotrophic mutant of the *M. tuberculosis* Erdman and H37Rv isolates by interrupting *leuD* with a kanamycin resistance cassette[3] (Figure 3A). However, these transformation events were infrequent, were not reproducible and led to a high number of integration events with low homology between the targeting substrate and the bacterial chromosome.[67] While these events are rare in mycobacteria, they are common in eukaryotes, suggesting that slow-growing species of

mycobacteria such as *M. tuberculosis* may possess an unusually high level of illegitimate recombination, which is perhaps accompanied by a decreased level of homologous recombination.[67]

2.b Phagemid systems

The phagemid or phasmid is a hybrid between a phage and a plasmid, and it produces a cloning vector that grows as a plasmid in *E. coli* and is packaged as single-stranded DNA in viral particles for delivery to *M. tuberculosis.*[76] Phagemids contain an origin of replication (ori) for double-stranded replication as well as an f1 ori that enables single-stranded replication and packaging into phage particles (Figure 4). These phagemids introduce genes into the mycobacteria chromosome by site-specific recombination between the phage and bacterial attachment sites. The recombinants can be introduced into mycobacteria, where the shuttle phasmid that carries the gene(s) of interest can lysogenize and be stably maintained as a prophage (Figure 4). In 1987, Jacobs et al. constructed the first recombinant shuttle phagmids as chimeras containing mycobacteriophage DNA inserted into an *E. coli* cosmid. These shuttle vectors permitted the first introduction of foreign DNA into *M. smegmatis* and *Mycobacterium bovis* BCG but not to *M. tuberculosis.*[76] The further use of phagmids derived from the temperate L1 phage demonstrated that the *aph* kanamycin resistance gene could be used as an antibiotic selectable marker for mycobacteria.[74,113] Conditionally replicating shuttle phasmids derived from mycobacteriophages D29 and TM4 were shown to deliver transposons into the *M. tuberculosis* Erdman, H37Rv and CDC1551 strains.[73] In addition, the use of phagemids in gene replacement experiments with *M. tuberculosis* H37Rv was found to enhance homologous recombination and reduce illegitimate recombination.[70] This method has also been successfully used to create gene mutations in the *M. tuberculosis* H37Rv,[77,78,114-118] Erdman[10,75,119,120] and Beijing F2 strains.[77]

2.c UV-irradiation of double-stranded DNA

This technique is based on the UV treatment (25, 100 or 300 mJ) of plasmid DNA to mutate a target gene prior to its electroporation into *M. tuberculosis* (Figure 5). Allelic exchange was employed with UV-irradiated double-stranded DNA to mutate 11 amino acid biosynthesis genes[71] in *M. tuberculosis* H37Rv[41,69-72] and the *tlyA* and *plcABC* genes (encoding haemolysin and three phospholipases) in *M. tuberculosis* Erdman.[46,72,121] These studies demonstrated that the use of UV-irradiated double-stranded DNA enhanced the frequency of recombination.[72] UV pre-treatment was the most successful and technically the simplest method available for

generating a large number of transformants; however, recombination with a DNA-damaged vector may introduce secondary mutations into the mycobacterial chromosome.

2.d The screening marker *lacZ*

Cells containing the *lacZ* gene produce beta-galactosidase,[122] which converts X-galactose into a blue product that is visible with the naked eye. This screening method is widely used to detect the expression of a piece of DNA that has been inserted into a *lacZ*-encoding plasmid (Figure 5A). The inclusion of *lacZ* in a suicide vector facilitates the identification of colonies carrying plasmids that have integrated by single crossover events (Figure 5A). If no double crossover events have occurred, blue colonies can be selected and plated for a second recombination event (Figure 5A). However, *lacZ* screening cannot be used to determine the frequency of homologous recombination.

2.e The counter-selectable marker *sacB*

The *Bacillus subtilis sacB* gene encodes secreted levansucrase (sucrose: 2,6-b-D-fructan 6-b-D-fructosyltransferase), which catalyzes the hydrolysis of sucrose and the synthesis of levans, which are lethal to mycobacteria in the presence of sucrose (Figure 5B).[51] This marker has been used extensively for the construction of both unmarked and marked mutations in the *M. tuberculosis* MT103,[84,88] Erdman,[73,123] CSU93,[123] CDC1551,[73] 210[81] and H37Rv strains.[44,69,72,80,82,83,85,87,89,90,124-130] Using a sucrose counter-selectable suicide vector enables the positive selection of double recombinants, which considerably facilitates allelic exchange (Figure 5B). This marker is generally preferred over antibiotic sensitivity markers, but its great disadvantage is that spontaneous *sacB* mutants arise at a high frequency.[131]

2.f The counter-selectable marker *rpsL+*

This system is based on the expression of the dominant negative selectable marker *rpsL+*, which codes for ribosomal protein S12 and confers streptomycin susceptibility to a streptomycin-resistant host. Two successive components are employed for this selection: a kanamycin-resistance gene and a streptomycin-sensitive allele of the *rpsL* gene.[94] Thus, gene replacement occurs by placing the gene for positive selection inside of the target gene and by placing the gene for negative selection outside of the target gene (Figure 5C). This technique successfully generated mutants in the *M. tuberculosis* H37Rv ompATb,[93] ahpC[40] and sigM strains (reviewed in[26]). However, this system is limited because streptomycin is also used as a chemotherapeutic agent against tuberculosis.

2.g The counter-selectable marker *galK*

This system is based on the expression of the *E. coli* marker *galK* encoding galactokinase, which catalyzes the phosphorylation of d-galactose into d-galactose-1-phosphate. This enzyme also efficiently phosphorylates a galactose analogue, 2-deoxy-galactose (2-DOG), to 2-deoxy-galactose-1-phosphate, a toxic substance for mycobacteria.[92] Gene replacement is achieved by placing the *galK* gene within the target gene and then by growing the transformed mycobacteria on plates containing 2-DOG (Figure 5D). This system was used to delete the nonessential *M. tuberculosis ligD* gene, which demonstrated that gene replacement using this counter-selectable marker was more efficient than that using the *sacB* marker. Furthermore, the combination of both markers was 100% effective at selecting for recombination events.[92] Using the *galK*/*sacB* system, resistant colonies can be directly screened by Southern blot analysis, which circumvents the need for the two-step screening strategy used with *sacB*.[51]

2.h Recombineering systems

The use of the screening markers *lacZ*, *rpsL*+ or *sacB* as well as specialized phagemids has facilitated mutant recovery but also requires multiple steps of transformation and selection or the construction of different suicide vectors or phagemids. To overcome these obstacles, a method was developed to enhancing mycobacterial recombination frequencies using the mycobacteriophage-encoded recombinant system Che9c, and this greatly simplified the genetic manipulation of both fast- and slow-growing mycobacteria strains. Recombineering (recombination-mediated genetic engineering) is a genetic and molecular biology technique that is based on the use of homologous recombination systems. Initially developed in *E. coli*,[95] this technique was then applied to slow- and fast-growing mycobacteria including *M. tuberculosis*. It is used to modify the DNA of *Mycobacterium* spp. in a precise and simple manner using the mycobacteriophage Che9c-encoded homolog of RecE and RecT (genes 60 and 61) for double-stranded DNA (dsDNA) manipulation and the Che9c 61 gene for single-stranded DNA (ssDNA) manipulation[96-98] (Figure 3B). This technique represents a simple and efficient approach for performing gene replacement in *Mycobacterium* spp.[97] Recently, detailed reviews highlighted the importance of this technique and the various ways in which it can be used.[29,132]

3. Key mycobacterial vector systems

Most of the methods used to promote allelic exchange require a carrier, such as a plasmid, and the carrier must be maintained by the host, either through its insertion into the chromosome or the presence of a selectable marker. In most cases, genetic modifications to *M. tuberculosis* require transit through an easily manipulated bacterial species, such as *E. coli,* to construct the suitable vector. The vector must replicate in both bacterial species and is introduced to *M. tuberculosis* by electroporation.[133] Many studies used different key vectors or derivatives of these vectors for the genetic manipulation of *M. tuberculosis.*[37,74,88,134-138] Previously used vectors include replicating vectors derived from the pAL5000 and pMF1 plasmids present in *Mycobacterium fortuitum*[139,140] and integrating vectors, which incorporate a phage integrase, encoding the target gene and a phage attachment site (attP) derived from mycobacteriophage L5[113,137] (Table 1). Both systems incorporate a mycobacterial promoter, such as Hp60 or Ag85a[140] or other inducible promoters (reviewed in[141]), and these vectors require selection by kanamycin,[140] hygromycin (Hyg) or gentamycin (Gm),[137] as no replicating vector is stable in the absence of antibiotic selection.[142]

Engaging genetic engineering techniques

Of 11 techniques currently available to modify and to transform *M. tuberculosis,* deleting or muting one gene can be realized by eight different techniques (Table 2), whereas mutant libraries could be realized by two techniques only and gene addition by one technique only (Table 2) with specific advantages and disadvantages (Table 3). These techniques could be used in a triaging strategy incorporating mutant libraries to identify interesting loci further analysed by one of the deleting/muting techniques. Among three mutagenesis techniques, signature-tagged mutagenesis allows to specifically mutate a target gene using systems with different tags, faciliting mutant screening using only one animal or cell model (Table 3). Among techniques used in allelic exchange, some techniques are easier to manage but less effective than others such as counter-selectable marker *galK* and *rpsL*+. Indeed, combinations of two techniques such as the counter-selectable markers *sacB* or *galK* and the marker *lacZ* could be more efficient than using single technique.[51] Moreover, among all studied techniques, the phagemid systems need many steps to obtain one gene mutation while recombineering systems are more rapid and yield high efficiencies of chromosomal recombineering[96-98] (Table 3). Comparing these different techniques, we observed they could be grouped into three clusters (Figure 6): one cluster formed by transposon mutagenesis, a rapid technique which presents an important efficiency and productivity to modify many genes in parallel, used for slow- and rapid-growing mycobacteria, reportedly used in

identification of genes implicated in drug resistance or virulence;[12,14,50] a second cluster comprises four techniques based with the same system: counter-selectable markers *sacB*, *galK*, *rpsL*+ and the marker *lacZ*; these latter do not require a lot of materials and a good lab equipment, allowing rapid screening and do not require a large inoculum and can be used for slow- and rapid-growing mycobacteria. Future application was showed using just counter-selectable marker *sacB*;[72,73,81,127] a third cluster includes recombineering systems, signature-tagged mutagenesis, site-directed mutagenesis, linear DNA substrates (long and short), phagemids systems and UV-irradiated of double-stranded DNA: these techniques present many varied advantages with the more important, efficiency and productive technique is recombineering systems and will be a key tool necessary for simple construction of potential vaccine strains and the identification of virulence genes.[16-18,19,44,80,127]

Applications to the study of *M. tuberculosis*

1. Defining *M. tuberculosis*-host interactions

Several animal and cellular (e.g., macrophage) models for tuberculosis[143-145] have been improved upon through *M. tuberculosis* genetic engineering. The mariner-derived transposon Himar1 was used to detect genes involved in the survival of *M. tuberculosis* CDC1551 in mouse,[101,102] guinea pig[101] and primate models.[100] Likewise, the MycoMarT7 transposon systems were used to identify genes required for *M. tuberculosis* H37Rv adaptation and survival in macrophages,[52] and transposon mutagenesis was used to screen mutants implicated in intracellular macrophage survival[12,50] and the inhibition of macrophage apoptosis.[48] Additionally, STM has been used to identify *M. tuberculosis* virulence genes as well as genes implicated in *M. tuberculosis* survival in mouse[53-57] and/or macrophage models.[53,57,111]

2. Analyzing antibiotic resistance

Treatments of tuberculosis patients and control over the propagation of *M. tuberculosis* have been hampered by the emergence of bacilli that are resistant to both first- and second-line antituberculosis drugs.[146] Antituberculosis drug resistance is associated with deletions or point mutations in drug-activating genes, genes encoding drug targets and the promoters of the drug target genes.[147] Real-time PCR, microarray hybridization, sequencing, heteroduplex analysis and QIAplex are used to detect such events.[148-150] The genetic tools reviewed herein have been used to trace drug resistance[81,98,125,147] and to characterize new antimycobacterial drug targets: for example, site-directed mutagenesis allowed to discover that diarylquinoline targeted the proton pump of the *M. tuberculosis* adenosine triphosphate synthase;[98,125,147,151]

phagemid systems were used to study *embB*, which is involved in ethambutol resistance,[77] the counter-selectable marker *sacB* was used to study the DnaE2 polymerase[125] and pyrazinamidase[124] and recombineering techniques helped to characterize new antimycobacterial drug targets as well as mutations conferring resistance to isoniazid, rifampicin, ofloxacin and streptomycin.[98]

3. Engineering antigenic proteins for vaccine production

The only vaccine currently available, Bacillus Calmette Guérin (BCG), consists of an attenuated *Mycobacterium bovis* strain that was developed in 1924.[152] Clinical trials in developing countries have reported highly variable protection efficacy ranging from 0 to 90%.[152-154] Moreover, while BCG confers moderate protection from infantile tuberculosis, it confers only limited protection against adult pulmonary TB.[19] Recombinant DNA techniques have been used to construct BCG variants that induce a more effective protective response against *M. tuberculosis*. One strategy was the development of BCG variants that had been engineered to produce and secrete cytokines,[155] and another was the construction of BCG recombinants that overexpressed the *M. tuberculosis* 30-kDa major secretor protein antigen, which demonstrated increased protective power in animal models.[156] Additionally, several studies demonstrated that recombinant BCG that was engineered to secrete *Listeria monocytogenes* listeriolysin stimulated T CD8+ and T CD4+ cells involved in the protection against tuberculosis.[157] A third strategy was the generation of attenuated *M. tuberculosis* recombinants, such as mutants carrying inactivated *phoP* or *mce*. Such mutants induced significantly greater protection than wild-type BCG in guinea pigs and mice infected with high titers of *M. tuberculosis*.[44,158]

Future developments

1. Genetic engineering and model organisms

Both animals and macrophage cell lines are used as models to assess the effects of *M. tuberculosis* transformation. We demonstrated that free-living amoeba acted as a training field for the resistance of mycobacteria to macrophage killing,[159,160] and several studies used free-living amoeba to investigate the phagocytosis and intracellular survival mechanisms of *Legionella pneumophila*.[161] Our laboratory extended this model to study *M. tuberculosis*-amoeba interactions.[162] Recently, we observed that the co-culture of amoeba with *Mycobacterium smegmatis* constituted a simple model to rapidly (< 5 days) probe for the mycobacterial factors implicated in the intracellular growth of mycobacteria.[163] Furthermore,

it could be interesting to use this macrophage-like organism to study the *M. tuberculosis* proteins implicated in drug resistance, virulence and pathogenesis.

2. Gene addition in *M. tuberculosis*

The various techniques reviewed herein have been used to create mutations or deletions within *M. tuberculosis* genes. For example, several studied showed that expressing phenolic glycolipid from *M. tuberculosis* HN878 (belonging to the W-Beijing family) in the *M. tuberculosis* H37Rv strain inhibited the innate immune response and increased virulence in infected mice and rabbits.[164,165] Conversely, Fallow and collaborators (2010) showed that the expression of DosR from *M. tuberculosis* H37Rv in *M. tuberculosis* W/Beijing lineage established the natural expression profile of DosS/DosT-DosR two-component system under standard *in vitro* conditions.[166] However, techniques summarised in this review have seldom been used to add genes previously absent from the wild-type *M. tuberculosis* despite the fact that this technique may be able to reveal the function of many heterologous proteins. For example, the expression of porin MspA from the fast-growing *M. smegmatis* in *M. tuberculosis* increased its antibiotic susceptibility.[163] Thus, it would be interesting to confirm these observations in cellular models as well as animal models. Indeed, the addition of genes rather than the deletion of genes may be another way to modify the behaviour of this pathogenic organism.

Conclusions

In this review, we described a variety of methods and techniques that were used to generate genetic and molecular modifications to *M. tuberculosis*. These techniques greatly improved our understanding of the mechanisms implicated in *M. tuberculosis* virulence and its resistance to antituberculous drugs. *M. tuberculosis* genetic engineering has been used mainly to modify or delete genes based on the concept that reducing the genome would decrease virulence. However, the analysis of several bacterial genomes has contradicted this hypothesis and has instead revealed that highly virulent organisms have a smaller genome size than their less virulent counterparts.[167-171] Indeed, genome analyses from obligate intracellular pathogenic organisms indicated that these bacteria have small genomes that were derived from their larger, free-living bacterial ancestors. This reductive evolution has been associated with metabolic parasitism during adaptation to an intracellular habitat.[169] Thus, gene loss is thought to be a feature of intracellular pathogenic bacterial evolution.[167,170-173] Additionally, recent analysis of the *M. tuberculosis* genome indicated that ancestors of this pathogenic

organism likely acquired genes by lateral gene transfer.[20,23,24] Genetic engineering allowed to transfer genes from one strain to another strain of *M. tuberculosis*. All together, *M. tuberculosis* genetic engineering provided new insights into tuberculosis pathogenesis, and tuberculosis fighting by new antituberculous drugs and vaccines.

Competing interests

The authors declare that they are inventors of a patent regarding the gene addition for reducing mycobacteria virulence.

Table 1. Plasmid vectors used for allelic exchange in *M. tuberculosis*.

Vectors	Origin	Marker genes	References
pMY10	pAL5000	KanR	[138]
pDC100	pAL5000	KanR	[138]
pMH94	L5	KanR	[142]
pMV261	pAL5000	KanR	[174]
pMV361	L5	KanR	[174]
pMD31	pAL5000	KanR	[135]
p16R1	pAL5000	KanR	[136]
pYUB854	pAL5000	HygR	[74]
pYUB870	pAL5000	KanR	[74]
pBP10	pMF1	KanR	[134]
pYUB415	L5	HygR, KanR	[88]
pYUB412	L5	HygR	[37]
pAPA3	L5	GmR	[7]
pML1342	L5	HygR	[137]

KanR: Kanamycin resistance; HygR: Hygromycin resistance; GmR: Gentamicin resistance

Table 2. Genetic engineering techniques and their possible applications

	Mutant libraries	Modify specific gene in a defined site	Gene deletion or mutation	Gene addition
Transposon mutagenesis	+	-	-	-
Signature-tagged mutagenesis	+	-	-	-
Site-directed mutagenesis	-	+	-	-
Linear DNA substrates (long and short)	-	+	+	-
Phagemids systems	-	+	+	-
UV-irradiated of double-stranded DNA	-	-	+	-
Marker lacZ	-	+	+	-
Counter-selectable marker *sacB*	-	+	+	-
Counter-selectable marker *rpsL*+	-	+	+	-
Cunter-selectable marker *galK*	-	+	+	-
Recombineering systems	-	+	+	+

Table 3. Advantages and disadvantages of different techniques used to modify *M. tuberculosis* genome.

Techniques	Advantages	Disadvantages
Transposon mutagenesis	Modify specific gene using transposon systems Modify many genes in the same time Construction of a mutant libraries Important efficiency	Require an effective methods to analyse mutants
Signature-tagged mutagenesis	Modify specific gene using transposon systems with different tags Construction of a mutant libraries Specific mutation in a target gene Important efficiency Injection of "a pool" of different random mutants to the same animal or cell model for screening	-
Site-directed mutagenesis	Creates a mutation at a defined site Used commonly in protein engineering	Sequence of target gene is needed No mutant libraries
Linear DNA substrates (long and short)	Gene modification by replacing target gene by resistance markers Transfer to mycobacteria by electroporation	Transfer events were infrequent Not reproducible technique No specific integration events
Phagemids systems	Create specific gene mutant Reduce illegitimate recombination Enhance homologous recombination	Many steps to obtain one gene mutation Mycobacteriophage creation which need recognition in this field
UV-irradiated of double-stranded DNA	Simple methods to generate a large number of transformants	UV-irradiation can create a damage in used vector Introduction of secondary not specific mutation
Marker *lacZ*	Create mutation in a specific gene Colonies screening facilitate by using suicide vector Can be used with *sacB* marker: combination of selection	Low homologous recombination frequency Low efficiency Multiple transformation and selection steps
Counter-selectable marker *sacB*	Create mutation in a specific gene Construction of both unmarked and marked mutations Colonies screening facilitate by using suicide vector	Spontaneous *sacB* mutants arise at a high frequency Low efficiency Multiple transformation and selection steps
Counter-selectable marker *rpsL+*	Create mutation in a specific gene Colonies screening facilitate by using negative selectable marker	Limited technique because the streptomycin used for selection can be used as a chemotherapeutic agent against tuberculosis
Counter-selectable marker *galK*	Create mutation in a specific gene More effective than *sacB* marker Can be used with *sacB* marker: combination of selection	-
Recombineering systems	Modify DNA precisely using the mycobacteriophage-encoded recombinant system Che9c Create knockout mutant High efficiencies of chromosomal recombineering	-

Figures legends

Figure 1. Number of publications concerning *M. tuberculosis* in the last fifteen years. Nine sequenced *M. tuberculosis* strain genomes are represented at the top of bar, and the year of their publication is indicated.

Figure 2. Mutagenesis in *M. tuberculosis*. Similarities and differences between transposon (A), signature-tagged (B) and site-directed (C) mutagenesis. A) Transposon systems were used to create a mutant library and each single mutant was selected using animal or cell models. B) Transposon systems with different tags were used to create a signature-tagged mutant library and after "a pool" of different random mutants were injected to the same animal or cell model for screening. C) After creation of a mutation at a defined site (represented in red) the gene was cloned in an expression vector and transformed to *Escherichia coli* for screening. n: nucleus, l: lysosome, $Gene^R$: antibiotic resistance gene.

Figure 3. Allelic exchange using (A) short and long linear DNA and (B) mycobacteriophage Che9c proteins. (A) The disrupted target gene replaces the intact gene in the genome of *M. tuberculosis* after homologous recombination between the upstream and downstream regions. (B) Gene mutation or deletion is performed in an *M. tuberculosis* strain carrying the pJV53 plasmid, which encodes for the mycobacteriophage proteins 60 and 61 that facilitate recombineering. $Gene^R$: antibiotic resistance gene.

Figure 4. Generating recombinant DNA using a phagemid vector. The expression vector contains the upstream and downstream regions of the target gene, the antibiotic resistance gene ($Gene^R$), a lambda cos site and a unique PacI site. This unique site is used in the ligation with the shuttle phage (L1, TM4 or D29). The conversion into mycobacteriophages containing the phagemid occur in *M. smegmatis* at the permissive temperature of 30°C, and the infection of *M. tuberculosis* for gene mutation will be at the non-permissive temperature 37°C.

Figure 5. Different steps for allelic exchange in the *M. tuberculosis* genome using different markers: (A) *lacZ*, (B) *sacB*, (C) *rpsL* and *galK* (E). In all cases, the suicide vector contains the upstream and downstream regions of the target gene, the antibiotic resistance gene ($Gene^R$) and the specific marker was prepared and transformed into *M. tuberculosis*. To generate a large number of transformants the suicide vectors can be treated with UV light

prior to their electroporation into the bacteria.

Figure 6. Chip-like form obtained after comparison between different techniques used to modify and to transform *M. tuberculosis*. 10 criteria was used: (A) duration of manipulator formation, (B) materials and laboratory equipments, (C) screening methods, (D) inoculum, (E) versatility, (F) efficiency and productivity, (G) duration of experiment, (H) steps of experiment, (I) publications and (J) applications. Each variable for each technique was indicated with a note varying from -1, 0 or 1. TM: transposon mutagenesis, STM: signature-tagged mutagenesis, SDM: site-directed mutagenesis, SLDNAs: linear DNA substrates (long and short), Ph: phagemids systems, UV: UV-irradiated of double-stranded DNA, lacZ: marker *lacZ*, sacB: counter-selectable marker *sacB*, rpsL+: counter-selectable marker *rpsL*+, galK: counter-selectable marker *galK* and RS: recombineering systems.

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Figure 1
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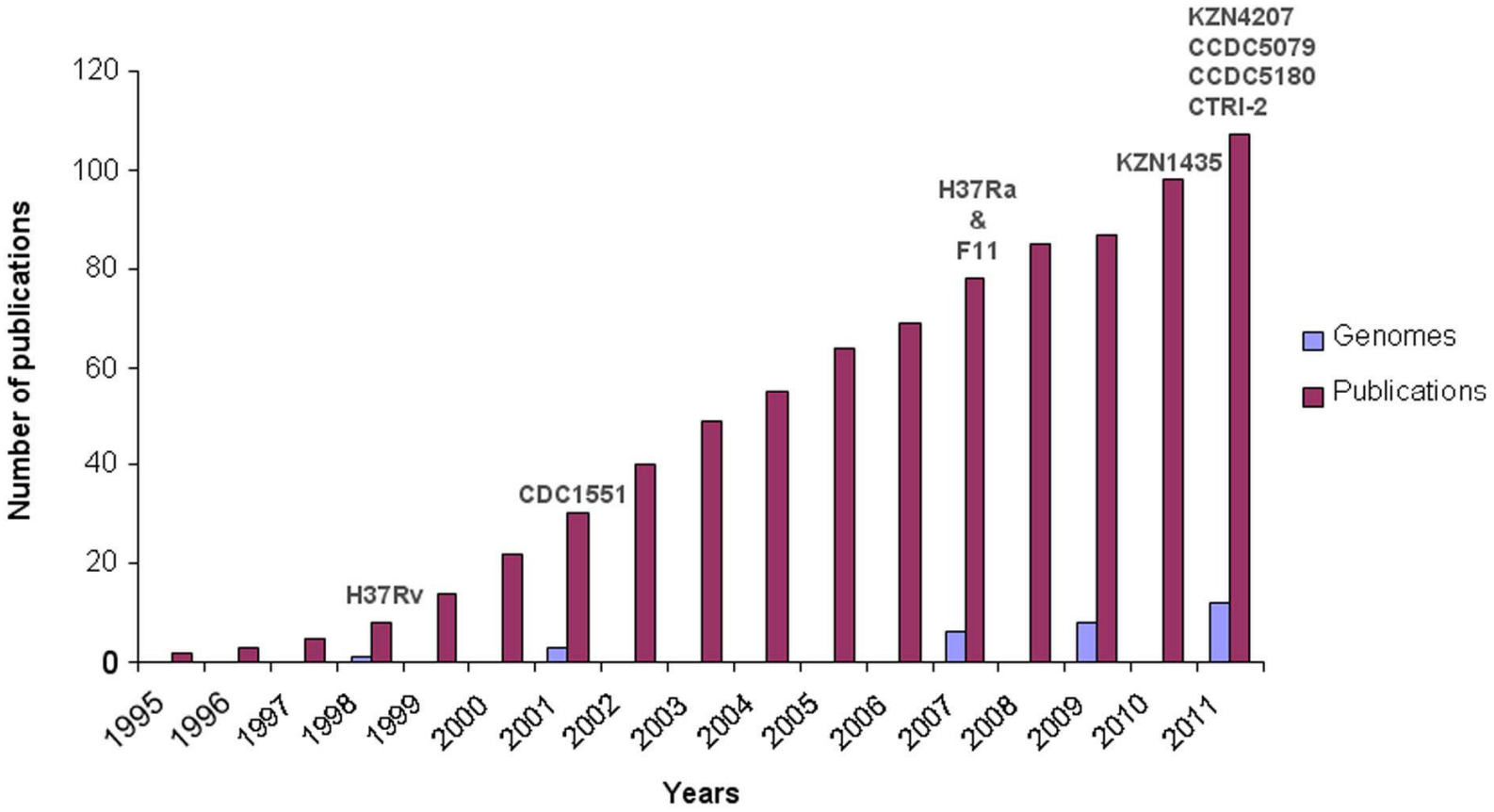

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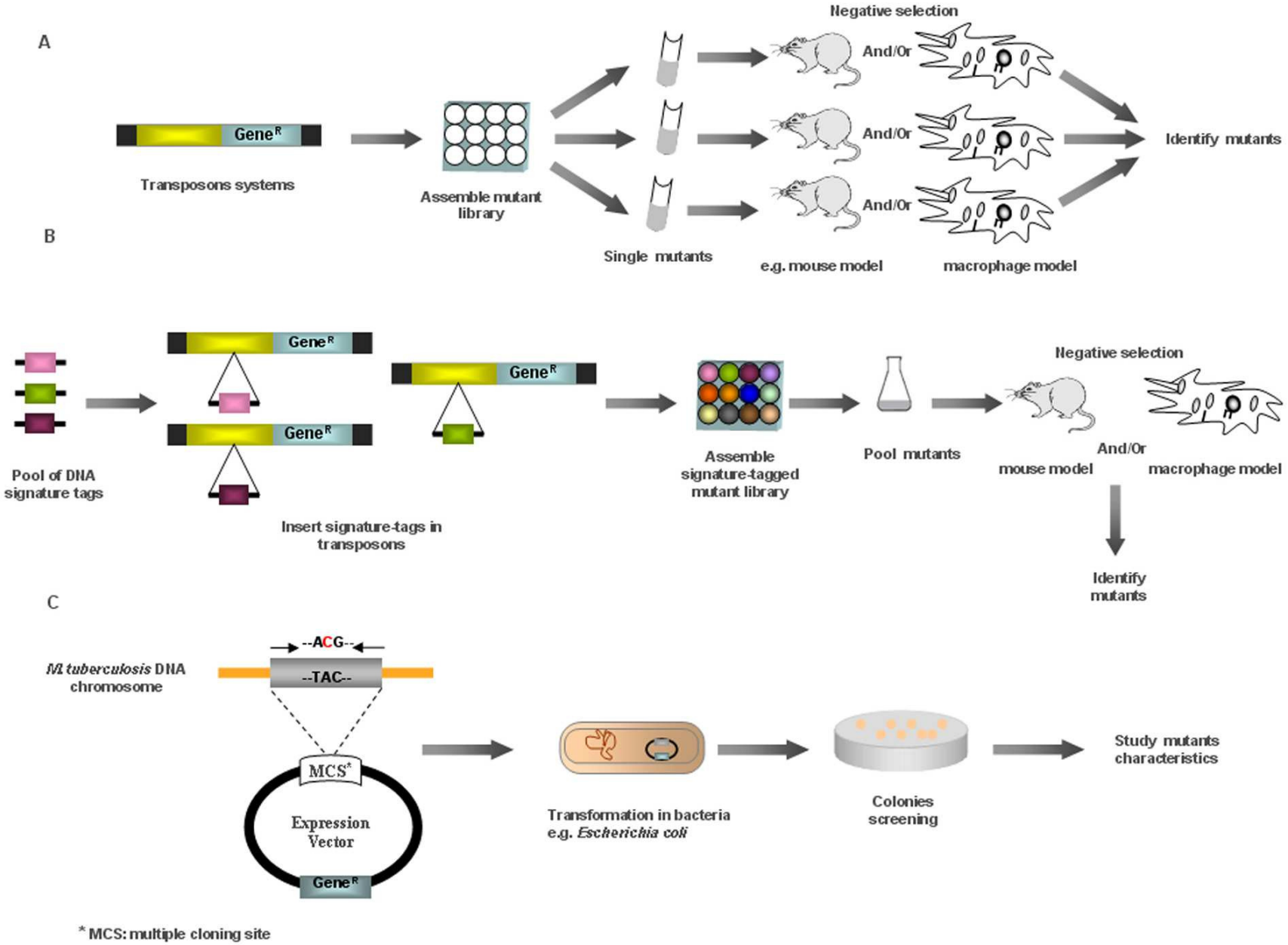

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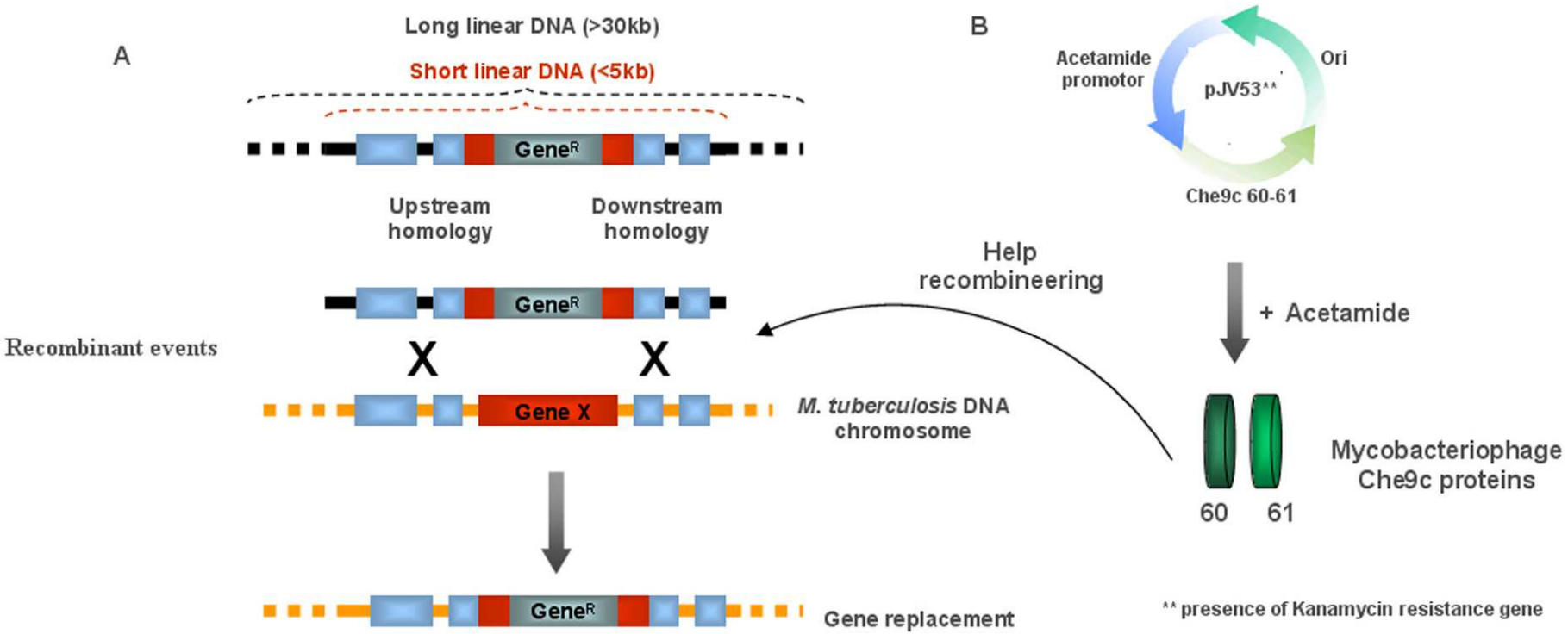

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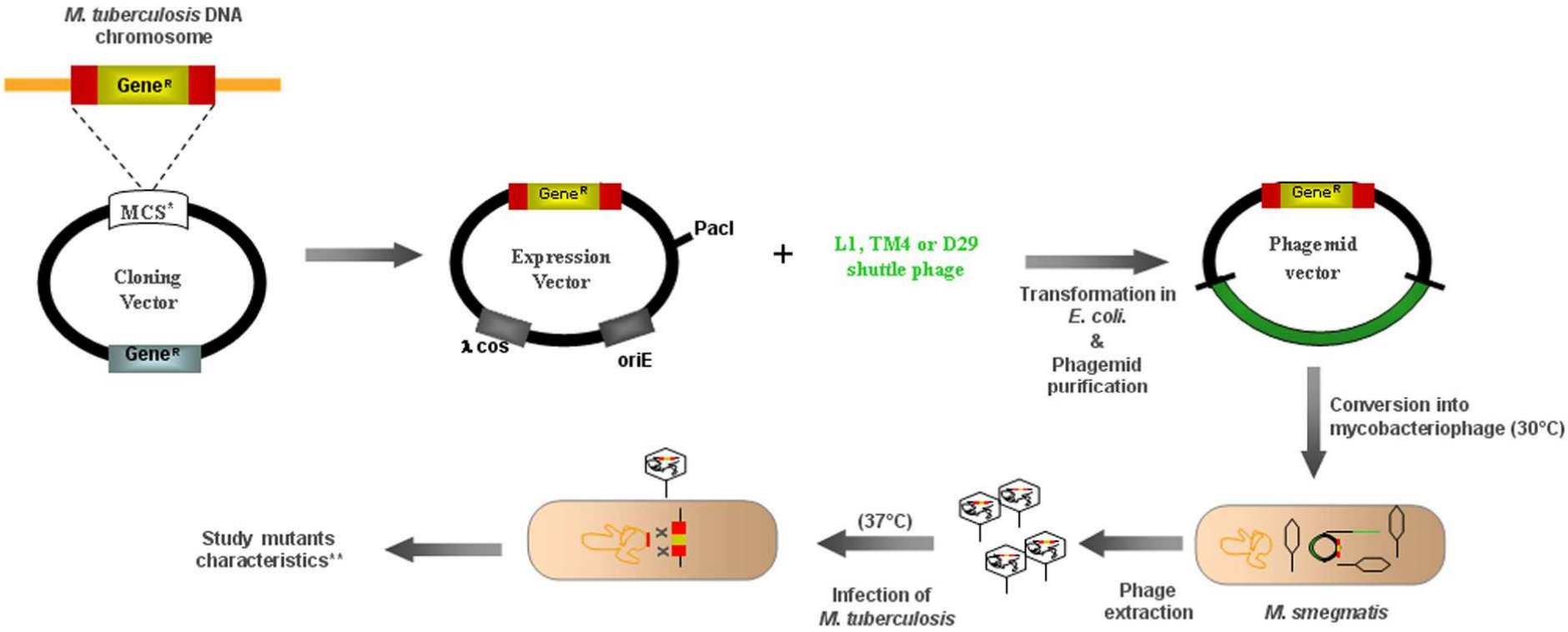

* MCS: multiple cloning site

** In some case the mutants characteristic can be studied in animal or cell models

Figure 5

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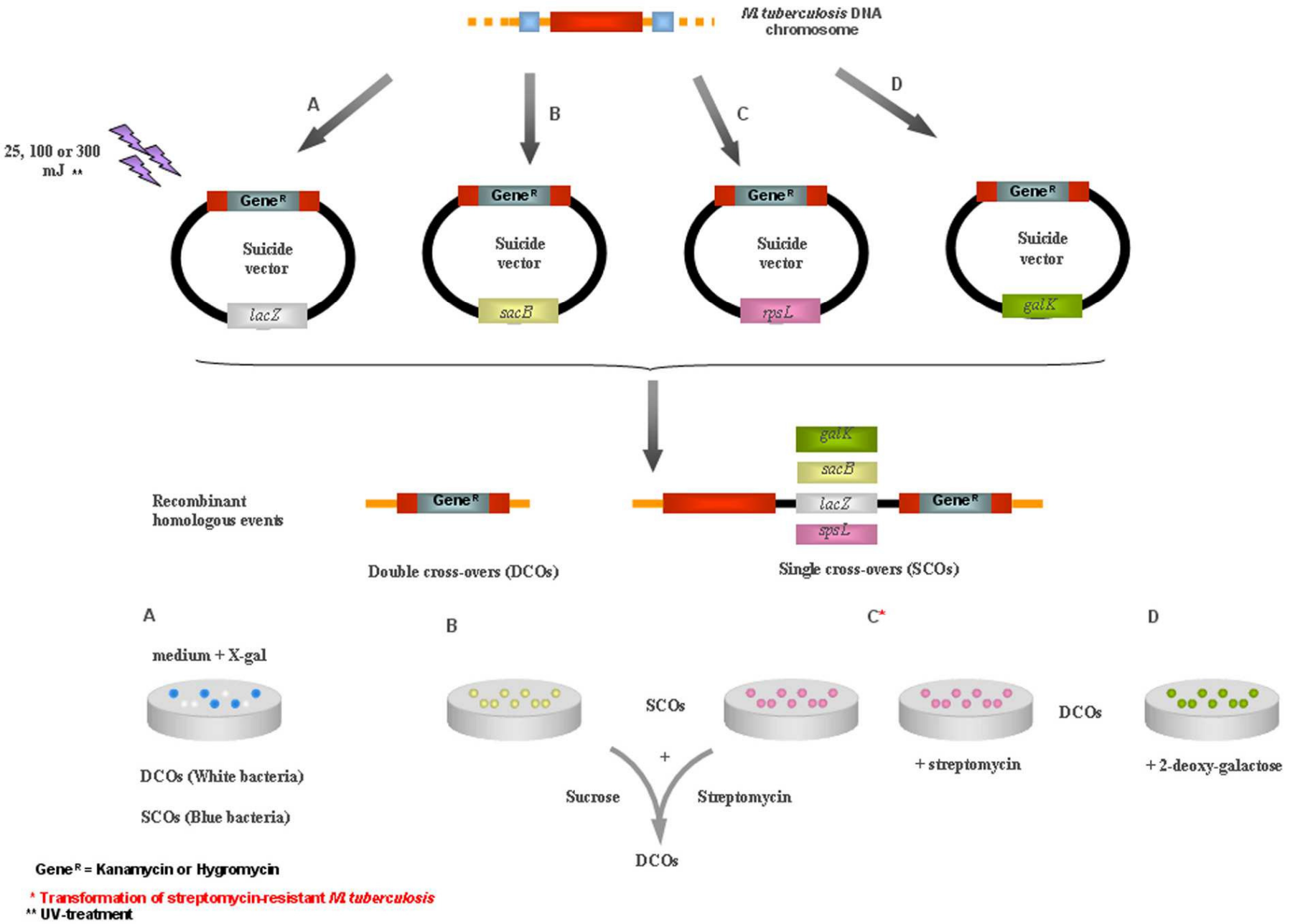

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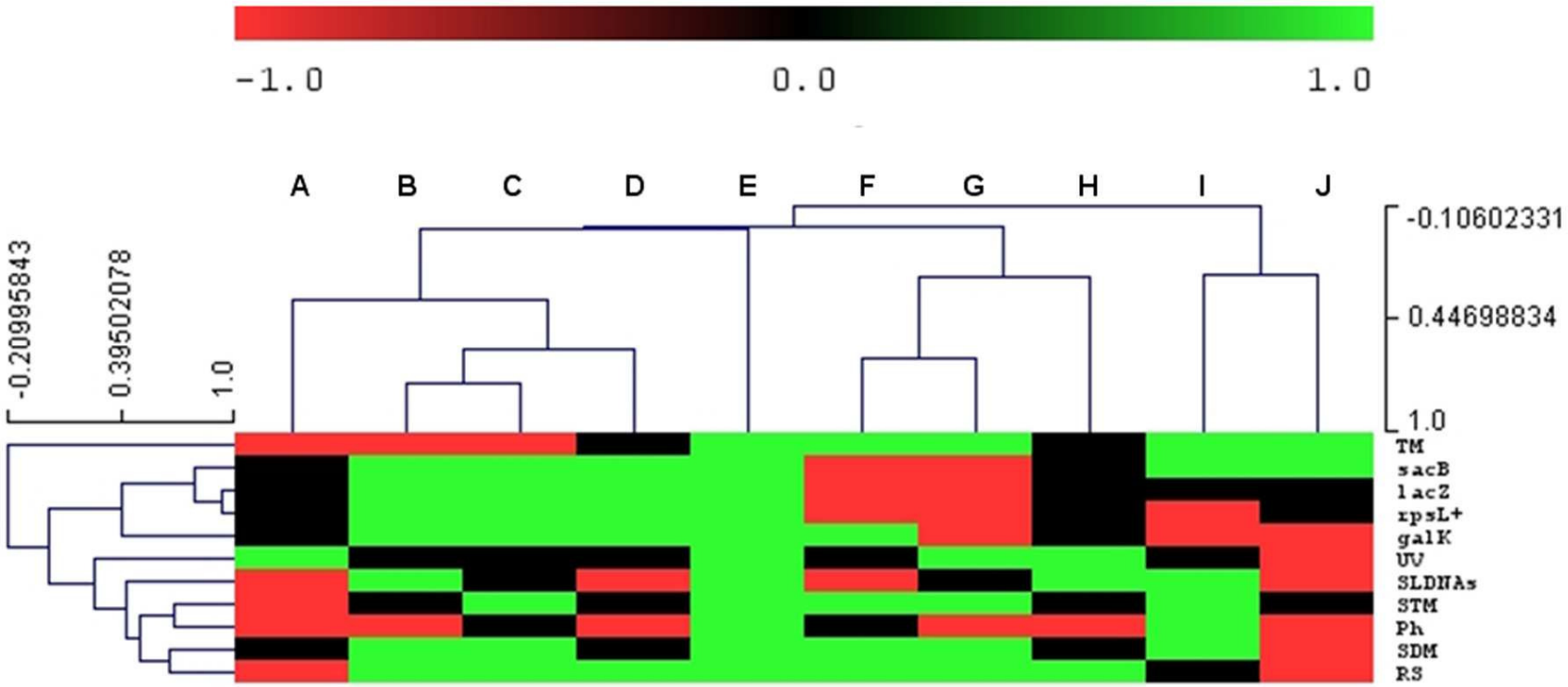

Chapitre II

The genealogic tree of mycobacteria reveals a long-standing sympatric life into free-living protozoa

Otmane Lamrabet, Vicky Merhej, Pierre Pontarotti, Didier Raoult, Michel Drancourt

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Chapitre II – Avant propos

Les protozoaires libres permettent le transfert horizontal de l'ADN et des gènes avec et entre les micro-organismes qu'ils hébergent. Ils sont des hôtes des mycobactéries pour lesquelles la ou les sources des gènes transférés restent inconnues. Dans cette étude, nous avons cherché à l'aide de BLASTp des gènes homologues entre les génomes de 15 mycobactéries, de 34 bactéries résistantes à l'amibe et du protozoaire libre *Dictyostelium discoideum*. Les analyses phylogénétiques de ces séquences ont révélé que huit cadres ouverts de lecture (ORF) des mycobactéries ont probablement été acquis par transfert horizontal à partir des bêta-et gamma-Protéobactéries et des Firmicutes, mais les histoires des transferts n'ont pas pu être établies de manière fiable dans les détails. Un autre ORF codant pour une pyridine nucléotide désulfite oxidoreductase (pyr-redox) place les mycobactéries non-tuberculeuses dans un clade avec *Legionella* spp., *Francisella* spp., *Coxiella burnetii* et deux ciliés *Tetrahymena thermophila* et *D. discoideum* avec une fiabilité élevée. Enfin, la coculture de *Mycobacterium avium* et *Legionella pneumophila* dans l'amibe *Acanthamoeba polyphaga* a montré que ces deux bactéries pouvaient vivre de façon sympatrique dans les amibes. Cette expérience renforce la possibilité d'un échange intra-amibien du gène pyr-redox. En conclusion, les résultats de cette étude soutiennent l'hypothèse que les protistes sont des sources et des lieux de transfert des gènes chez les mycobactéries.

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The Genealogic Tree of Mycobacteria Reveals a Long-Standing Sympatric Life into Free-Living Protozoa

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Abstract

Free-living protozoa allow horizontal gene transfer with and between the microorganisms that they host. They host mycobacteria for which the sources of transferred genes remain unknown. Using BLASTp, we searched within the genomes of 15 mycobacteria for homologous genes with 34 amoeba-resistant bacteria and the free-living protozoa *Dictyostelium discoideum*. Subsequent phylogenetic analysis of these sequences revealed that eight mycobacterial open-reading frames (ORFs) were probably acquired via horizontal transfer from beta- and gamma-Proteobacteria and from Firmicutes, but the transfer histories could not be reliably established in details. One further ORF encoding a pyridine nucleotide disulfide oxidoreductase (pyr-redox) placed non-tuberculous mycobacteria in a clade with *Legionella* spp., *Francisella* spp., *Coxiella burnetii*, the ciliate *Tetrahymena thermophila* and *D. discoideum* with a high reliability. Co-culturing *Mycobacterium avium* and *Legionella pneumophila* with the amoeba *Acanthamoeba polyphaga* demonstrated that these two bacteria could live together in amoebae for five days, indicating the biological relevance of intra-amoebal transfer of the pyr-redox gene. In conclusion, the results of this study support the hypothesis that protists can serve as a source and a place for gene transfer in mycobacteria.

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Introduction

Massive sequencing revealed that bacterial genomes have undergone a mosaic evolution, combining variable proportions of vertically acquired DNA from previous generations and horizontally acquired DNA from other organisms present in their environment [1]. Therefore, the evolution of bacterial genomes cannot be represented by trees alone but rather must be represented by more complex structures such as rhizomes illustrating the various, multiple sources of DNA that have been combined in one particular bacterial species [2]. Therefore, to a certain extent, a bacterial genome sheds light on the particular environment in which that bacterium's ancestors used to live and on the amount of DNA exchange with neighbor organisms [3]. Accordingly, genome sequencing revealed that contrary to previous conjecture, current *Mycobacterium* organisms are the result, in part, of horizontal genetic transfer from unidentified Eukarya and from environmental alpha- and gamma-Proteobacteria and Actinobacteria, as demonstrated for *Mycobacterium tuberculosis* [4–7]. However, the places in which *Mycobacterium* ancestors came in contact with other organisms for these genetic transfer events remained unknown.

Recent studies have shown that free-living protozoa, amoebae in particular, are indeed places in which horizontal genetic transfer occurs [8]. Free-living amoebae host numerous amoeba-resistant bacteria [3,9–13], fungi [14], giant DNA viruses [15] and virophages [16], all of which live in sympatry in the free-living protozoa. Moreover, free-living protozoa are "melting pots" in which microorganisms exchange DNA including genes by horizontal gene transfer (HGT) [3,17–19], as illustrated for *Rickettsia bellii* [20], *Candidatus Amoebophilus* asiaticus [21] and the recently found transfer of a *Acanthamoeba polyphaga* Mimivirus protein to *Legionella pneumophila* [22]. DNA can also be transferred from the protozoa themselves to the microorganisms, as in the cases of the *A. polyphaga* Mimivirus [15,23], *Legionella drancourtii* [22,24] and *Chloroflexus aurantiacus* [25]. Genetic transfers can also occur in the reverse direction, from the microorganisms to free-living protozoa, as in the case of *Tetrahymena thermophila*, which acquired bacterial genes involved in the catabolism of complex carbohydrates, contributing largely to its capacity to colonize the rumen [26]. There have also been documented transfers from bacteria to animals [27].

Non-tuberculous mycobacteria share aquatic and terrestrial ecological niches with free-living protozoa including ciliates, flagellates and amoebae [19,28–30]. Co-culture experiments further showed that non-tuberculous mycobacteria could be phagocytosed by the ciliate *Tetrahymena pyriformis* [28], the social amoeba *Dictyostelium discoideum* and the free-living amoeba (FLA) *Acanthamoeba polyphaga* [19,31–33] and further reside in amoebal cysts, which act as a "Trojan horse" for such amoeba-resistant mycobacteria [29,33,34]. *M. tuberculosis* complex organisms can also be phagocytosed by amoebae [35–37], and it was recently observed that, except for *Mycobacterium canetti*, *M. tuberculosis* complex members can also reside within amoebal cysts [37].

We speculated that free-living protozoa may have been places in which gene transfers into mycobacteria occurred. We performed

extensive bioinformatics comparisons of available mycobacteria genomes with those of amoeba-resistant bacteria and free-living protozoa to test this hypothesis, and we used co-culture experiment to confirm its biological relevance.

Materials and Methods

Bacterial genome sequences and homologous gene determination

The protein complement of *M. tuberculosis* H37Rv (NC_000962), *M. tuberculosis* CDC1551 (NC_002755), *Mycobacterium bovis* (NC_002945), *Mycobacterium avium* subsp. *hominissuis* 104 (NC_008595), *M. avium* subsp. *paratuberculosis* K10 (NC_002944), *M. avium* subsp. *avium* (NZ_ACFI00000000), *Mycobacterium intracellulare* (NZ_ABIN00000000), *Mycobacterium abscessus* (NC_010397), *Mycobacterium smegmatis* mc^2 155 (NC_008596), *Mycobacterium marinum* (NC_010612), *Mycobacterium ulcerans* Agy99 (NC_008611), *Mycobacterium gilvum* PYR-GCK (NC_009338), *Mycobacterium sp.* JLS (NC_009077), *Mycobacterium vanbaalenii* PYR-1 (NC_008726) and *Mycobacterium leprae* TN (NC_002677) was downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) (Table 1).

Each mycobacterial open reading frame (ORF) was then compared with the complete genomes of *D. discoideum* (NC_007087-92) and 34 amoeba-resistant bacteria [38] (Table S1) using the BLASTp program. The 100 hit sequences exhibiting a significant alignment (E-value$<1.10^{-4}$) and a hit sequence with coverage $\geq$80% and similarity $\geq$30% were selected for further phylogenetic analyses. The conserved domains of selected ORFs were searched with InterProScan (http://www.ebi.ac.uk/Tools/InterProScan).

Phylogenetic analysis and molecular data

For each set of 100 hits, the amino acid sequences were aligned using MUSCLE algorithm [39]. The alignments produced were then manually refined in order to remove regions that contain gaps or are highly divergent with the BioEdit program v7.0.9 [40].

The corrected alignments were then used for maximum likelihood (ML) and Bayesian inference (BI). ML was constructed using PHYML [41] in the PHYLIP package version 3.5c with 100 and 1,000 randomizations of input order. The substitution model was set to WAG and enabled the optimization options for tree topology, branch lengths, and rate parameters. To test the robustness of inferred topologies, posterior probabilities were determined by a Bayesian Markov chain Monte Carlo (MCMC) method implemented in the program MR BAYES V3.0 [42]. One million generations were run using the WAG matrix and model parameters (gamma shape and proportion invariant), and the trees were sampled every 100 generations. The posterior probability stabilized after 100,000 generations, so all parameter estimates before generation 100,000 were omitted. The tree with maximum posterior probability was assessed using a consensus of the final 100 000 trees. Bootstrap support of >75% and posterior probability of >90% were considered to identify supported nodes.

Substitution rates were calculated by dating the nodes in the 16S rRNA gene sequence-based phylogeny. Distances or numbers of substitutions per site separating pairs of species were estimated from the absolute numbers of differences between pairs of nucleotide sequences. We converted these data into measures of time divergence using the constant rate of 16S rRNA divergence of 0.01–0.02 per 50 million years found by Moran *et al.* [43]. All distance calculations were based on the same 1,440 sites, for which there were no missing data.

The species tree of mycobacteria was constructed based on the 16S rRNA gene sequences. The 16S rRNA sequences from the 15 studied *Mycobacterium* spp. were retrieved from NCBI database and aligned using MUSCLE. The phylogenetic relationships were inferred using the Neighbor-joining method.

Table 1. Workflow summarizing the steps followed in the identification of HGT genes in mycobacteria.

1.	The proteomes of 15 *Mycobacterium* spp., *D. discoideum* and 34 amoeba-resistant bacteria (Table S1) were downloaded from the National Center for Biotechnology Information (NCBI).
2.	Search for homologous genes of mycobacterial open reading frames (ORFs) in the genomes of *D. discoideum* and 34 amoeba-resistant bacteria using the BLASTp program from NCBI (E-value<1.10–4, similarity >30% and coverage >80%).
3.	Search for the homologous sequences of the mycobacterial ORFs found in step 2 in the NR database using BLASTp (E-value<1.10–4, similarity >30% and coverage >80%).
4.	Selection of ORFs from mycobacteria found in step 3 presenting significant homology with *D. discoideum* and 34 amoeba-resistant bacteria in the first 100 hits.
5.	Infer phylogenetic relationships between the protein sequences found in steps 4 using MUSCLE for alignment and two construction methods (Maximum Likelihood within the PHYML program and M. Bayes).
6.	Analysis of the trees generated in step 5, looking for possible HGT between *Mycobacterium* spp. and amoebae and/or amoeba-resistant bacteria.

More details can be found in the materials and methods.
doi:10.1371/journal.pone.0034754.t001

Co-culture experiments

The *A. polyphaga* Linc-AP1 strain (a gift from T. J. Rowbotham, Public Health Laboratory, Leeds, United Kingdom) was grown at 28°C for 3 days in 150-cm^3 culture flasks (Corning, New York, USA) containing 30 ml of peptone-yeast extract-glucose (PYG) broth [44–46]. When the average amoeba concentration reached 5×10^5 cells/ml, amoebae were centrifuged at 500 g for 10 min, and the pellet was suspended twice in 30 ml of Page's modified Neff's amoeba saline (PAS) (solution A-NaCl 1.20 g; $MgSO_4.7H_20$ 0.04 g; Na_2HPO_4 1.42 g; KH_2PO_4 1.36 g/100 ml of glass distilled water; solution B-$CaCl_2.2H_2O$ 0.04 g/100 ml of distilled water; amoeba saline, 10 ml of solution A+10 ml of solution B+980 ml distilled water) [44,46,47]. Liquid medium-cultured *M. avium* subsp. *avium* CIP104244^T [33] and *L. pneumophila* strain Lens [12] organisms were washed two times with sterile phosphate-buffered saline (PBS), and the pellet was suspended in PAS. This inoculum was vortexed to minimize mycobacterial clumping. Ten milliliters of the amoebal suspension in PAS ($\sim10^5$ amoeba/ml) was inoculated with $\sim10^6$ *L. pneumophila*/ml or $\sim10^6$ *M. avium*/ml (MOI = 10) or co-infected with both bacteria. As controls, *A. polyphaga*, *L. pneumophila* and *M. avium* were cultured separately in PAS. After a 3-h incubation at 32°C, the coculture was washed three times with PAS to remove any remaining extracellular or adherent mycobacteria, and it was incubated in 10 ml PAS for 5 days at 32°C. At 0, 3 and 5 days of co-culture, *A. polyphaga* monolayers were lysed with 0.1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich Logistic Gmbh, Lyon, France) for 30 min and passed through a 26-gauge needle to ensure complete lysis of the amoebae. The lysate (100 µl) was plated onto 7H10 agar for *M. avium* or Buffered Charcoal Yeast Extract (BCYE) agar plates for *L. pneumophila* and incubated for 5 to 15 days at 35 or 37°C to determine the number of colonies (CFU) of intracellular *M. avium* and *L. pneumophila*. All experiments were performed in triplicate.

Statistical analyses

All statistical analyses mentioned in this study were performed using the chi2-square test with a significance level of $p = 0.05$.

Results

Identification of genes homologous to amoeba and amoeba-resisting bacteria in mycobacterial genomes

We searched for homologous sequences for the 65,812 ORFs of the 15 studied mycobacterial genomes in a database of free living protozoa and amoeba-resisting bacteria using a BLASTp. We found a total of 11,783 that have homologous sequences in the free living protozoa *D. discoideum* and/or amoeba-resisting bacteria (E-value$<$1.10–4, similarity $>$30% and coverage $>$80%). We found a total of 88 mycobacterial ORFs (0.13%) that present significant homology in the genome of the free-living protozoa *D. discoideum*. The number of ORFs with significant homology ranged from 4 genes in *M. leprae* to 29 genes in *M. smegmatis*. When comparing the 15 genomes of *Mycobacterium* spp. with the 34 available genomes of amoeba-resisting bacteria we could identify a total of 11,695 ORFs (17.8%) with significant homology in amoeba-resisting bacterial genomes. The number of mycobacterial ORFs with significant homology in the amoeba-resisting bacteria ranged from 365 for *M. leprae* to 1,208 for *M. smegmatis*. The closely related homologous genes were found in beta-Proteobacteria (30.5% ORFs), gamma-Proteobacteria (18.3% ORFs), Firmicutes (17.6% ORFs), Bacteroidetes (10.8% ORFs), delta-Proteobacteria (7.8% ORFs), Chlamydiae (6.7% ORFs) and alpha-Proteobacteria (8.3% ORFs) (Figure S1).

Phylogenetic analyses and horizontal transfer history

We searched for homologous sequences for the 11,783 ORFs in the NR database. We selected the only queries that contain free living protozoa *D. discoideum* and/or amoeba-resisting bacteria in the 100 first hits. This analysis yielded 151 sets of 100 homologous genes including sequences from free living protozoa *D. discoideum* and/or amoeba-resisting. We made 151 phylogenetic trees on the basis of these 151 gene sequences. Eight out of the 151 gene-trees showed *Mycobacterium* species in a clade with amoeba-resisting bacteria (Fig. S2, S3, S4, S5, S6, S7) and one gene (encoding for pyr-redox) showed *Mycobacterium* species in a clade with *D. discoideum* and amoeba-resisting bacteria (Fig. 1) (Table S2). Mycobacterial sequences clustered with gamma-Proteobacteria in 2/9 trees; with Archaea, gamma-Proteobacteria and Planctomyces in 1/9 trees; with Bacteroidetes and gamma-Proteobacteria in 1/9 trees; with Firmicutes spp. in 2/9 trees; with beta-Proteobacteria in 2/9 trees; and with Eukarya in 1/9 trees (Fig. S2, S3, S4, S5, S6, S7).

The gene encoding for hypothetical hydrolase placed *M. marinum* and *M. ulcerans* in a clade with *Methanosarcina acetivorans*, *Desulfovibrio salexigens*, *Planctomyces limnophilus* and *Vibrio cholerae* (Fig. S2). The gene encoding for hypothetical protein MT3512 placed *M. tuberculosis* H37Rv in a clade with *Gramella forsetii* and *Francisella tularensis* (Fig. S3). The gene encoding for amidase placed *M. marinum* in a clade with *Legionella* spp. (Fig. S4). The gene encoding for Two ORFs encoding for Acetyl CoA hydrolase in *M. marinum*, *M. ulcerans* and transcriptional regulator in *M. smegmatis*, placed these mycobacteria in clade with *Burkholderia* spp. (Fig. S5). Two ORFs encoding for sulphate transporter in *tuberculosis*, *M. bovis* and betalactamase in *M. abscessus*, placed these mycobacteria in clade with *Bacillus* spp. (Fig. S6). Finally, the gene encoding for amino acid permease placed *M. smegmatis* in a clade with *Pseudomonas putida* (Fig. S7). Further phylogenetic analyses of an ORF encoding a pyridine nucleotide disulfide oxidoreductase (pyr-redox) placed *M. marinum*, *M. ulcerans*, *M. avium*, *M. intracellulare*, *M. abscessus*, *Mycobacterium parascrofulaceum* and *M. smegmatis* in a clade with *Legionella* spp., *Francisella* spp., *Coxiella burnetii*, *T. thermophila* and *D. discoideum* with a high reliability (Fig. 1). The different construction methods showed that *Mycobacterium* spp. formed a highly supported group (bootstrap values, 94–95%) with gamma-Proteobacteria (*Legionella* spp., *C. burnetii* and *Francisella* spp.), *D. discoideum* and *T. thermophila*. In addition, we observed that *Legionella* spp. did not cluster with the other gamma-Proteobacteria but rather with *Mycobacterium* spp., *D. discoideum* (amoeba) and *T. thermophila* (ciliates) (Fig. 1). The phylogenetic construction using M. Bayes gave the same topology. The tree topology is the same when carrying out with 100 or 1,000 bootstrap replicas in what concerns the place of mycobacteria in a highly supported clade with amoeba and amoeba-resistant bacteria. Interestingly, the pyr-redox sequences matched with genes encoding for a monooxygenase with coverage of 60% and identity 25% in *Rhodococcus* and coverage of 58% and identity of 24% in *Nocardia*. These results suggest that the HGT event of pyr-redox concerns only the mycobacteria genus.

Characteristics and functions of the horizontally transfered genes

Our findings showed that environmental mycobacteria and mycobacteria from *M. tuberculosis* complex are all affected by HGT. However, the source organisms differ between the 2 groups of mycobacteria: *M. tuberculosis* complex underwent HGT from Firmicutes, Bacteroidetes and gamma-Proteobacteria spp. while the environmental mycobacteria acquired their 7 ORFs from Firmicutes, beta- and gamma-Proteobacteria, Archaea and Eukarya (Table S2).

The nine transferred genes identified here account for 0.02–0.09% of the mycobacterial genomic content. From the nine HGT, four candidates encode for proteins involved in metabolism and five genes encode for proteins involved in information storage and processing (Table S2). Among the five genes encoding for information storage and processing, two genes encode for amidase proteins that hydrolyse the $CO\text{-}NH_2$ bond with production of NH_3, one gene encodes for a betalactamase implicated in the bacterial resistance to beta-lactam antibiotics, one gene encodes for one transcriptional regulator and one gene encodes for a hypothetical protein, characterized by the presence of a formyl_trans_N domain and belonging to the transferase family. Among the genes encoding for metabolic proteins, two genes are implicated in transporter of different substrates across the membrane including the sulfate transporter and the amino acid permease and two genes encode for the Acetyl-CoA hydrolase and a pyridine nucleotide disulfide oxidoreductase.

The gene length of these ORFs varies from 702 to 1,485 pb. These ORFs are widely distributed across the genomes of *Mycobacterium* spp. The detailed observation of the regions surrounding these HGT candidates, i.e. 10 genes upstream and downstream, revealed the presence of 4 transposases in 3 mycobacterial genomes *M. ulcerans*, *M. smegmatis* and *M. avium* (Table S3). The GC content of 5 transferred genes significantly differ from the GC content of the genome in *M. tuberculosis*, *M. bovis*, *M. smegmatis*, *M. ulcerans* and *M. marinum* ($p<0.05$) (Table S3). Only 2 out of 9 transfered genes present both a GC% significantly differing from that of the mycobacterial host genome and transposase gene in the close vicinity.

Co-culture experiments

We co-cultured the amoeba *A. polyphaga* with both *L. pneumophila* and *M. avium*, and we observed that *L. pneumophila and M. avium*

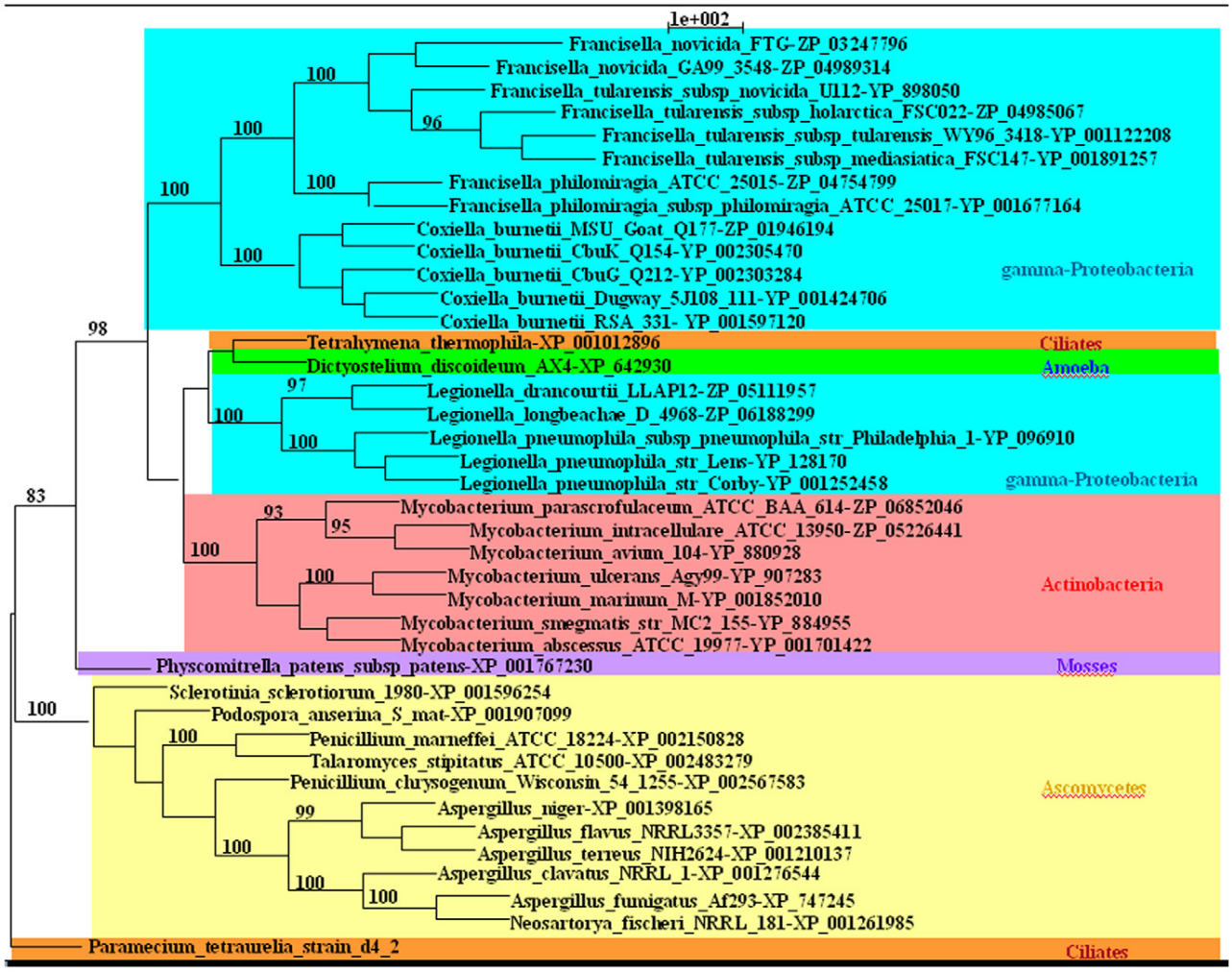

Figure 1. Phylogeny as inferred from the pyr-redox gene. The phylogenetic tree was obtained using maximum likelihood with the amino acid dataset. Numbers at the nodes represent bootstrap percentages. Only high bootstraps (>75) are indicated.
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could indeed live together in amoebae for at least five days. We first observed that the number of *A. polyphaga* trophozoites infected with *M. avium*, *L. pneumophila* or both strains increased significantly ($p \leq 0.05$) over the course of the experiments. The quantification of the colony forming units (CFU) of *M. avium* and *L. pneumophila* when co-cultured with amoebae yielded $1.66 \times 10^6 \pm 1.68 \times 10^5$ CFU/mL at day 0, $1.52 \times 10^9 \pm 2.5 \times 10^8$ CFU/mL at day 3 and $1.65 \times 10^9 \pm 2.76 \times 10^8$ CFU/mL at day 5 for *L. pneumophila* and $1.99 \times 10^5 \pm 1.63 \times 10^4$ CFU/mL at day 0, $5.2 \times 10^6 \pm 7.07 \times 10^5$ CFU/mL at day 3 and $2.05 \times 10^7 \pm 1.48 \times 10^7$ CFU/mL at day 5 for *M. avium* (Fig. 2).

Discussion

Our phylogenetic analyses identified eight mycobacterial genes that have close phylogenetic relationships with bacteria other than *Actinobacteria* spp. Given that most of these species are amoeba-resistant, the phylogenies were highly suggestive of possible HGT within amoeba. Nonetheless, the lack of information about the direction of the transfer hampered the elucidation of the HGT history. Furthermore, we found one gene encoding for pyr-redox that gave insight into the history of the HGT events in relation with the mycobacterial lifestyle within free-living protozoa.

It has been previously shown that the *Mycobacterium* spp. and gamma-Proteobacteria studied herein are able to live alone in amoebae [9,29,38,48] as well as in ciliates [12] or together in *Acanthamoeba castellanii* [49]. We therefore co-cultured the amoeba *A. polyphaga* with both *L. pneumophila* and *M. avium*, and we observed that *L. pneumophila* and *M. avium* could indeed live together in amoebae for at least five days. Thus, our data expand the previous demonstration of intra-amoebal surviving of both *Legionella* and mycobacteria in amoeba *A. castellanii* to another species of amoeba, *A. polyphaga*. This sympatric lifestyle, *i.e.*, various microorganisms living together, provides opportunities for DNA exchange and gene transfer within amoebae [3,15,23]. This hypothesis agrees with the current model for the evolution of mycobacteria, which postulates that the ancestor of mycobacteria was an environmental organism living in an aquatic habitat [50]. Recent genome analysis of the environmental *Mycobacterium indicus pranii*, a member of the *M. avium* complex, further supports this hypothesis in which the most recent common ancestor of mycobacteria gave rise to waterborne *M. marinum* and *M. ulcerans* on one branch, the *M.*

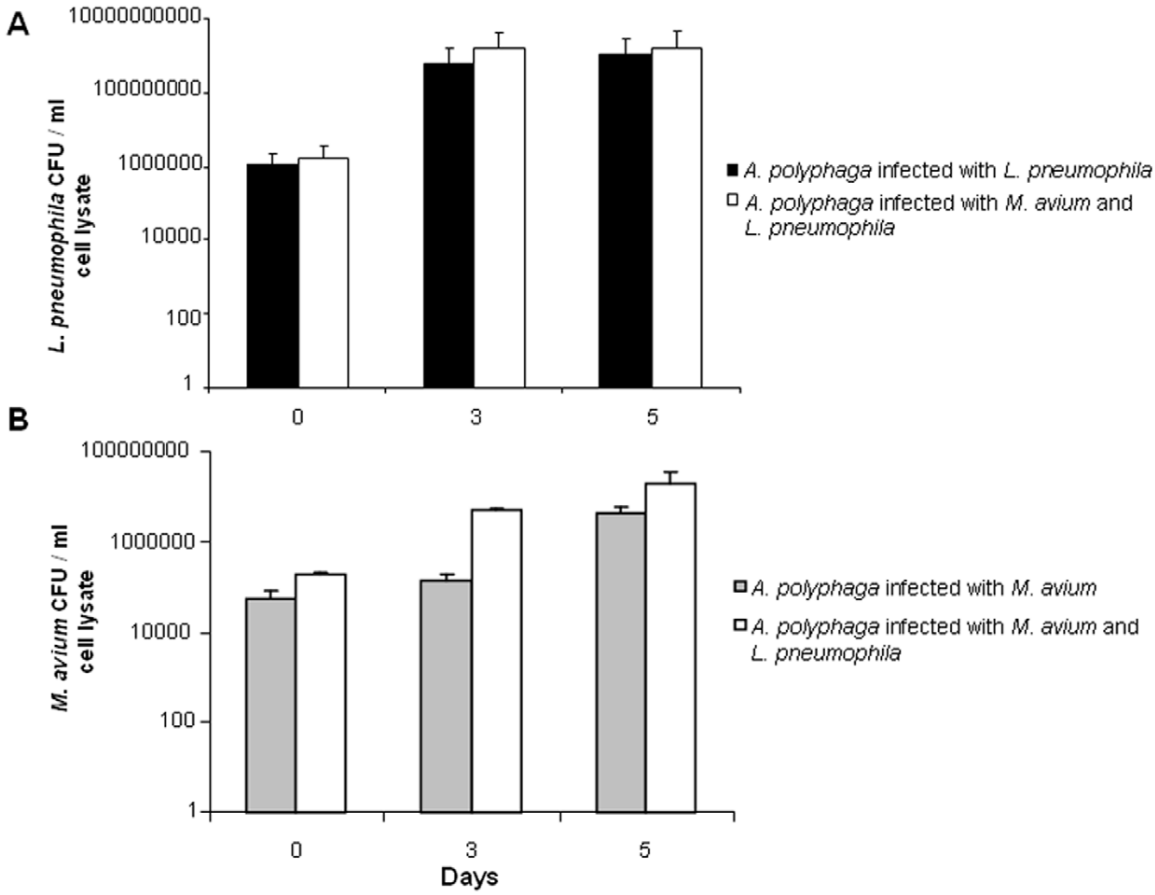

Figure 2. *A. polyphaga* co-cultured with *M. avium* and *L. pneumophila* for 5 days. A) The number of *L. pneumophila* colonies was obtained after plating the lysate of *L. pneumophila* and *A. polyphaga* culture or *L. pneumophila, M. avium* and *A. polyphaga* co-culture in BCYE agar medium. B) The number of *M. avium* colonies was obtained after plating the lysate of *M. avium* and *A. polyphaga* culture or *M. avium, L. pneumophila* and A. polyphaga co-culture in 7H10 agar medium. Data points are the means of triplicate wells, and the standard errors are represented by error bars.
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avium complex on a second branch and the *M. tuberculosis* complex on a third branch [50].

Life in free-living amoebae has been demonstrated to protect amoeba-resistant organisms, such as environmental mycobacteria and *Legionella*, against adverse environmental conditions [3,9,38,48], to increase their resistance to some antibiotics [48,51,52] and to enhance their virulence [36,48,51]. The pyr-redox gene studied herein is present in *Mycobacterium* spp. that have been shown to survive in amoebal cysts. The significant association between the presence of pyr-redox and the survival in amoebal cysts (chi2-square, $p = 0.002$) highlights the possible role of this protein in the intraamoebal lifestyle and life inside macrophages [19,48]. During phagocytosis, amoebae and macrophages produce the oxygen metabolites nitric oxide and hydrogen peroxide, which generate a toxic environment that can kill phagocytized bacteria [53–55]. *Mycobacterium* spp. deploy multiple strategies to resist to this oxidative stress, including the expression of catalase/peroxidase [56] and superoxide dismutase [57], a thiol-based detoxification response [58] and the pyr-redox response [59,60]. Pyr-redox complements the anti-oxidative arsenal of mycobacteria during their survival in amoebae and macrophages.

Phylogenetic trees have indicated that phylogenetically distant organisms have acquired the pyr-redox via HGT, but the source of this transfer is ambiguous (Fig. 3). According to one scenario, pyr-redox was acquired by *Mycobacterium* spp., *D. discoideum* and *T. thermophila* from gamma-Proteobacteria, specifically from *Legionella* spp. (Fig. 3). This result agrees with previously published observations that genes acquired by HGT in Actinobacteria mostly originated from beta- and gamma-Proteobacteria [6]. The genes acquired by HGT in fungi [61] and HGT in ciliates such as *T. thermophila* [26,62] mostly originated from bacteria. *D. discoideum* may have transferred the pyr-redox gene to *Mycobacterium* spp. or may have been the place for transfer. According to a second scenario, there were multiple gene losses in *Legionella* spp., *D. discoideum* and *T. thermophila* and recent acquisitions from *Mycobacterium* spp. (Fig. 3). Whereas several studies have demonstrated HGT between mycobacteria [63], HGT originating from *Mycobacterium* spp. has never been reported. Considering the paraphyly of *Tetrahymena* spp. and *Dictyostelium* spp., we have to postulate a minimum of two independent HGT events in both scenarii: the first one event from *Legionella* spp. or mycobacteria (ancestors) into amoebae and amoeba-resistant bacteria and the second event from *Legionella* spp. or mycobacteria (ancestors) into *T. thermophila*. Alternatively, the scenarii might have required a single ancient HGT event in a certain common ancestor of eukaryotes and subsequent multiple losses from organisms except *T. thermophila* and *D. discoideum*.

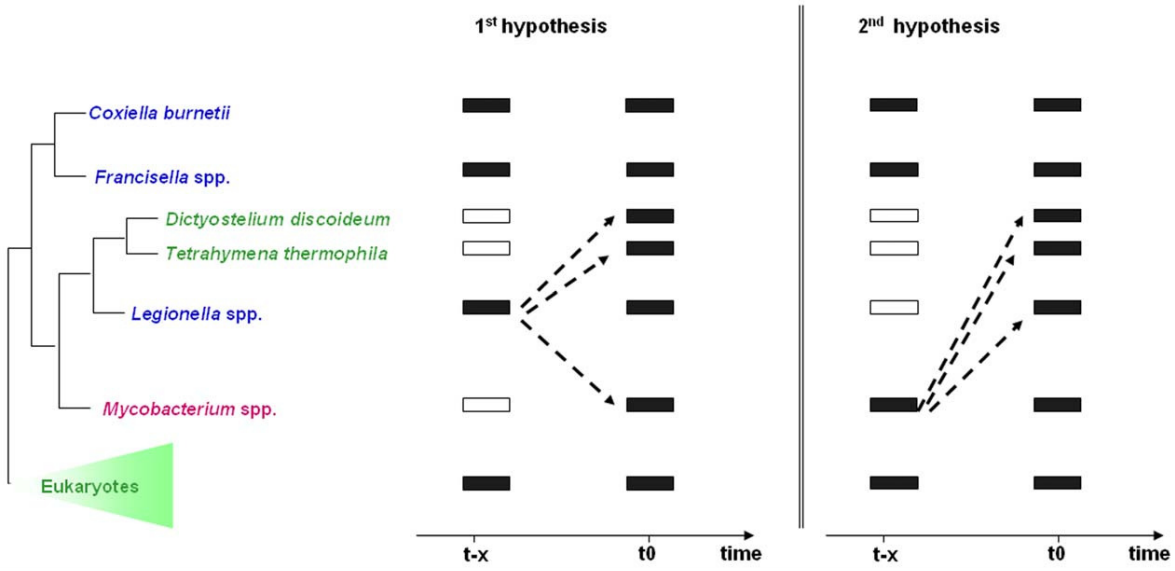

Figure 3. Schematic representation of two alternative explanations of the evolutionary history of the pyridine nucleotide disulfide oxidoreductase gene. Dashed arrows indicate the possible lateral transfer of the gene. First hypothesis: The gene encoding pyr-redox exists in the gamma-Proteobacteria species and is absent in *D. discoideum* and *T. thermophila*. This gene was acquired from *Legionella* spp. by *Mycobacterium* spp., *D. discoideum* and *T. thermophila*. Second hypothesis: The gene encoding pyr-redox was lost from *Legionella* spp., *D. discoideum* and *T. thermophila* and was acquired later from *Mycobacterium* spp. T0 corresponds to the time of observation and T-x to the time when the event occurred.
doi:10.1371/journal.pone.0034754.g003

Both scenarii involve HGT from a bacterium to a eukaryote (*Tetrahymena* spp. and *Dictyostelium)* following the loss of the eukaryotic pyr-redox gene from these genomes. Indeed, the gene encoding pyr-redox might have become disused or lost its functional importance, allowing the loss of the gene. The second scenario requires additional losses from *Legionella* genomes that occurred before the HGT and is less parsimonious than the first one. Thus, the scenario that postulates HGT from *Legionella* or *Dictyostelium* into mycobacteria seems to be more likely. The molecular clock showed that mycobacteria and amoeba-resistant gamma-Proteobacteria exchanged the pyr-redox gene between 33 and 267 Million Years Ago, after the separation of gamma-Proteobacteria spp. and before the radiation of *Legionella* spp. (Fig. 4). This range provides an estimated time-frame for the intracellular association of mycobacteria within amoebae and subsequent horizontal gene transfers. The pyr-redox gene has been found in 5/21 annotated *Mycobacterium* genomes, with the notable exception of the *M. tuberculosis* complex members. The genome of *M. tuberculosis* has been shown to exhibit the highest ratio of eukaryotic-prokaryotic gene fusion [64], but this observation was made before protist genomes were sequenced. The most parsimonious scenario suggests that the pyr-redox gene was acquired by an ancestor of all mycobacterial species, followed by a loss by the *M. tuberculosis* complex members *M. leprae*, *M. vanbaalenii*, *M. gilvum* and *Mycobacterium* sp. JLS.

In conclusion, our phylogenetic analyses found 8 ORFs most likely acquired through HGT in *Mycobacterium* spp. and one further pyr-redox ORF that elucidates the history of the HGT events in relation with the mycobacterial lifestyle within free-living protozoa. The experimental data reported herein support these genome-based analyses. Amoebae or other phagocytic organisms may have been the places in which the gene exchanges occurred. Thus, *Mycobacterium* spp. have followed an evolutionary strategy similar to that of other intracellular bacteria: they interfere with host cellular processes through the expression of genes horizontally acquired from the host. HGT may have contributed to the adaptation of mycobacteria to an intracellular lifestyle.

Supporting Information

Figure S1 Putative sources of homologous ORFs from bacteria other than Actinobacteria in the mycobacterial genome.
(PDF)

Figure S2 Extended phylogenetic tree showing representatives of the conserved hypothetical hydrolase. Phylogenetic trees showing HGT events as generated by the Maximum Likelihood method. Numbers at nodes are bootstrap percentages based on 100 resamplings. The scale bar represents the number of estimated changes per position for a unit of branch length. *Mycobacterium* spp. are colored in red.
(PDF)

Figure S3 Extended phylogenetic tree showing representatives of hypothetical protein MT3512. Phylogenetic trees showing HGT events as generated by the Maximum Likelihood method. Numbers at nodes are bootstrap percentages based on 100 resamplings. The scale bar represents the number of estimated changes per position for a unit of branch length. *Mycobacterium* spp. are colored in red.
(PDF)

Figure S4 Extended phylogenetic tree showing representatives of amidase. Phylogenetic trees showing HGT events as generated by the Maximum Likelihood method. Numbers at nodes are bootstrap percentages based on 100 resamplings. The scale bar represents the number of estimated changes per position for a unit of branch length. *Mycobacterium* spp. are colored in red.
(PDF)

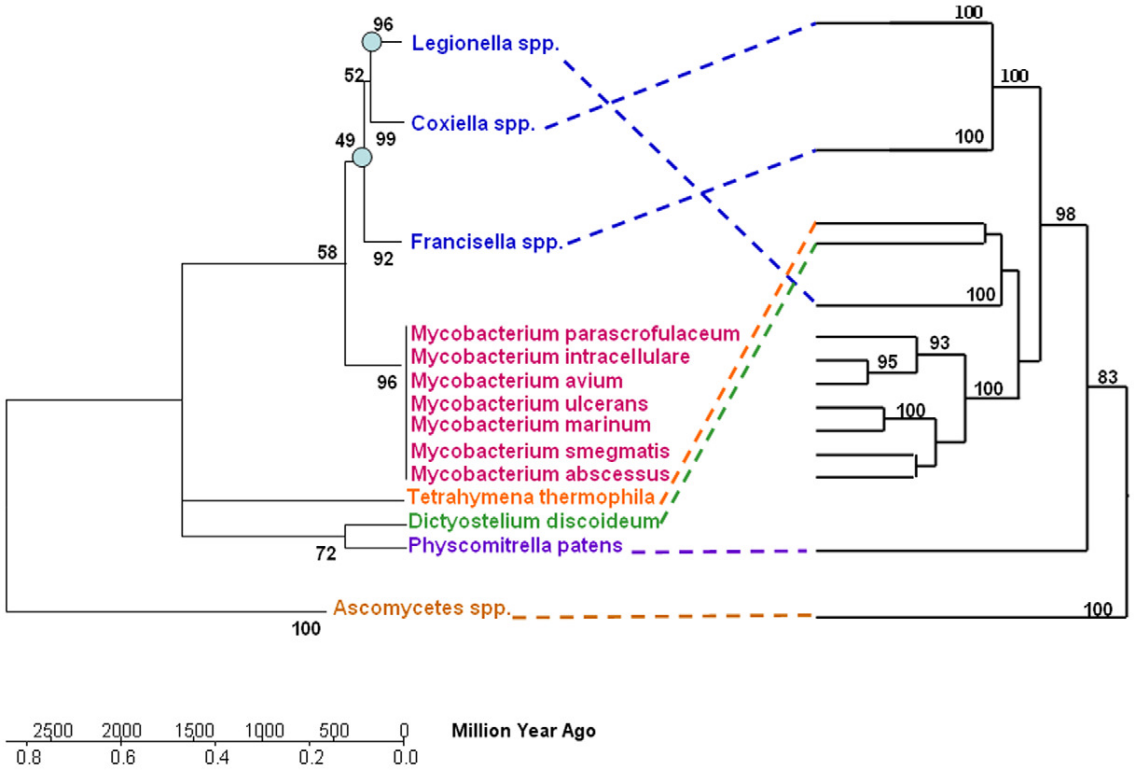

Figure 4. Schematic representation of HGT and molecular clock. The left tree shows the relationships and approximate dates of divergence of the species. The phylogeny was reconstructed based on 16S and 18S rDNA sequences, and the expected divergence was calculated as a function of time, 1–2% per 50 MYA. The right tree shows the relationships among species based on pyr redox protein. The circles on the nodes indicate the radiation of *Legionella* spp. (33 MYA) and the separation of gamma-Proteobacteria spp. (267 MYA).
doi:10.1371/journal.pone.0034754.g004

Figure S5 Extended phylogenetic tree showing representatives of A) acetyl CoA hydrolase and B) transcriptional regulator. Phylogenetic trees showing HGT events as generated by the Maximum Likelihood method. Numbers at nodes are bootstrap percentages based on 100 resamplings. The scale bar represents the number of estimated changes per position for a unit of branch length. *Mycobacterium* spp. are colored in red.
(PDF)

Figure S6 Extended phylogenetic tree showing representatives of A) sulfate transporter and B) beta-lactamase. Phylogenetic trees showing HGT events as generated by the Maximum Likelihood method. Numbers at nodes are bootstrap percentages based on 100 resamplings. The scale bar represents the number of estimated changes per position for a unit of branch length. *Mycobacterium* spp. are colored in red.
(PDF)

Figure S7 Extended phylogenetic tree showing representatives of amino acid permease. Phylogenetic trees showing HGT events as generated by the Maximum Likelihood method. Numbers at nodes are bootstrap percentages based on 100 resamplings. The scale bar represents the number of estimated changes per position for a unit of branch length. Mycobacterium spp. are colored in red.
(PDF)

Table S1 Genome sequence of amoeba-resistant bacteria utilized in this study.
(DOC)

Table S2 Genes probably transferred by horizontal gene transfer.
(DOC)

Table S3 Description of the nine probably transfered genes.
(DOC)

Author Contributions

Conceived and designed the experiments: DR MD. Performed the experiments: OL VM PP. Analyzed the data: OL VM PP MD DR. Contributed reagents/materials/analysis tools: DR PP. Wrote the paper: OL PP MD.

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Chapitre III

Acanthamoeba polyphaga-Enhanced Growth of *Mycobacterium smegmatis*

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Chapitre III – Avant propos

Mycobacterium smegmatis est une mycobactérie à croissance rapide rarement responsable des infections opportunistes chez les patients. Elle est présente dans des environnements (sols, sources d'eau) où les amibes libres résident également, mais les données concernant les interactions entre *M.* smegmatis et les amibes ont été contradictoires de la destruction des mycobactéries à leur survie.

A l'aide de la microscopie optique et électronique et de la culture basée sur l'énumération microbienne, nous avons étudié la capacité de trois souches différentes de *M. smegmatis* (*M. smegmatis* mc^2 155, *M. smegmatis* ATCC 19420^T et *M. smegmatis* ATCC 27204) à survivre dans les trophozoites et les kystes de l'amibe *Acanthamoeba polyphaga*. Nous avons observé que *M. smegmatis* pénètre, survit dans les trophozoites *A. polyphaga* et entraine la lyse des amibes et la libération des mycobactéries vivantes après cinq jours de coculture, sans la formation de kyste. Nous avons également observé une augmentation de quatre fois la croissance des trois souches de *M. smegmatis* en présence de l'amibe. D'autre part, nous avons observé que le surnageant et le culot des amibes lysés entraine une augmentation significative de la croissance des trois souches de *M. smegmatis* testées.

La coculture *M. smegmatis*-amibe entraine une augmentation de la croissance de *M. smegmatis*, leur réplication et la lyse des amibes. Ce système de coculture illustre un paradigme inhabituel dans les

interactions entre les mycobactéries et les amibes par ce que les mycobactéries ont été essentiellement considérées comme des organismes résistants aux amibes. Ce système de coculture pourrait être utilisé comme un modèle simple et rapide pour cibler les facteurs impliqués dans la croissance intracellulaire des mycobactéries.

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Acanthamoeba polyphaga-Enhanced Growth of *Mycobacterium smegmatis*

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Abstract

Background: *Mycobacterium smegmatis* is a rapidly-growing mycobacterium causing rare opportunistic infections in human patients. It is present in soil and water environments where free-living amoeba also reside, but data regarding *M. smegmatis*-amoeba relationships have been contradictory from mycobacteria destruction to mycobacteria survival.

Methodology/Principal Findings: Using optic and electron microscopy and culture-based microbial enumeration we investigated the ability of *M. smegmatis* mc^2 155, *M. smegmatis* ATCC 19420^T and *M. smegmatis* ATCC 27204 organisms to survive into *Acanthamoeba polyphaga* trophozoites and cysts. We observed that *M. smegmatis* mycobacteria penetrated and survived in *A. polyphaga* trophozoites over five-day co-culture resulting in amoeba lysis and the release of viable *M. smegmatis* mycobacteria without amoebal cyst formation. We further observed that amoeba-co-culture, and lysed amoeba and supernatant and pellet, significantly increased five-day growth of the three tested *M. smegmatis* strains, including a four-fold increase in intra-amoebal growth.

Conclusions/Significance: Amoebal co-culture increases the growth of *M. smegmatis* resulting in amoeba killing by replicating *M. smegmatis* mycobacteria. This amoeba-*M. smegmatis* co-culture system illustrates an unusual paradigm in the mycobacteria-amoeba interactions as mycobacteria have been mainly regarded as amoeba-resistant organisms. Using these model organisms, this co-culture system could be used as a simple and rapid model to probe mycobacterial factors implicated in the intracellular growth of mycobacteria.

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Introduction

Mycobacteria are mycolic-acid containing, high GC% bacterial organisms belonging to the phylum *Actinobacteria*. They are recovered from soil and fresh water environments where free-living amoeba (FLA) are also living [1,2,3]. Co-isolation of mycobacteria and FLA collected from such environmental sources has been reported [4,5]. Several experiments further demonstrated the ability of most environmental mycobacteria to survive in the amoebal trophozoites and to further reside into the amoebal cysts [6,7,8]. We recently showed that this holds true also for some of the *Mycobacterium tuberculosis* complex mycobacteria [9]. FLA have been therefore regarded as ''Trojan horses'' for such amoeba-resistant mycobacteria. Indeed, intra-amoebal survival has been demonstrated for 37 different *Mycobacterium* species and intra-amoebal surviving became a dogma for amoeba-mycobacteria interactions except for *Mycobacterium bovis* BCG which is killed by the FLA *Acanthamoeba castellanii* [8] and *Mycobacterium canettii* which bypasses amoebal encystement [9].

Amoeba-resistant mycobacteria include both slow-growing mycobacteria, i.e. mycobacteria sub-culturing over more than seven days and fast-growing mycobacteria which produce visible colonies in less than seven days [10]. Whereas fast-growing mycobacteria are comprised of both harmless organisms and opportunistic pathogens, slow-growing mycobacteria are comprised of some of the most successful bacterial human pathogens such as *M. tuberculosis* complex organisms causing tuberculosis [11], *Mycobacterium leprae* causing leprosy [12] and *Mycobacterium ulcerans* causing the Buruli ulcer [13]. Although several experimental studies have demonstrated the interactions of slow-growing mycobacteria, such as *Mycobacterium avium* complex members, with amoebae [6,8,9,14], the interactions of fast-growing mycobacteria with amoebae remain poorly understood [14,15,16]. For example, conflicting results have been published regarding *Mycobacterium smegmatis*, ranging from its survival in the amoeba [15,16] to its destruction by amoebae [14,17].

M. smegmatis is the prototypical species of the so-called *M. smegmatis* group, which also contains *Mycobacterium wolinskyi* and *Mycobacterium goodii* [18]. Organisms of this group have seldom been associated with human infection, including orthopedic device infection and bacteremia [19,20]. In the present work, we utilized *M. smegmatis* as a model organism to study the interactions of fast-growing mycobacteria with *Acanthamoeba polyphaga* which, together with *Acanthamoeba castellanii*, is one of two FLA routinely used to probe bacteria-FLA interactions [21] at large and more specifically mycobacteria-FLA interactions [22].

Materials and Methods

Mycobacterium strains

M. smegmatis mc^2 155 (ATCC 700084; a gift from Stéphane Canaan, Laboratoire d'Enzymologie Interfaciale et Physiologie de la Lipolyse CNRS UPR 9025, Marseille, France), *M. smegmatis* ATCC 19420^T and *M. smegmatis* ATCC 27204 purchased from German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany) were used in this study. *M. smegmatis* organisms were cultured in Middelbrook 7H9 liquid medium (Sigma-Aldrich Logistic Gmbh, Lyon, France) and sub-cultured in Middlebrook and Cohn 7H10 agar (Becton Dickinson, Le Pont de Claix, France) at 37°C. Under these culture conditions, the three *M. smegmatis* strains yielded smooth colonies within three days.

Microscopic detection of *A. polyphaga* infected with mycobacteria

A. polyphaga Linc-AP1 strain (a gift from T. J. Rowbotham, Public Health Laboratory, Leeds, United Kingdom) was grown at 28°C for 4 days in 150-cm^3 culture flasks (Corning, New York, USA) containing 30 mL of peptone-yeast extract-glucose (PYG) broth. When average amoeba concentration reached 5×10^5 cells/mL, amoebae were centrifuged at 500 g for 10 min and the pellet was suspended twice in 30 mL Page's modified Neff's Ameoba Saline (PAS) (Solution A-NaCl 1.20 g; $MgSO_4.7H_2O$ 0.04 g; Na_2HPO_4 1.42 g; KH_2PO_4 1.36 g/100 mL of glass distilled water. Solution B-$CaCl_2.2H_2O$ 0.04 g/100 mL of distilled water. Amoeba saline, 10 mL of solution A+10 mL of solution B+980 mL distilled water). Liquid medium-cultured *M. smegmatis* organisms were washed twice with PBS and the pellet was suspended in PAS. This inoculum was strongly vortexed to minimize mycobacterial clumping and the inoculum was determined by optic microscopy counting after Ziehl-Neelsen staining. Ten milliliters of the amoebal suspension in PAS (10^5 amoeba/mL) were inoculated with 10^6 mycobacteria/mL to achieve a MOI of 10 mycobacteria/amoeba. As controls, *A. polyphaga* and *M. smegmatis* were cultured separately in PAS. After incubation for 6 h at 32°C, the co-culture was washed three times with PAS to remove any remaining extracellular or adherent mycobacteria, and it was incubated in 10 mL PAS for 5 days at 32°C. After gentle shaking and cytocentrifugation at 100 g for 10 min, mycobacteria were detected inside amoebal trophozoites by Ziehl-Neelsen staining. Also, the presence of viable mycobacteria inside amoebal trophozoites was documented by sub-culturing. At 0, 24, 48, 72, 96 and 120 h time points, *A. polyphaga* monolayer were lysed with 0.1% Sodium dodecyl sulfate (SDS) (Sigma-Aldrich Logistic Gmbh) for 30 min and passed through a 26-gauge needle to ensure complete lysis of the amoebae. The lysate (100 µL) was plated onto 7H10 agar and incubated for four days at 37°C to determine the number of colonies (CFU) of intracellular *M. smegmatis*. The viability of amoeba, with and without bacteria, was done using Trypan Bleu coloration 0.4% (Sigma-Aldrich, Taufkirchen, Germany) and counting in the Glasstic slide chamber (HycoR, Garden Grove, California USA). Experiments were done in triplicate.

Encystment of infected amoeba

Fifty milliliters of a 48-hour amoebal co-culture (concentration, 5×10^5 amoebal cells/mL of PAS) were put in a 175-cm^3 culture flask (Corning) and infected with 5 mL (concentration, 10^7 mycobacteria cells/mL of PAS) of *M. smegmatis* suspension in PAS for 6 hours (time point, 0). The co-culture was washed twice with PAS to remove any remaining extracellular or adherent mycobacteria and it was incubated in 50 mL PAS for 5 days. In parallel, at different time points after infection (each 24 hours), ten milliliters of co-culture was taken, the supernatant was discarded and the amoebal monolayer was rinsed twice with encystment buffer (0.1 M KCl, 0.02 M Tris, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$) before being incubated (at 32°C for 3 days) in fresh encystment buffer (0.1 M KCl, 0.02 M Tris, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$). As control, *A. polyphaga* was cultured in encystment buffer. The process of excystment was verified by light microscopic examination of Ziehl-Neelsen smears. After 3 days, the number of cysts and trophozoites at different time points was determined by microscopic observation.

Moreover, the cysts corresponding to the time point 0 were then centrifuged at 1,000 g for 10 min and washed three times with PAS before using it for electron microscopic observation. Experiments were done in triplicate.

Culture of *M. smegmatis* with amoeba debris

A. polyphaga and *M. smegmatis* were prepared as described before. After washing with PAS, 10 mL of *A. polyphaga* cells suspension ($\sim5\times10^5$ amoeba/mL) were lysed (1 min at liquid nitrogen and 1 min at 37°C for three times) and centrifuged at 800 g for 10 min. 10^3 mycobacteria/mL was separately incubated with amoeba lysis pellet and supernatant for 5 days at 32°C. *M. smegmatis* were observed in the culture at each time point by Ziehl-

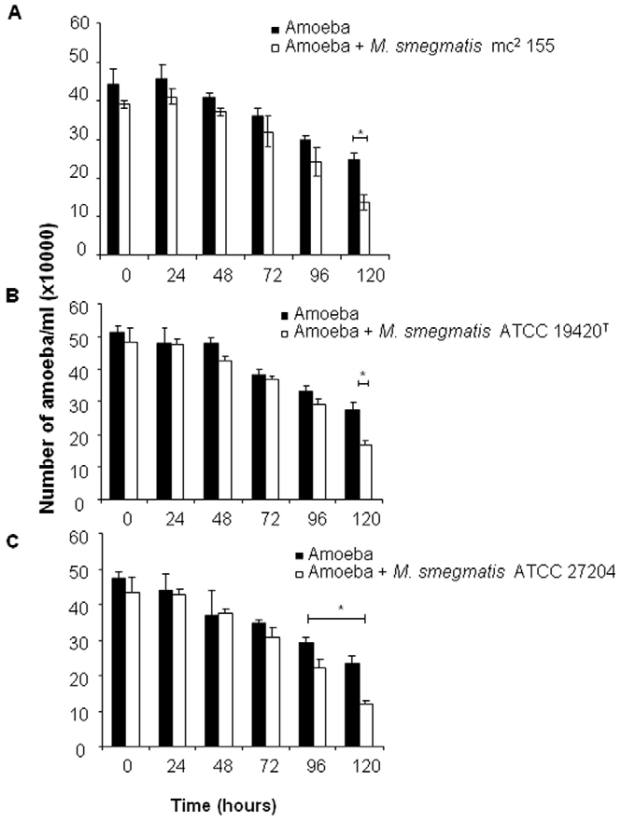

Figure 1. Amoeba increases the growth of *M. smegmatis*. Counting of amoeba alive with and without *M. smegmatis* mc^2 155 (A), *M. smegmatis* ATCC 19420^T (B) and *M. smegmatis* ATCC 27204 (C) in PAS. Asterix represent significant variation ($p\leq0.05$). Each bar represents the mean of triplicate wells, and the standard errors are represented by error bars.
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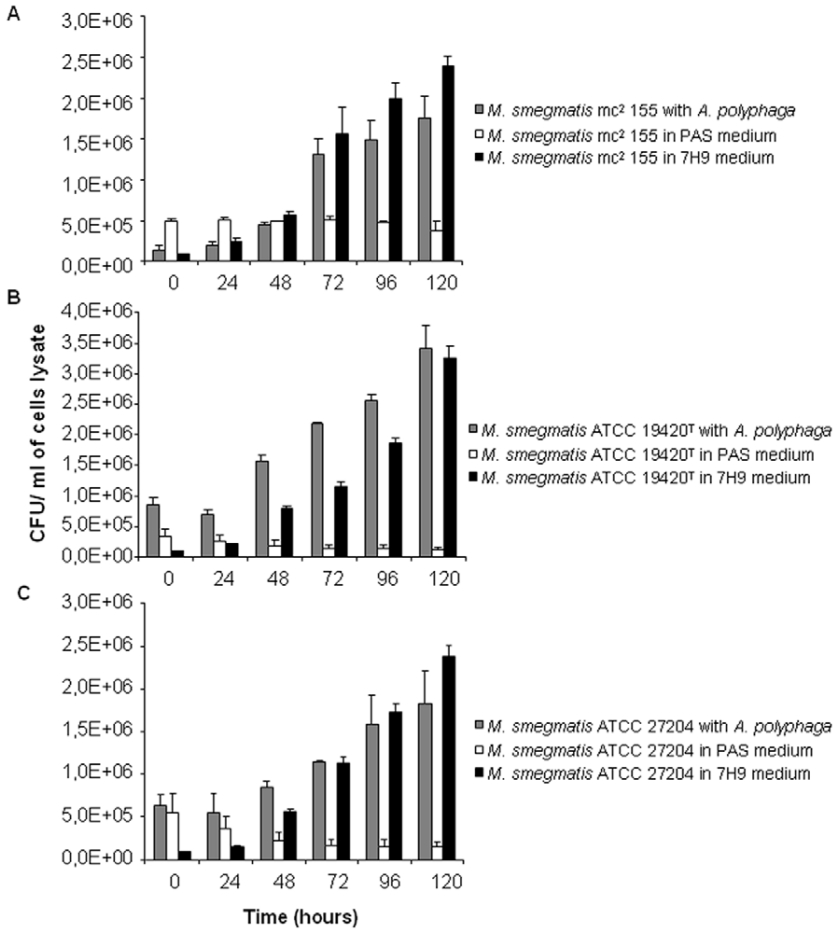

Figure 2. Growth of *M. smegmatis* within *A. polyphaga* trophozoites. *M. smegmatis* co-cultures with free-living amoeba *A. polyphaga* (gray bar) and alone in PAS medium (white bar) and in 7H9 complete medium (black bar). Three *M. smegmatis* organisms were tested: (A) *M. smegmatis* mc^2 155, (B) *M. smegmatis* ATCC 19420^T and (C) *M. smegmatis* ATCC 27204. Each bar represents the mean of triplicate wells, and the standard errors are represented by error bars.
doi:10.1371/journal.pone.0029833.g002

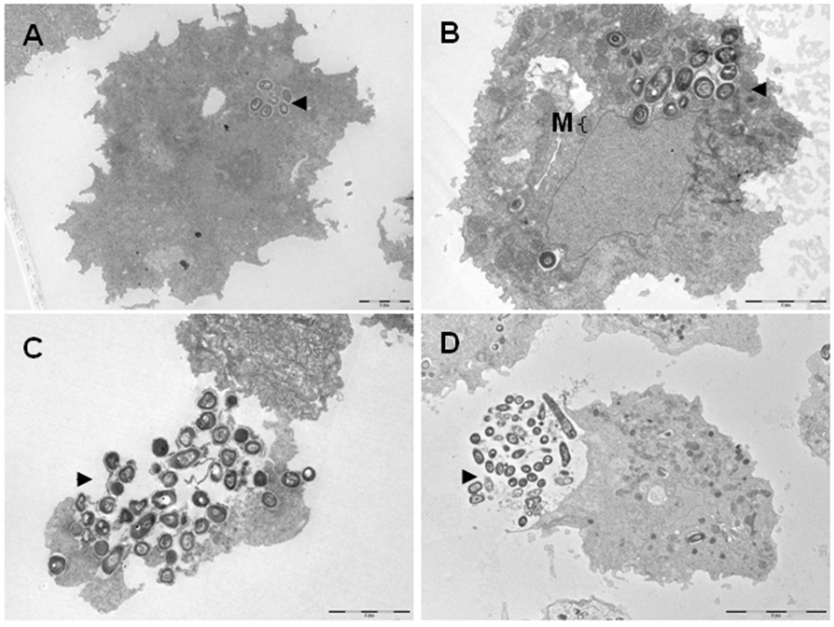

Figure 3. *M. smegmatis* is internalized into amoeba. Transmission electron-microscopy observation of *M. smegmatis* mc^2 155 (▶) co-cultivated with *A. polyphaga* trophozoites at (A) 0 hour, (B) 48 hours, (C) 72 hours and (D) 120 hours **m**: mitochondria. Scale bar: 2 μm (A, B, C) and 5 μm (D).
doi:10.1371/journal.pone.0029833.g003

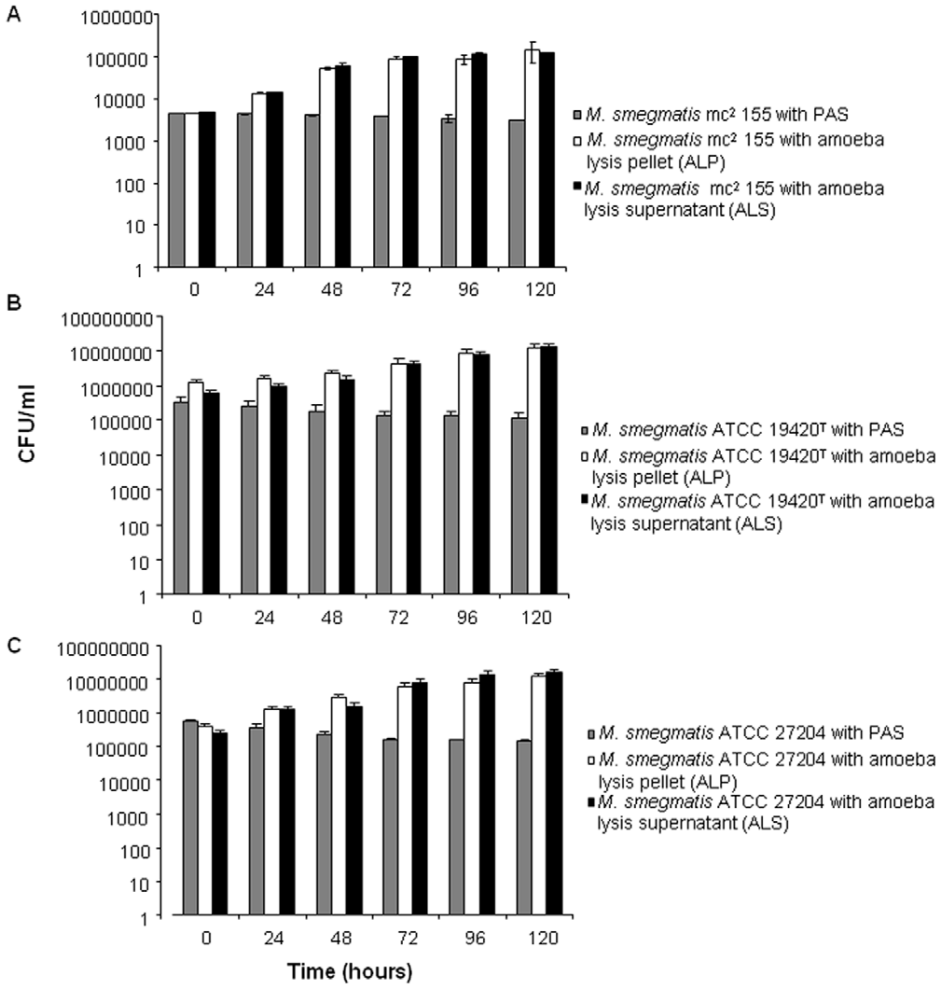

Figure 4. Growth of *M. smegmatis* in the presence of amoeba lysis. Three *M. smegmatis* organisms were tested: (A) *M. smegmatis* mc^2 155, (B) *M. smegmatis* ATCC 19420^T and (C) *M. smegmatis* ATCC 27204. *M. smegmatis* strains cultured with amoeba lysis pellet (white bar) and supernatant (black bar). PAS medium was used as negative control (gray bar). Each bar represents the mean of triplicate wells, and the standard errors are represented by error bars.
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Neelsen staining. As controls, *M. smegmatis* were cultured in PAS. Experiments were done in triplicate.

Ultrastructural study

Amoebal cysts and monolayers inoculated with mycobacteria were washed twice with sterile PAS to eliminate non-ingested mycobacteria. Samples were fixed in 2% glutaraldehyde and 0.1 M cacodylate buffer overnight, then in 2% glutaraldehyde and 0.33% acroleine in 0.07 M cacodylate buffer for one hour. After washing in 0.2 M cacodylate buffer, the preparation was post-fixed in 1% osmium tetraoxide in 0.1 M potassium ferrycyanure for one hour and dehydrated in an ascending

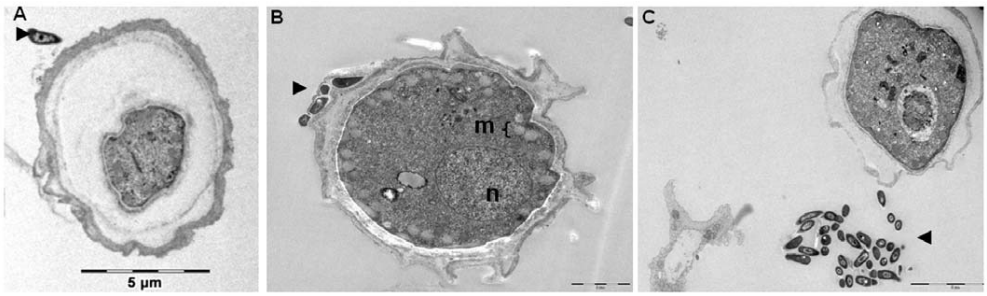

Figure 5. Transmission electron-microscopy observation of *A. polyphaga* cysts. (A) The mature form of cyst. *M. smegmatis* mc^2 155 (▶) exit from *A. polyphaga* pre-cyst (B) and present in the outside of pre-cyst (C); **n:** nucleus, **m:** mitochondria. Scale bar: 5 µm (A, C) and 2 µm (B).
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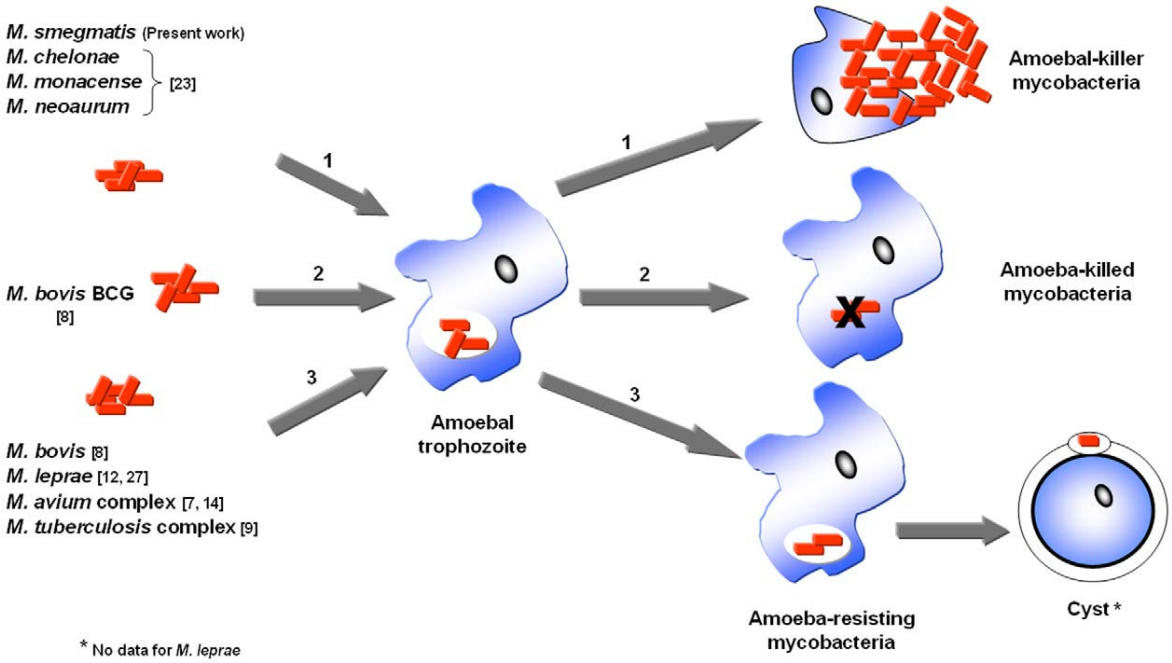

Figure 6. Different forms of amoeba-mycobacteria interactions.
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series of ethanol concentrations, up to 100% ethanol. The samples were then successively incubated (for 45 min) in a 3:1, 2:2, 1:3 (vol/vol) ethanol-Epon suspension, then in 100% Epon overnight with continuous shaking. Samples were embedded in an Epon 812 resin (Fluka, St Quentin Fallavier, France) and then incubated for three days at 60°C. Ultrathin sections (70 nm) were cut from the blocks using an ultracut microtome (Reichert-Leica, Marseille, France) before being deposited on Formvar-coated copper grids (Sigma-Aldrich). Ultrathin sections were stained for 10 min with 5% uranyl acetate and lead citrate before being examined using a transmission electron microscope (Morgani 268D; Philips, Eindhoven, Netherlands).

Results

M. smegmatis - A. polyphaga trophozoites co-culture

We first observed that the number of both non-infected and infected *A. polyphaga* trophozoites incubated into PAS at 32°C decreased over the time with the number of infected-amoeba decreasing significantly more than the number of non-infected amoeba ($p \leq 0.05$) at day four of co-culture for *M. smegmatis* ATCC 27204 and at day five of co-culture for *M. smegmatis* mc^2 155 and *M. smegmatis* ATCC 19420^T, in triplicate experiment (Figure 1). In parallel, we observed that the three tested *M. smegmatis* strains survived but did not multiply over five-day incubation in PAS at 32°C (Figure 2). At six-hour incubation, *M. smegmatis* mc^2 155-*A. polyphaga* co-culture yielded 72% infected amoeba presenting at least one vacuole containing mycobacteria (Figure 3). Such vacuoles were surrounded by several mitochondria and displayed morphological features consistent with mycobacterial division, i.e. two organisms tightly attached by one extremity into a single vacuole (Figure 3). For the three tested *M. smegmatis* strains, quantification of colony forming units (CFU) co-cultured with amoeba indicated a significant increase ($p < 0.05$) in the number of mycobacteria organisms starting at day 2 (Figure 2). To understand whether this significant increase in the growth of *M. smegmatis* co-cultured with *A. polyphaga* necessitated viable amoeba, we further cultured each one of the three tested *M. smegmatis* strains in PAS enriched in an amoeba lysis pellet (ALP) or an amoeba lysis supernatant (ALS). Regardless of the *M. smegmatis* strain, we observed that growth of *M. smegmatis* organisms was significantly increased ($p \leq 0.05$) by the addition of ALP or the addition of ALS to the PAS (Figure 4).

Interaction of *M. smegmatis* mc^2 155 with *A. polyphaga* cysts

We further infected *A. polyphaga* trophozoites with *M. smegmatis* mc^2 155 organisms for 6 hours, and then incubated in encystement buffer for 3 days noted as days 0–3. A sample was then taken every 24 hours and microscopic examination disclosed cystic formation in 43% of *M. smegmatis*-infected amoebae at day 0 (6 hours of infection); 38% at day 1; 19% at day 2 and 8% at day 3. Non-infected, negative control amoeba yielded 46% encystment at day 0; 52% at day 1; 71% at day 2 and 78% at day 3. This difference in the percentage of encysted amoeba was statistically significant from day 0 to day 3 in triplicate experiment ($p \leq 0.05$). Electron microscopy further identified mature cysts by the presence of condensation of indistinct components implicated in the metabolism and replication in the middle of this form (Figure 5A), and pre-cysts identified by the presence of the nucleus and mitochondria scattered into the cytoplasm (Figure 5B). Careful electron microscopy observation of 500 cysts formed at day 3 failed to reveal any *M. smegmatis* organisms into *A. polyphaga* cysts (Figure 5C). In one case only the *M. smegmatis* organism was observed to have moved from the endocyst of a pre-cyst present in the earlier phase of encystation after three-day encystment (Figure 5B). Experimental encystment of *A. polyphaga* co-culture yielded no intracystic mycobacteria after a three-day encystement.

Table 1. Described interactions of rapid and slow-growing mycobacteria with FLA.

Bacterial species	Growing mycobacteria		Described interaction with protozoa	References
	Rapid	Slow		
Mycobacterium abscessus	✓		IC survival and multiplication (Ap)	[23]
Mycobacterium avium		✓	IC multiplication (Ac), IK survival (Ap)	[14]
Mycobacterium avium subsp. avium		✓	IC multiplication (Ap), IK survival (Ap)	[6,7]
Mycobacterium avium subsp. paratuberculosis		✓	IC multiplication (Ap), IK survival (Ap)	[31]
Mycobacterium aurum	✓		IC multiplication (Ap), IK survival (Ap)	[6]
Mycobacterium bohemicum		✓	IC and IK survival (Ap)	[6]
Mycobacterium bovis		✓	IC survival (Ac)	[8]
Mycobacterium bovis BCG		✓	No survival (Ac)	[8]
Mycobacterium chelonae	✓		IC survival and multiplication (Ap)	[23]
Mycobacterium fortuitum subsp. fortuitum	✓		IC multiplication (Ac)	[15]
Mycobacterium fortuitum	✓		IC multiplication (Ac), IC and IK survival (Ap)	[6]
Mycobacterium gastri		✓	IC and IK survival (Ap)	[6]
Mycobacterium goodii	✓		IC and IK survival (Ap)	[6]
Mycobacterium gordonae		✓	IC and IK survival (Ap)	[6]
Mycobacterium gilvum	✓		?	-
Mycobacterium immunogenum	✓		IC and IK survival (Ap)	[6]
Mycobacterium intracellulare		✓	IC and IK survival (Ap)	[6]
Mycobacterium kansasii		✓	IC multiplication (Ac), IC and IK survival (Ap)	[6,32]
Mycobacterium lentiflavum	✓	✓	IC and IK survival (Ap)	[6]
Mycobacterium leprae		✓	IC survival (*A. culbertsoni*)	[12,16]
Mycobacterium mageritense	✓		IC and IK survival (Ap)	[6]
Mycobacterium malmoense		✓	IC and IK survival (Ap)	[6]
Mycobacterium marinum		✓	IC multiplication (Ac), IC and IK survival (Ap)	[6,33]
Mycobacterium massiliense	✓		IC and IK survival (Ap)	[18]
Mycobacterium mucogenicum	✓		IC and IK survival (Ap)	[6]
Mycobacterium peregrinum	✓		IC and IK survival (Ap)	[6]
Mycobacterium phlei	✓		IC and IK survival (Ac)	[15]
Mycobacterium porcinum	✓		IC and IK survival (Ap)	[6]
Mycobacterium septicum	✓		IC and IK survival (Ap)	[6]
Mycobacterium scrofulaceum		✓	IC multiplication (Tp), IK survival (Tp)	[34]
Mycobacterium simiae		✓	IC and IK survival (Ap), IC survival (Ac)	[6,15]
Mycobacterium smegmatis	✓		IC survival and multiplication (Ap)	Present work
Mycobacterium szulgai		✓	IC and IK survival (Ap)	[6]
Mycobacterium tuberculosis		✓	IC survival (Ap)	[9]
Mycobacterium terrae		✓	IC and IK survival (Ap)	[6]
Mycobacterium tusciae		✓	IC and IK survival (Ap)	[6]
Mycobacterium ulcerans		✓	IC survival (Ac, Ap)	[15,35]
Mycobacterium xenopi		✓	IC multiplication (Ap), IK survival (Ap)	[9,26]

IC, intracellular; IK, intracyst; Ap, *Acanthamoeba polyphaga*; Ac, *Acanthamoeba castellanii*; Tp, *Tetrahymena pyriformis*.
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Discussion

The data presented in this study were interpreted as authentic because negative controls remained negative in each experimental step. In this work, two model organisms have been used in order to set-up a standardized co-culture system. Moreover, similar results were obtained when testing three different strains of *M. smegmatis*, including one type strain as well as *M. smegmatis* mc^2 155 (ATCC 700084). Indeed, *M. smegmatis* mc^2 155 strain, the only *M. smegmatis* strain with available genome sequence, has particular parietal features which may not be found in other *M. smegmatis* strains. This could have biased results. We herein show that this was not the case. Moreover, *M. smegmatis* mc^2 155 has known genetics and it has been previously used in 37/46 (80%) studies dealing with *M. smegmatis* – macrophage/amoeba interactions (Table S1). In addition, *M. smegmatis* mc^2 155 is commonly used as a model strain for the cloning genes from harmful mycobacteria [17]. Likewise, *A. polyphaga* has been extensively used for studying amoeba-mycobacteria interactions [6]. The co-culture system herein reported is therefore a standardized system which could be

reproduced in other laboratories. We observed that *M. smegmatis* organisms readily penetrated into *A. polyphaga* trophozoites, a reproducible result obtained by using a low (1:10) multiplicity of infection (MOI). We further observed that such intra-amoebal mycobacteria survived into *A. polyphaga* trophozoites, a fact documented by microscopic observations. Previously published data regarding the *M. smegmatis*-amoeba relationships have been conflicting: some studies reported that *M. smegmatis* survived within *A. castellanii* [15,16], whereas other studies found the opposite [14,17]. These discrepancies may be explained by the fact that a 30-minute amoeba-*M. smegmatis* co-culture used in some studies may be insufficient for the mycobacteria to penetrate into the amoeba. Thus, our data expand the previous demonstration of intra-amoebal surviving of *M. smegmatis* in amoeba *A. castellanii* to another species of amoeba, *A. polyphaga*.

We further observed that *M. smegmatis* organisms multiply within amoeba during the time of the experiment and that *M. smegmatis* lysed the amoeba at the 4–5 days p.i. peak of its intra-amoebal growth. Amoebal lysis has been previously reported for the rapidly growing *Mycobacterium chelonae*, *Mycobacterium abscessus*, *Mycobacterium monacense* and *Mycobacterium neoaurum* [23]. Also, 63 of 454 non-mycobacterial strains isolated from water yielded complete and rapid lysis of amoebae [23]. These bacteria were organisms closely related to *Clostridium haemolyticum*, *Methylobacterium sp.*, *Pseudomonas aeruginosa* and *Bradyrhizobium japonicum* [24,25].

Interestingly, we further observed that pelleted debris of lysed amoeba and the supernatant of such lysed amoeba also significantly enhanced the growth *M. smegmatis* mycobacteria, regardless of the strain under study. This observation reminds recent observations made when co-culturing *Salmonella enterica* Typhi with *A. castellanii* [21] and suggests that amoeba contain one or several currently uncharacterized growth-promoting factors or nutriments for *M. smegmatis*. Determining such factors was beyond the scope of present study, but further culture-based experiments incorporating fractions of amoeba supernatant are warranted to precise the nature of these factors.

We further observed that *M. smegmatis* moved out of the *A. polyphaga* pre-cyst before its maturation; this observation extended previous data found for other rapidly growing mycobacteria such as *Mycobacterium septicum* [6]. This observation contrasts with previous observations that slowly growing mycobacteria survived within the amoebal exocyst [26]. It was observed that 92% of *M. avium*-infected trophozoites evolved into mature cysts whereas we observed that only 8% of *M. smegmatis*-infected trophozoites produced mature cysts at the same time [26]. Accordingly, forced encystment of *M. smegmatis*-infected *A. polyphaga* amoeba yielded no mycobacteria in the cysts. Taken together, these data suggest that fast-growing mycobacteria rapidly escape the encystment to infect new amoebal trophozoites. Interestingly, we recently observed that *M. canettii* was the only tested *M. tuberculosis* complex member to by-pass the *A. polyphaga* encystement [9]. Exactly as for *M. smegmatis*, *M. canettii* also massively invaded the amoeba host [9].

Previously published findings [15,16] coordinated with herein presented results, suggest that rapidly growing mycobacteria should be regarded as amoeba-killing mycobacteria contrary to slowly growing mycobacteria (Figure 6). Indeed, most previous experimental studies of amoebae-mycobacteria interactions focused on slowly growing mycobacteria (Table 1). It has been observed that these species, such as *M. bovis* [8], *M. tuberculosis* [9], *M. leprae* [12,27], *Mycobacterium xenopi* [26] and members of the *M. avium* complex [7,14], can survive and/or multiply within trophozoites.

We previously proposed that amoeba are a training field for macrophage resistance of mycobacteria [28]. Several studies used amoeba to investigate the phagocytosis and intracellular survival mechanisms of pathogens including *Legionella pneumophila* [29], *Yersinia pseudotuberculosis* [23] and *P. aeruginosa* [30]. *M. smegmatis* has been used to develop genetic engineering of mycobacteria and the *M. smegmatis*-amoeba co-culture developed here could therefore be used as a simple and rapid first-line system to scan mycobacterial factors implicated in the intracellular growth of mycobacteria.

In conclusion, the spectrum of interactions between amoeba and environmental mycobacteria may be wider than previously appreciated. It includes mycobacteria such as *M. leprae* surviving in amoeba [12,27], mycobacteria such as *M. avium* and *M. tuberculosis* multiplying in amoeba as opportunistic organisms [9,7,14] and mycobacteria such as *M. chelonae* [23] and *M. smegmatis* killing the amoeba (Figure 6).

Supporting Information

Table S1 The *M. smegmatis* strain used in 46 published studies on *M. smegmatis* – macrophage/amoeba interactions.
(XLS)

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Author Contributions

Conceived and designed the experiments: MD. Performed the experiments: OL FMM. Analyzed the data: OL MD. Contributed reagents/materials/analysis tools: MD. Wrote the paper: OL MD.

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Chapitre IV

Mycobacterium gilvum illustrates size-dependant mycobacteria amoeba relationships

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Chapitre IV – Avant propos

Les amibes libres sont bactéricides, mais certaines mycobactéries à croissance rapide sont des organismes résistants aux amibes qui peuvent survivre dans ces différentes formes : trophozoites et/ou kystes. Cette capacité n'a pas été étudiée pour la mycobactérie à croissance rapide *Mycobacterium gilvum*.

Nous avons étudié la capacité de *M. gilvum* à survivre dans les trophozoites de l'amibe *Acanthamoeba polyphaga* en utilisant la microscopie optique et électronique et la culture à base des dénombrements microbiens. Six heures post-infection, nous n'avons observé que 29% des cellules d'*A. polyphaga* qui étaient infectées par *M. gilvum*. Des observations extensives en microscopie électronique ont permis de montrer l'absence de *M. gilvum* dans les kystes d'amibes.

Les résultats de ce travail illustrent pour la première fois une relation entre la taille des mycobactéries et leurs interactions avec les amibes. Les mycobactéries d'une taille inferieure à 2 µm (dans cette étude *M. gilvum* mesurée 1,4 ± 0,25 µm) ne croissent pas à l'intérieur des trophozoites d'amibes et ne les tuent pas. Par ailleurs, la taille des mycobactéries à croissance rapide est corrélée à leur position sur un arbre construit à partir de la séquence du gène *rpoB*, mais les mécanismes sous-jacents à cette observation restent à déterminer.

Mycobacterium gilvum illustrates size-dependant mycobacteria amoeba relationships

Running title: *Mycobacterium gilvum*-amoeba relationships

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ABSTRACT

Mycobacteria are isolated from soil and water environments where free-living amoebae are living. Free-living amoebae are bactericidal, yet some rapidly-growing mycobacteria are amoeba-resistant organisms surviving in the amoebal trophozoites and cysts. Such capacity has not been studied for the environmental rapidly-growing *Mycobacterium gilvum*. We investigated the ability of *M. gilvum* to survive in the trophozoites of *Acanthamoeba polyphaga* strain Linc-AP1 by using optic and electron microscopy and culture-based microbial enumerations in the presence of negative controls. We observed that 29% of *A. polyphaga* cells were infected by *M. gilvum* mycobacteria 6-hour post-infection. *M. smegmatis* mycobacteria survived, did not multiply and did not kill the amoebal trophozoites until five-day co-culture. Extensive electron microscopy observation found no *M. gilvum* organisms into the amoebal cysts. *M. gilvum* illustrates that mycobacteria-amoeba relationships depend on mycobacteria size. Mycobacteria measuring < 2 µm (*M. gilvum* measured in this study was 1.4 ± 0.25 µm) significantly ($P < 0.05$) do not grow within and do not kill amoebal trophozoites. Size of rapidly-growing mycobacteria correlates with their *rpo*B gene sequence-based phylogeny but the mechanisms underlying such observation remains to be determined.

BACKGROUND

Non-tuberculous mycobacteria are environmental organisms (31, 7) found in soil (29), marine environment (25) and fresh water (11, 12). They are recovered from water samples also colonized by free-living amoeba (FLA) (13, 4, 1). Despite the fact that FLA are bactericidal, several non-tuberculous mycobacteria were found to be amoeba-resistant, surviving within FLA trophozoites and cysts (14, 21). Latter act as "Trojan horses" protecting environmental mycobacteria from unfavorable conditions (27, 1, 2).

Amoeba-resistant mycobacteria include slowly-growing mycobacteria (SGM) such as *Mycobacterium avium* (3) and *Mycobacterium tuberculosis* complex mycobacteria (33, 27); and more than 25 different species of rapidly-growing mycobacteria (RGM) (1, 21). The outcome of such rapidly-growing, amoeba-resistant mycobacteria depends on the mycobacterial species: some *Mycobacterium* species such as *Mycobacterium septicum* survive without multiplication into the trophozoites (1), while other species such as *Mycobacterium smegmatis* and *Mycobacterium chelonae* multiply within the trophozoite (21, 30). Also, some mycobacteria such as *Mycobacterium canettii* escape the FLA before encystment (27) whereas the majority of *Mycobacterium* species encyste with the amoeba (27, 3). The determinants of such contrasted amoeba-mycobacteria relationships are poorly known, except for the role of mycobacterial cellulases (28).

Mycobacterium gilvum (formerly *Mycobacterium flavescens*) is an environmental mycobacteria, isolated from river sediments based on its ability to degrade polycyclic aromatic hydrocarbons such as pyrene, as a sole source of carbon and energy (6, 9). It is able to form biofilm and its resists to ampicillin, being susceptible to other antibiotics including isoniazid (32). *M. gilvum* has rarely been isolated as an opportunistic pathogen (32) and no study issued regarding *M. gilvum* amoeba relationships.

We therefore studied the relationships between *M. gilvum* with the FLA *Acanthamoeba polyphaga* trophozoites and cysts and derived features characterizing amoeba-mycobacteria relationships.

MATERIALS AND METHODS

Mycobacterium and *A. polyphaga* strains.

The type strains of *Mycobacterium senegalense* DSM-43656^T, *Mycobacterium conceptionense* DSM-45102^T, *Mycobacterium rhodesiae* DSM-44223^T, *Mycobacterium thermoresistibile* DSM-44167^T, *Mycobacterium chelonae* DSM-43804^T, *Mycobacterium smegmatis* DSM-43756^T and *M. gilvum* DSM-45363^T were purchased from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). Mycobacteria were cultured in Middlebrook 7H9 liquid medium (Sigma-Aldrich, Lyon, France) and subcultured at 37°C on Middlebrook and Cohn 7H10 agar (Becton Dickinson, Le Pont de Claix, France) for three days. This strain developed smooth colonies after three days culture. *A. polyphaga* Link-AP1 strain (23) was cultured in peptone-yeast extract-glucose (PYG) medium at 32°C for 3 day as described previously (27, 21). In brief, *A. polyphaga* amoebae were suspended twice in Page's modified Neff's Amoeba Saline (PAS) to obtain 5.10^5 cells/mL and 10 mL of such suspension was dropped into falcon 50 mL (Becton Dickinson, Le Pont de Claix, France).

M. gilvum-amoeba coculture.

The liquid culture of *M. gilvum* was washed two times with sterile phosphate-buffer saline (PBS) and the pellet was suspended in PAS. Each 10 mL of the amoebal culture was inoculated with 1 mL of a 10^6 mycobacteria/mL suspension (multiplicity of infection (MOI) =1:10). As a control, *A. polyphaga* and *M. gilvum* were cultured separately. After 6 h incubation at 32°C, the coculture was washed two times with PAS to remove any remaining extracellular or adherent mycobacteria (21). After washing, the coculture was incubated in 10 mL of PAS for 5 days at 32°C. The presence of intra-amoebal mycobacteria was determined by shaking, 10 min centrifugation at 100 g and observation using a light microscope after Ziehl-Neelsen staining. In

addition, the presence of viable mycobacteria inside amoebal trophozoites was assessed as previously described (21). In brief at 0, 24, 48, 72, 96 and 120 h post-inoculation time points, the *A. polyphaga* monolayer was lysed with 0.1% sodium dodecyl sulfate (Sigma-Aldrich) for 30 min and passed through a 26-gauge needle to ensure complete lysis of the amoebae. A 100 µL volume of lysate was plated onto 7H10 agar and incubated for four days at 37°C to determine the number of colonies (CFU) of intracellular mycobacteria. Experiments were done in triplicate.

Encystment of *M. gilvum*-infected amoeba.

Amoebae were cultured with encystement buffer as described previously (21, 27). In brief, 10 mL of amoebal coculture (5.10^5 cells/mL of PAS) were infected with 1 mL (10^6 mycobacteria/mL of PAS) of *Mycobacterium* suspension in PAS for 6 hours. The supernatant was discarded and the amoebal monolayer was rinsed twice with encystment buffer before being incubated at 32°C for three days in fresh encystment buffer (21). Moreover, cysts corresponding to the time point 0 were centrifuged at 1,000 g for 10 min and washed three times with PAS before electron microscopy observation. Experiments were done in triplicate.

Ultrastructural studies.

Ultrastructural observations were done as previously described (21). In brief, amoeba monolayer previously infected by *M. gilvum* and amoebal cysts were washed three times with sterile PAS to eliminate noningested mycobacteria and fixed (21). Then, the samples were successively incubated for 45 min in a 3:1, 2:2, 1:3 (vol/vol) ethanol-Epon suspension, then in 100% Epon overnight with continuous shaking before being embedded in an Epon 812 resin (Fluka, St Quentin Fallavier, France) incubated for three days at 60°C. Ultrathin sections (70 nm) were cut from the blocks using an ultracut microtome (Reichert-Leica, Marseille, France) before

being deposited on Formvar-coated copper grids (Sigma-Aldrich). Ultrathin sections were stained for 10 min with 5% uranyl acetate and lead citrate before being examined using a transmission electron microscope (Morgani 268D; Philips, Eindhoven, the Netherlands). The size of *M. senegalense*, *M. conceptionense*, *M. rhodesiae*, *M. thermoresistibile*, *M. chelonae*, *M. smegmatis* and *M. gilvum* mycobacteria was measured by electron microscopic observation of 30 pictures of single bacteria to determine the median and standard deviation of cell length.

_rpo_B gene sequence-based phylogeny.

For phylogenetic analyses, the *rpo*B gene sequences of 31 RGM were downloaded from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). All sequences were aligned using MUSCLE algorithm (22). Alignments were refined with the BioEdit program v7.0.9 (16) in order to remove regions that contain gaps or are highly divergent. Refined alignments were analysed for maximum likelihood (ML). A ML tree was constructed using PHYML (15) in the PHYLIP package version 3.5c with 1,000 randomizations of input order. The substitution model was set to WAG and enabled the optimization options for tree topology, branch lengths and rate parameters. Bootstrap support of >75% and posterior probability of >90% were considered to identify supported nodes.

Statistical analyses

All statistical analyses mentioned in this study were performed using the chi2-square test with a significance level of $p = 0.05$.

RESULTS

Survival of *M. gilvum* into *A. polyphaga* trophozoites and cysts.

The number of non-infected (negative control) and infected *A. polyphaga* trophozoites incubated into PAS at 32°C did not change significantly over the time of the experiment. After six hours co-culture, 29% of *A. polyphaga* cells were found to be infected by mycobacteria as confirmed by Zielh-Neelsen staining. The number of *M. gilvum* per trophozoite varied from 1 to 46 (a mean of 17 ± 14 mycobacteria/trophozoite). Electron microscopy revealed mycobacteria into vacuoles surrounded by several mitochondria (Fig. 1). Numerating *M. gilvum* colony-forming units (CFU) co-cultured with amoeba yielded 2.4 X 10^5 ± 5.1 10^4 CFU/mL at day 0, 2.9 X 10^5 ± 4.7 10^4 CFU/mL at day 1, 1.7 X 10^5 ± 4.3 10^4 CFU/mL at day 2, 1.3 X 10^5 ± 3.4 10^4 CFU/mL at day 3, 1.5 X 10^5 ± 3.5 10^4 CFU/mL at day 4 and 1.3 X 10^5 ± 2.9 10^4 CFU/mL at day 5 (Fig. 2). No significant difference was observed in the number of mycobacteria with time ($p = 0.1$). Mycobacteria survived in PAS yet *M. gilvum* CFUs did not increase from day 0 to day 5 (negative control) (Fig. 2). Electron microscopy revealed pre-cysts and mature-cyst after 3-day co-culture. Careful electron microscopy observation of 300 cysts formed at that time failed to reveal any *M. gilvum* organism into *A. polyphaga* cysts.

RGM size measurement

When it was not available in literature, we measured the length of RGM mycobacterial cells using electron microscopy observation. In this study, measured length was 2 ± 0.3 µm for *M. senegalense*, 2.4 ± 0.8 µm for *M. conceptionense*, 2.1 ± 0.8 µm for *M. chelonae,* 2.6 ± 0.6 µm for *M. smegmatis,* 1.1 ± 0.2 µm *M. rhodesiae*, 0.9 ± 0.2 µm *M. thermoresistibile*, and 1.4 ± 0.25 µm for *M. gilvum* (Fig. S1). *rpo*B gene sequence-derived phylogenetic tree clusterized *M. gilvum*, *Mycobacterium* strain Spyr1 and *M. vanbaalenii* all measuring < 2 µm in one cluster with > 99%

191 bootstrap values and all RGM species measuring > 2 µm in another cluster with > 90% bootstrap
192 values comprising of one *M. chelonae-M. abscessus* subcluster and one *M. fortuitum* subcluster
193 (Fig. 3). The size of mycobacteria significantly correlated with intraamoebal growth and amoeba
194 killing with mycobacteria measuring < 2 µm are not growing within and are not killing amoebal
195 trophozoites and mycobacteria measuring > 2 µm being growing within and killing amoebal
196 trophozoites ($p < 0.05$) (Table 1).
197
198 **DISCUSSION**
199 Data herein presented were interpreted as authentic since negative controls remained
200 negative in each experimental step performed in triplicate. *A. polyphaga* and *Acanthamoeba*
201 *castellanii* are two FLA routinely used to probe mycobacteria-FLA interactions (10, 35, 24).
202 We observed that *M. gilvum* mycobacteria penetrated into *A. polyphaga* trophozoites, a
203 reproducible result obtained by using a low (1:10) multiplicity of infection (MOI). Previous
204 studies have showed that the majority of RGM penetrated into amoebal trophozoites (Table 1)
205 but our observation that *M. gilvum* could also be ingested by amoebal trophozoites has not been
206 previously reported. We further observed that such intra-amoebal *M. gilvum* mycobacteria
207 survived into the *A. polyphaga* trophozoites, a fact documented by both microscopic observations
208 and microbial enumerations. This observation agree with previous demonstrations of intra-
209 amoebal surviving of *Mycobacterium septicum*, *Mycobacterium abscessus* (1) and *M. smegmatis*
210 in *A. castellanii* (17,34) and *A. polyphaga* (21). Furthermore, *M. gilvum* mycobacteria were
211 observed into vacuoles, as previously observed for other RGM as *M. septicum*, *Mycobacterium*
212 *mucogenicum*, *Mycobacterium massilience* and *M. smegmatis* in *A. polyphaga* (1, 21). For
213 mycobacteria, intra-amoebal survival relies on mechanisms allowing them to also penetrate and
214 survive into macrophages, classifying them as pathogens (14). Data presented in this study

suggest that *M. gilvum* would be also an organism surviving in macrophages; accordingly, it had been rarely reported as an opportunistic pathogen in humans (32).

M. gilvum mycobacteria did not multiply within amoeba and did not kill the amoeba during the time of the experiment. This is contrary to other RGM such as *M. abscessus*, *M. chelonae*, *M. smegmatis*, *Mycobacterium monacens* and *Mycobacterium neoaurum* which all multiply within trophozoites and kill the amoeba after five-day co-culture (17, 30, 21). These data indicate that not all the RGM are amoebal killers suggesting that factors other than the rapid growth may be involved in the mycobacteria-amoeba interactions. Accordingly, we here observed that intraamoebal multiplication and amoeba killing significantly correlated with the size of mycobacteria. Indeed, *M. gilvum* and other small RGM *M. rhodesiae* and *M. thermoresistibile* measuring less than 2 µm do not kill amoeba contrary to bigger RGM species (This study). This puzzling observation could be extended to all SGM, which are all measuring less than 2 µm and do not kill amoeba; the notable exception being *M. canettii*, a species measuring more than 2 µm (27) which does not kill, but instead escapes out of amoeba. Size is relevant as *rpoB* gene sequence-based phylogenetic tree clusters RGM species herein studied according to their size.

Extensive electron microscope observation failed to reveal any *M. gilvum* organisms in cysts. This observation extends previous observations made for other RGM such *M. smegmatis* (21). Combining morphological and cultural data indicates that the majority of RGM bypass the amoebal cyst after they are phagocyted into the amoebal trophozoites. These data agree with the previous observations that all *M. canettii* organisms and the majority of *M. tuberculosis* organisms and non-tuberculous organisms such as *M. smegmatis* (21) moved out of the *A. polyphaga* pre-cyst before its maturation contrary to *M. avium* organisms (3). Indeed, we observed that *M. gilvum* genome encodes for a putative cellulose binding protein (CBD2) and

239 one candidate cellulase (Cel12), which are sugar-cleaving enzymes capable of hydrolyzing
240 cellulose, a major component of the amoebal cyst wall addition. This observation agrees with a
241 recent observation that the genome of several *Mycobacterium* spp. encode such cellulases (26,
242 28).

243 In conclusion, the spectrum of RGM-amoeba interactions may be wider than previously
244 reported. It may partly relied on the size of the RGM species and is comprising of (i) RGM
245 species smaller than 2 µm, surviving in amoebal trophozoites but not in the cysts such as *M.*
246 *septicum* (1) and *M. gilvum* (present work); (ii) RGM smaller than 2 µm, surviving into
247 trophozoites and cysts such as *M. fortuitum* (1, 8); and (iii) RGM longer than 2 µm, killing the
248 amoeba such as *M. chelonae* (30) and *M. smegmatis* (21) (Fig. 4).

ACKNOWLEDGMENTS

The authors acknowledge Audrey Borg and Audrey Averna for their technical help with the electron microscopy observations.

COMPETING INTERESTS

The authors declare that they have no competing interests.

256 **TABLE 1. Mycobacteria amoeba relationships.**

Growing mycobacteria	Mycobacteria	Amoeba-Killer	Length (µm)	Multiply in trophozoites	Reference(s)
Rapid	*M. abscessus*	+	> 2	+	30, 19
	M. chelonae	+	> 2	+	30, 18, 5
	M. smegmatis	+	> 2	+	21
	M. fortuitum subsp. fortuitum	+	> 2	+	17
	M. fortuitum	+	> 2	+	1
	M. gilvum	-	< 2	-	This study
Slow	*M. bovis*	-	< 2	-	27
	M. leprae	-	< 2	-	20
	M. avium	-	< 2	-	27
	M. tuberculosis	-	< 2	-	27
	M. canettii	-	> 2	-	27

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Figure legends:

Fig. 1. *M. gilvum* mycobacteria are internalized into amoeba. Transmission electron-microscopy observation of *M. gilvum* (►) co-cultivated with *A. polyphaga* trophozoites at (A) 0 hour (B) 72 hours. **m**: mitochondria. Scale bar: 2 µm (A, B).

Fig. 2. Growth of *M. gilvum* within *A. polyphaga* trophozoites. *M. gilvum* co-cultured with free-living amoeba *A. polyphaga* (black bar), cultivated in PAS medium (grey bar) and cultivated in 7H9 complete medium (white bar). Each bar represents the mean of triplicate wells. Standard errors are represented by error bars.

Fig. 3. *rpoB* gene sequence-based phylogenetic tree of *M. gilvum* and 30 other RGM species by using the maximum likelihood method. The support of each branch, as determined from 100 bootstrap samples, is indicated by the value (%) at each node. Only bootstrap values > 90% are indicated. RGM with a size < 2 µm are illustrated with one asterix, RGM with a size > 2 µm are illustrated by bold characters, RGM with an undetermined size are written in simple characters.

Fig. 4. The spectrum of RGM-amoeba interactions.

Supplemental Material:

Fig. S1. Size of *M. gilvum* (A)*, M. senegalense* (B)*, M. conceptionense* (C)*, M. rhodesiae* (D)*, M. thermoresistibile* (E), *M. chelonae* (F) and *M. smegmatis* (G) by electron microscopy.

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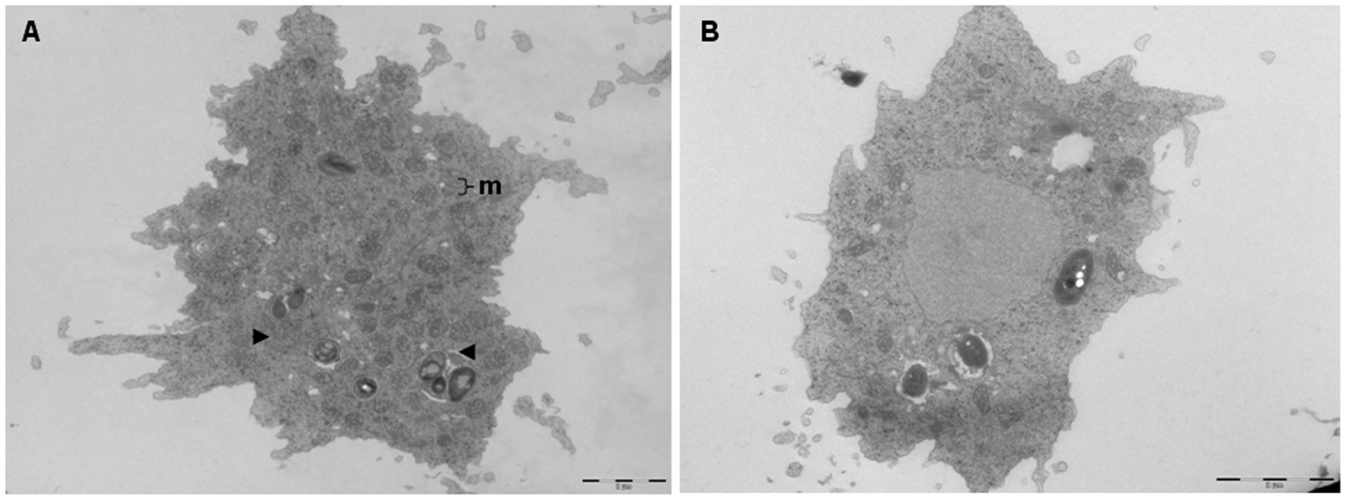
A
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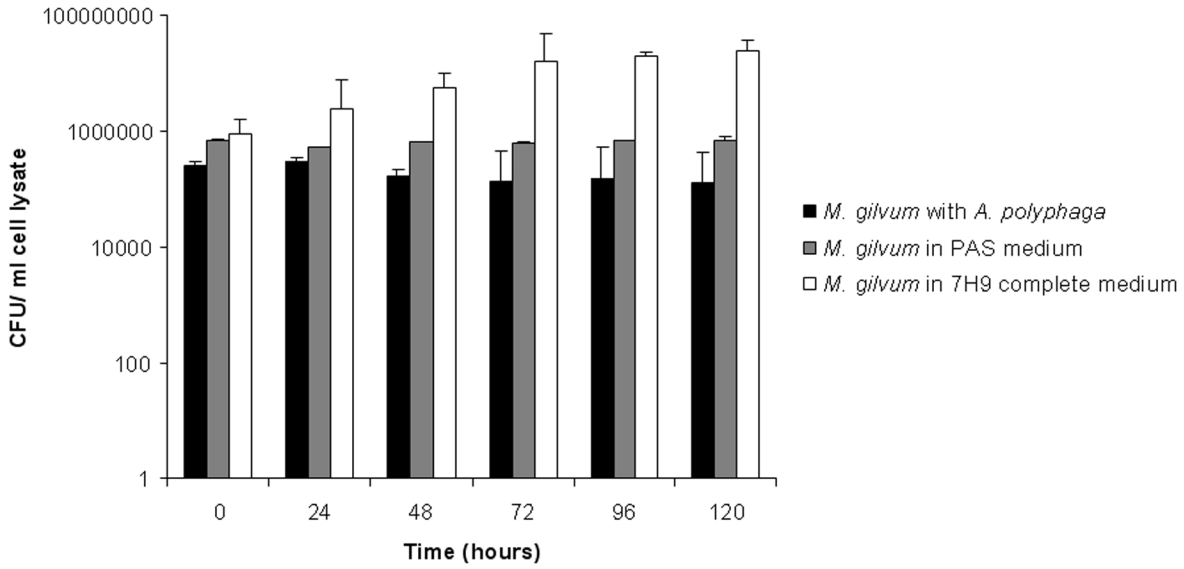
B


100000000
1000000
10000
100
1
CFU/ ml cell lysate
0
24
48
72
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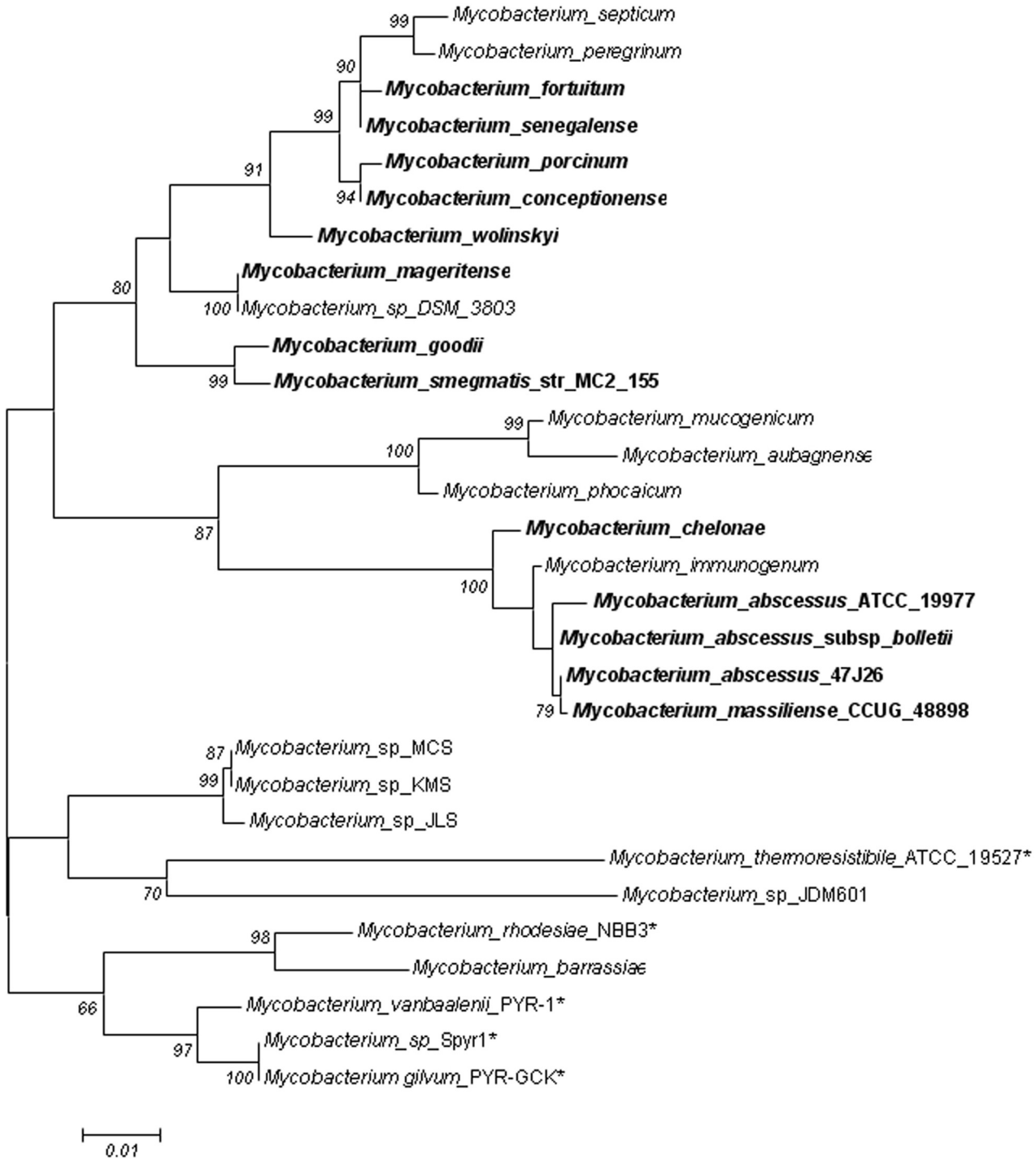
120
Time (hours)
M. gilvum with A. polyphaga
M. gilvum in PAS medium
M. gilvum in 7H9 complete medium


99
Mycobacterium_septicum
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Mycobacterium_wolinskyi
Mycobacterium_mageritense
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100
Mycobacterium_sp_DSM_3803
Mycobacterium_goodii
99
Mycobacterium_smegmatis_str_MC2_155
99
Mycobacterium_mucogenicum
100
Mycobacterium_aubagnense
Mycobacterium_phocaicum
87
Mycobacterium_chelonae
Mycobacterium_immunogenum
100
Mycobacterium_abscessus_ATCC_19977
Mycobacterium_abscessus_subsp_bolletii
Mycobacterium_abscessus_47J26
79
Mycobacterium_massiliense_CCUG_48898
87
Mycobacterium_sp_MCS
99
Mycobacterium_sp_KMS
Mycobacterium_sp_JLS
Mycobacterium_thermoresistibile_ATCC_19527*
70
Mycobacterium_sp_JDM601
98
Mycobacterium_rhodesiae_NBB3*
Mycobacterium_barrassiae
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Mycobacterium_vanbaalenii_PYR-1*
97
Mycobacterium_sp_Spyr1*
100
Mycobacterium gilvum_PYR-GCK*
0.01

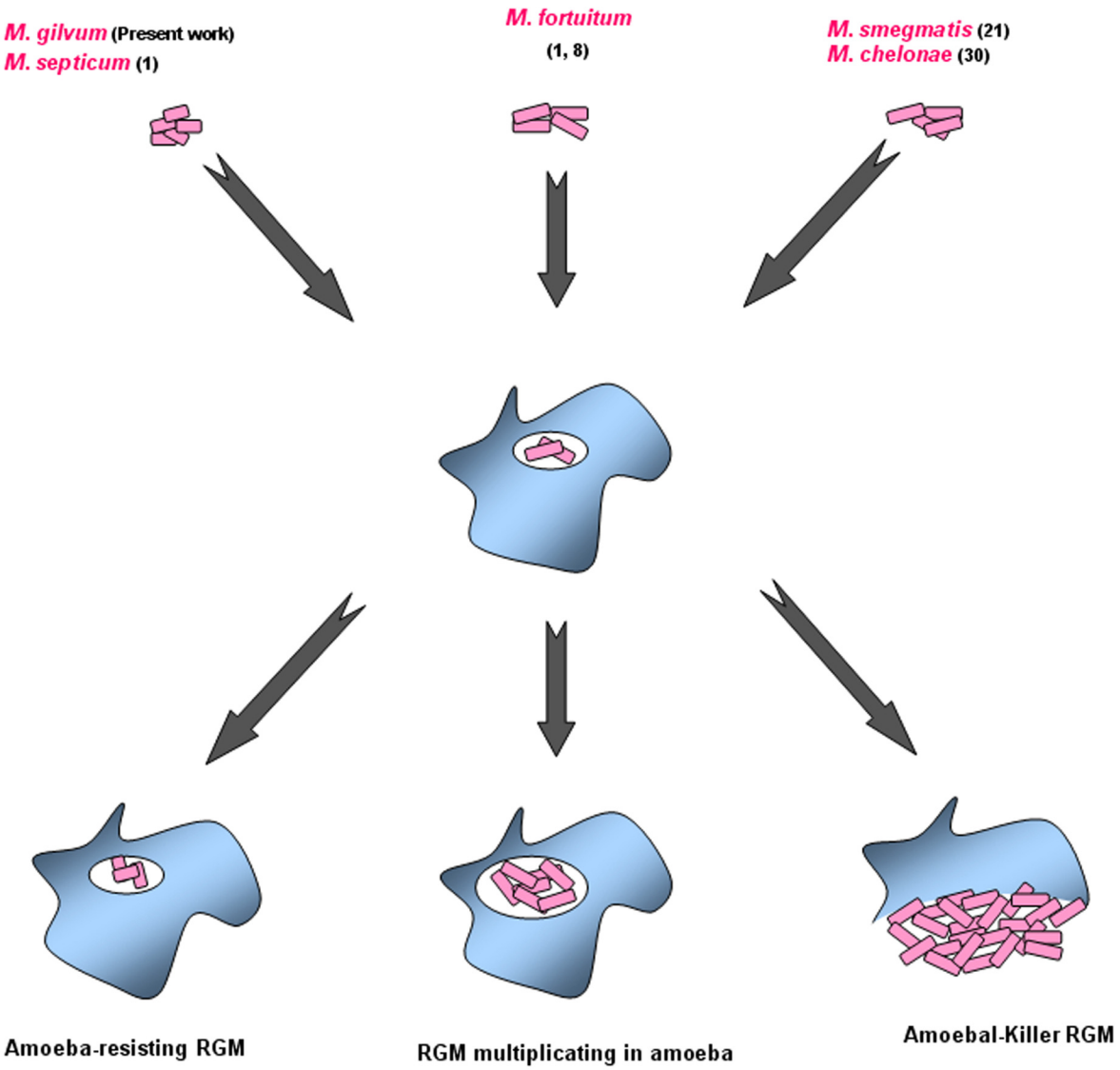
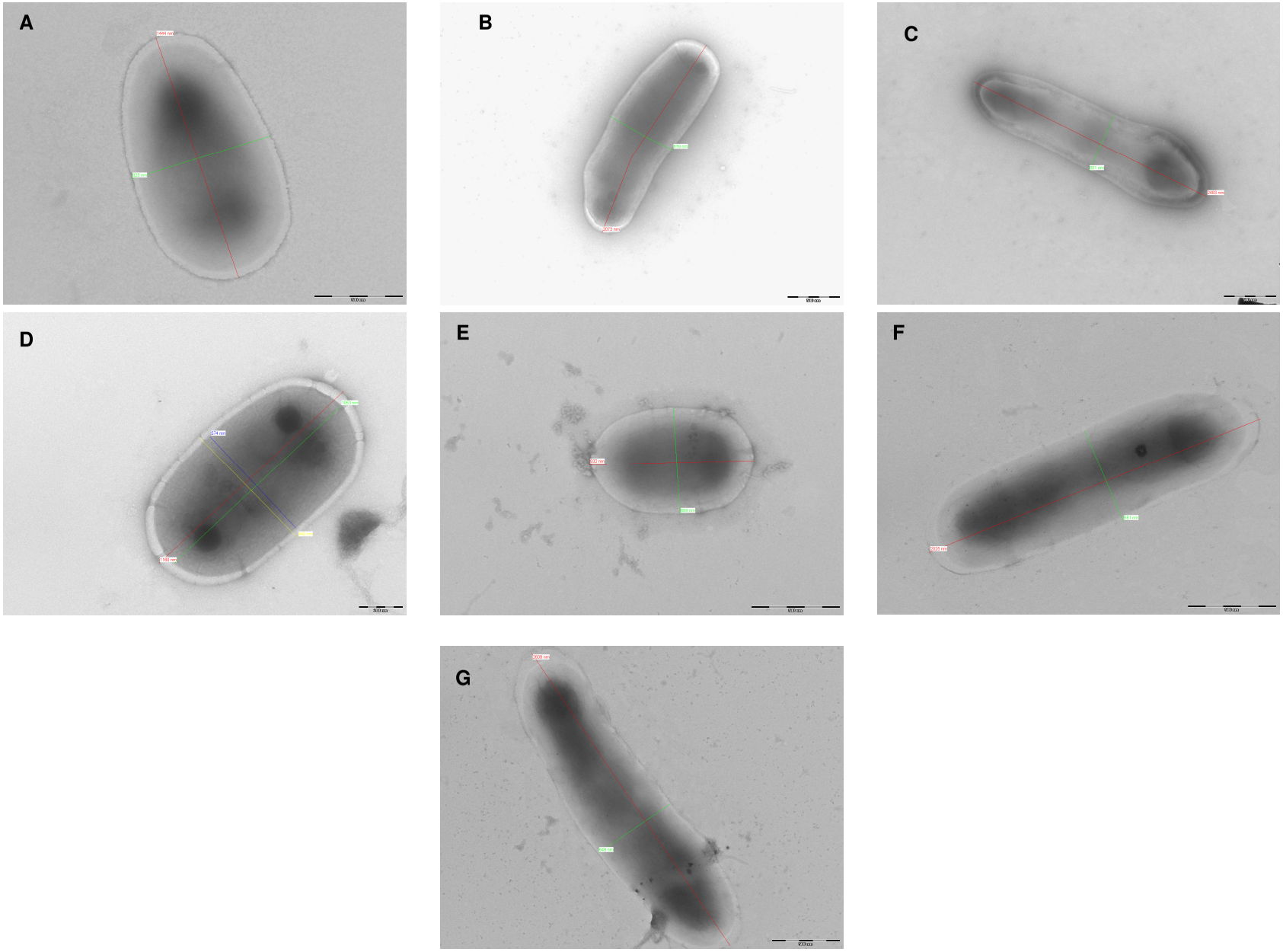

M. gilvum (Present work)
M. septicum (1)
M. fortuitum
(1, 8)
M. smegmatis (21)
M. chelonae (30)
Amoeba-resisting RGM
RGM multiplicating in amoeba
Amoebal-Killer RGM

Figure S1



Chapitre V

A) Adding *mspA* gene attenuated *Mycobacterium tuberculosis*: the "unbirthday" paradigm

Otmane Lamrabet, Hubert Lepidi, Claude Nappez, Didier Raoult,

Michel Drancourt

En cours de rédaction

Chapitre V-A) – Avant propos

La virulence des bactéries est généralement considérée comme résultante de l'acquisition des gènes étrangers éventuellement regroupés dans des îlots de pathogenicité. Dans ce travail, nous avons exprimé le gène *mspA* de *Mycobacterium smegmatis* chez *Mycobacterium tuberculosis* H37Rv et *Mycobacterium bovis* BCG utilisé comme un contrôle positif. Nous avons analysé l'effet de cette transformation sur la croissance en milieu axénique, la persistance intracellulaire dans différentes cellules phagocytaires (macrophages humains et souris, amibes *A. polyphaga*) et chez la souris.

Alors que la courbe de croissance de *M. bovis* BCG/pVVMspA en milieu axénique était similaire à celle précédemment publiée, la croissance de *M. tuberculosis* H37Rv exprimant la porine MspA a été significativement plus rapide que celle de la souche parentale ($p \leq 0,05$). Nous avons observé que les deux souches transformées survivent significativement moins dans les amibes et dans les cellules phagocytaires par rapport aux souches parentales ($p \leq 0,05$). Nous avons observé aussi que toutes les souris inoculées par voie intra-péritonéale avec les deux souches (*M. tuberculosis* H37Rv ou *M. tuberculosis* H37Rv/pVVMspA) survivent tout au long de l'expérience. À partir du 39ème jour après l'inoculation, nous avons constaté que le poids des souris inoculées avec 1.10^7 UFC/mL de *M. tuberculosis* H37Rv/pVVMspA est significativement supérieur à celui des souris inoculées avec *M. tuberculosis* H37Rv ($p \leq 0,05$). À partir

du 90ème jour après l'inoculation, l'examen macroscopique a montré un nombre de nodules significativement élevé ($p \leq 0,05$) dans les poumons des souris inoculées avec *M. tuberculosis* H37Rv/pVVMspA (52 ± 30 nodules) par rapport aux poumons des souris inoculées avec la souche parentale (2 ± 1 nodules). Enfin, nous avons observé que, quelque soit la dose d'infection, le nombre des mycobactéries dans la rate, le foie et les poumons des souris inoculées avec la souche parentale est significativement plus élevé chez les souris inoculées avec la souche *M. tuberculosis* H37Rv que chez les souris inoculées avec la souche transformé *M. tuberculosis* H37Rv/pVVMspA ($p \leq 0,05$). En conclusion, les données présentées dans notre travail contredisent le dogme que l'acquisition de l'ADN ou des gènes étrangers augmente la virulence des bactéries.

Introduction

Mycobacterium tuberculosis est le membre le plus important du complexe *Mycobacterium tuberculosis* et il comprend huit autres espèces étroitement liées : *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canetti*, *Mycobacterium caprae*, *Mycobacterium pinnipedi* et *Mycobacterium mungi* (Alexander *et al.,* 2011). Ces espèces sont responsables de la tuberculose chez les oiseaux et les mammifères ainsi que chez les humaines (Murray *et al*., 2007; Djelouadji *et al.*, 2008). Plusieurs études ont montré que les mycobactéries du complexe *M. tuberculosis* sont capables d'infecter, de survivre et de se multiplier dans les cellules eucaryotes et les amibes (McKinney et Gomez, 2003; Kusner DJ, 2005 ; Taylor *et al.*, 2003 ; Mba Medie *et al.*, 2011). A l'intérieur de leurs hôtes, les mycobactéries sont exposées à des dérivés réactifs d'oxygène et des dérivés réactifs d'azote, qui génèrent un environnement toxique pouvant potentiellement les tuer (Jordao *et al.*, 2008).

Les mycobactéries présentent une membrane externe épaisse, riche en lipide et formée par 30 à 40% de long cires d'acides gras : les acides mycoliques. Ces dernières sont les plus longues identifiées dans la nature (Rastogi *et al*., 2001). Cette membrane externe représente une barrière parfaite et efficace pour la protection contre les composés toxiques, les désinfectants chimiques et les antibiotiques (Brennan et Nikaido, 1995), mais elle laisse diffuser les nutriments de petites

tailles à travers des transporteurs membranaires appelés porines (Niederweis M, 2003; Niederweis M, 2008). Deux transporteurs ont été identifiés dans les parois cellulaires des mycobactéries: la porine MspA chez *Mycobacterium smegmatis* et la protéine de la membrane externe A (OmpA) chez *M. tuberculosis* (Senaratne *et al.*, 1998; Niederweis M, 2003). De nombreuses différences ont été présentées entre ces deux types de porines tels que leurs conductances (0,7 contre 4,6 ns) et leurs diamètres (1,4 contre 2,5 nm) (Niederweis M, 2003; Niederweis *et al*., 2010). En plus, il a été démontré que les mycobactéries à croissance lente ont moins de porines dans leur paroi cellulaire par rapport aux mycobactéries à croissance rapide (Niederweis 2008; Niederweis et al, 2010). L'expression de la porine MspA chez *Mycobacterium bovis* BCG et *M. tuberculosis* favorise leur croissance dans un milieu axénique et augmente leur sensibilité aux antibiotiques (Mailaender *et al*., 2004; Sharbati-Tehrani *et al*., 2004). Cependant, l'effet de l'expression de ce gène sur le comportement intracellulaire de *M. tuberculosis* n'a jamais été étudié avant notre travail de thèse.

Afin de tester cette hypothèse, nous avons exprimé le gène *mspA* de *M. smegmatis* chez *M. bovis* BCG utilisé comme un contrôle positif (Mailaender *et al*., 2004;. Sharbati-Tehrani *et al*., 2004) et chez *M. tuberculosis* H37Rv et nous avons utilisé différentes approches expérimentales pour étudier la croissance extracellulaires et intracellulaires de ces souches transformées. Nous avons analysé l'effet de cette transformation sur la croissance en milieu axénique, la

persistance intracellulaire dans différentes cellules phagocytaires (macrophages humains et souris, amibes *A. polyphaga*) et chez la souris.

Méthodologie

Toutes les manipulations impliquant des mycobactéries du complexe *M. tuberculosis* ont été effectuées sous une enceinte sécurisée dans un laboratoire de biosécurité de niveau type 3 (NSB3).

1) Expression du gène *mspA* chez *M. tuberculosis* H37Rv et *M. bovis* BCG.

Dans un premier temps, le gène codant pour la porine *mspA* à été amplifié par PCR standard à partir de l'ADN génomique de la souche *M. smegmatis* mc^2155 (ATCC 700084) (Niederweis *et al*., 1999) , puis il a été introduit dans le vecteur d'expression pVV16 (Stover *et al*., 1991) qui se caractérise par la présence de deux gènes codant pour des protéines de résistance à l'hygromycine et à la kanamycine (marqueurs de sélection) afin d'obtenir le plasmide pVVMspA. Ensuite, le plasmide pVVMspA a été introduit par électroporation dans *M. tuberculosis* H37Rv (ATCC 27294) et *M. bovis* BCG Tokyo (ATCC 35737) (Van Kessel et Hatfull, 2007). Enfin, la sélection des clones transformés a été réalisée en utilisant l'hygromycine et la kanamycine comme antibiotiques.

2) Croissance en milieu liquide de *M. tuberculosis* H37Rv et *M. bovis* BCG transformées ou non transformées.

M. tuberculosis H37Rv/pVV16 et *M. bovis* BCG/pVV16 (souches transformées contrôles n'exprimant pas la porine MspA) et *M. tuberculosis* H37Rv/pVVMspA et *M. bovis* BCG/pVVMspA (souches transformées exprimant la porine MspA) ont été mises en pré-culture dans 5 mL de milieu Middlebrook 7H9 (Becton Dickinson, Le Pont de Claix, France) pendant 15 jours jusqu'à l'obtention d'une densité optique (DO600) de 1. Ensuite, les mycobactéries ont été fortement mélangées sous agitation au vortex pour éliminer les amas cellulaires, récoltées et diluées dans 10 mL de milieu 7H9 frais. Les taux de croissance ont été déterminés pour les deux souches dans trois cultures indépendantes grâce à des mesures de DO600 dans un système BACTEC MGIT 960 (Becton Dickinson). Ce système entièrement automatisé est basé sur la détection de la consommation d'oxygène à partir des bactéries aérobies.

3) Coculture de *M. tuberculosis* H37Rv/pVV16, *M. tuberculosis* H37Rv/pVVMspA, *M. bovis* BCG/pVV16 et *M. bovis* BCG/pVVMspA avec l'amibe *A. polyphaga*:

Dix millilitres d'une suspension d'amibes (~ 10^5 amibes/mL) ont été inoculées avec ~ 10^6 mycobactéries/mL (MOI=10). Comme contrôles, *A. polyphaga* et les mycobactéries ont été cultivées séparément dans du tampon PAS (Page's modified Neff's amoeba saline) (Rowbotham TJ, 1983, La Scola *et al.*, 2001). Après 24h

d'incubation à 32 °C, la coculture a été lavée trois fois avec le PAS et incubée à 32 °C dans 10 mL du PAS pendant 10 à 15 jours.

4) Coculture de *M. tuberculosis* H37Rv/pVV16, *M. tuberculosis* H37Rv/pVVMspA, *M. bovis* BCG/pVV16 et *M. bovis* BCG/pVVMspA avec des macrophages humains (hMdMs) ou macrophages de souris (BMDMs):

Les BMDMs (Cook *et al*., 2007) et les hMdMs isolées à partir des leukopacks (Etablissement Français du Sang, Marseille, France) par un gradient Ficoll (MSL, Eurobio, Courtaboeuf, France) différenciés ont été ensemencées séparément (~ 10^5 cellules/puits) dans des plaques de culture de 24 puits dans un milieu RPMI 1640 contenant 10% du sérum de veau. Les BMDMs et les hMdMs ont été infectées séparément par *M. tuberculosis*, *M. tuberculosis* H37Rv/pVV16, *M. tuberculosis* H37Rv/pVVMspA, *M. bovis* BCG, *M. bovis* BCG/pVV16 et *M. bovis* BCG/pVVMspA pendant 24 heures, puis les macrophages ont été lavés pour éliminer les mycobactéries libres avant d'être incubés pendant différentes périodes de temps dans du milieu RPMI 1640 contenant 10% du sérum de veau. Comme contrôles, chaque type cellulaire ainsi que les mycobactéries ont été cultivées séparément.

5) Détermination du nombre des mycobactéries intracellulaires (UFC):

A un temps donné, les cellules infectées ont été lysées avec 0,1% de dodécyl sulfate de sodium (SDS) pendant 30 min puis passées à travers une aiguille de calibre 26 pour assurer leur lyse complète. Le lysat (500 µL) a été lavé trois fois avec du tampon phosphate salin (PBS) stérile, étalé sur milieu solide Middlebrook 7H10 (Becton Dickinson) et incubé pendant 20 jours à 37°C afin de déterminer le nombre de colonies des mycobactéries intracellulaires (UFC). Toutes les expériences ont été réalisées en triple exemplaire.

6) Croissance de *M. tuberculosis* H37Rv et *M. tuberculosis* H37Rv/pVVMspA dans un modèle souris.

Le travail avec les souris a été réalisé dans un laboratoire NSB 3 après approbation de l'échelon local du comité d'éthique pour l'expérimentation chez les animaux (Faculté de Médecine de la Timone, permis d'expérimentation: 13.385). Des souris BALB/c de 6 à 8 semaines (10 par groupe) ont été inoculées par voie intrapéritonéale par 100 µL de culture bactérienne. Deux inoculums différents de chacune des souches ci-dessus (souche parentale *M. tuberculosis* H37Rv et souche transformée *M. tuberculosis* H37Rv/pVVMspA) ont été utilisées: 1×10^7 UFC/mL et 1×10^5 UFC/mL. Comme contrôle, un groupe de souris a été inoculé avec 100 µL de PBS stérile. Le poids, la performance et la survie des souris ont été suivis pendant 90 jours. Le foie, la rate et les poumons sont analysés au 60ème et au 90ème jour après l'inoculation et le nombre des mycobactéries (UFC) a été déterminé.

Après 90 jours, des souris BALB/c préalablement inoculées avec l'une des deux souches (4 souris par groupe) ont été réinoculées avec 1×10^7 UFC/mL de la souche parentale *M. tuberculosis* H37Rv. Comme contrôle, deux groupes de souris saines (5 souris par groupe) ont été inoculés séparément avec 100 µL de PBS stérile et avec 1×10^7 UFC/mL de la souche sauvage *M. tuberculosis* H37Rv. Le poids, la performance et la survie des souris ont été suivis pendant 60 jours.

Résultats & conclusions

Clonage et expression du gène *mspA* chez *M. tuberculosis* H37Rv et *M. bovis* BCG.

Nous avons obtenu par PCR un fragment d'ADN de 822 pb contenant le gène qui code pour la porine MspA puis nous l'avons cloné dans le plasmide pVV16 (5792 pb) sous le contrôle du promoteur *hsp60* et nous avons obtenu le plasmide pVVMspA. Le séquençage de l'insert n'a pas révélé de remplacement d'acides nucléiques dans la séquence de *mspA*. Nous avons ensuite introduit par électroporation le plasmide pVVMspA et le plasmide contrôle pVV16 dans *M. bovis* BCG et *M. tuberculosis* H37Rv et la présence du gène *mspA* dans les transformants a été vérifiée par PCR en utilisant des amorces spécifiques (Tableau 1) (Figure 1). Afin de conserver le plasmide, les mycobactéries transformées ont été maintenues en permanence dans un milieu avec la kanamycine et l'hygromycine B.

Par la suite, nous avons validé par RT-PCR l'expression du gène *mspA* chez les souches transformées *M. bovis* BCG/pVVMspA et *M. tuberculosis*/pVVMspA (Figure 2). Ces résultats ont montré la transcription du gène *mspA* à la fois par *M. bovis* BCG et *M. tuberculosis* H37Rv.

La porine MspA est caractérisée par sa stabilité à la dénaturation par la chaleur (Niederweis *et al*., 1999). Nous avons effectué un SDS-PAGE avec des extraits protéiques préparés avec une extraction sélective à haute température et nous avons détecté un signal clair pour la forme tétramérique de la porine MspA dans les extraits de *M. smegmatis* (contrôle positif) et les souches : *M. bovis* BCG/pVVMspA et *M. tuberculosis* H37Rv/pVVMspA (Figure 2C). En plus, après chauffage à 100 °C de la protéine tétramérique dans 80% de diméthylsulfoxyde (DMSO), nous avons observé la forme monomère de la porine MspA (~ 20 kDa) dans les mêmes extraits protéiques (Figure 2D). Les spots des protéines ont été excisés du gel et analysés par spectrométrie de masse MALDI-TOF. Les protéines ont été identifiées avec une couverture de séquences allant de 21% à 29% et une similitude avec la séquence protéique MspA de *M. smegmatis* à 20 kDa.

La porine MspA augmente la croissance axénique des mycobactéries.

Nous avons observé que *M. bovis* BCG qui exprime la porine MspA présente 4 jours de moins dans la phase de latence par rapport à

la souche contrôle *M. bovis* BCG/pVV16. Le temps de génération de *M. bovis* BCG/pVV16 et de *M. bovis* BCG/pVVMspA étaient respectivement de 22 h 56 min ± 30 min et 20 h 45 min ± 18 min (Figure 3A). En ce qui concerne *M. tuberculosis*, nous avons observé que le temps de génération de la souche contrôle *M. tuberculosis* H37Rv/pVV16 et de la souche *M. tuberculosis* H37Rv/pVVMspA étaient respectivement de 19 h 10 min ± 10 min et 17 h 16 min ± 12 min (Figure 3B). Ainsi, nous avons constaté que les souches qui expriment la porine MspA croissent nettement plus rapide que les souches contrôles ($p \leq 0,05$). Cette expérience, réalisée trois fois a montré que l'expression de la porine MspA augmente le taux de croissance axénique de *M. tuberculosis* H37Rv et *M. bovis* BCG.

Croissance des mycobactéries exprimant la porine MspA en coculture avec des cellules eucaryotes.

Nous avons observé que les deux souches *M. tuberculosis* H37Rv/pVV16 et *M. tuberculosis* H37Rv/pVVMspA ont survécu tout au long de l'expérience de la coculture avec l'amibe *A. polyphaga*, mais le nombre des mycobactéries *M. tuberculosis* H37Rv/pVVMspA diminue significativement ($p \leq 0,05$) après 8 jours de coculture. Au 12ème jour, le nombre de colonies était de $2 \times 10^5 \pm 6 \times 10^4$ chez *M. tuberculosis* H37Rv/pVV16 et de $8 \times 10^3 \pm 5 \times 10^3$ chez *M. tuberculosis* H37Rv/pVVMspA (Figure 4A). Ces données indiquent une diminution d'environ deux logs de la croissance intra-amibienne

de *M. tuberculosis* H37Rv/pVVMspA par rapport à la souche *M. tuberculosis* H37Rv/pVV16.

En utilisant des macrophages BMDM comme cellules eucaryotes, nous avons observé que les deux souches *M. tuberculosis* H37Rv/pVV16 et *M. tuberculosis* H37Rv/pVVMspA ont survécu tout au long de l'expérience (14 jours). Nous avons observé qu'entre le 4ème et le 14ème jour la souche contrôle *M. tuberculosis* H37Rv/pVV16 a cru significativement plus que la souche qui exprime MspA ($p \leq 0,05$) (Figure 4B). Au 14ème jour, le nombre de colonies de *M. tuberculosis* H37Rv/pVV16 était de $1 \times 10^6 \pm 7 \times 10^5$ et de $4 \times 10^5 \pm 1 \times 10^5$ pour *M. tuberculosis* H37Rv/pVVMspA ($p \leq 0,05$).

En utilisant des macrophages hMdM comme cellules eucaryotes, nous avons observé qu'à partir du 4ème jour et jusqu'à la fin de l'expérience, la souche contrôle *M. tuberculosis* H37Rv/pVV16 a cru significativement plus que la souche *M. tuberculosis* H37Rv/pVVMspA ($p \leq 0,05$) (Figure 4C). Au 14ème jour, le nombre de colonies était de $2 \times 10^4 \pm 2 \times 10^3$ chez *M. tuberculosis* H37Rv/pVV16 et de $5 \times 10^3 \pm 7 \times 10^2$ chez *M. tuberculosis* H37Rv/pVVMspA.

Concernant *M. bovis* BCG et en utilisant des amibes comme cellules eucaryotes, nous avons observé que le nombre des mycobactéries *M. bovis* BCG/pVV16 et *M. bovis* BCG/pVVMspA diminue au cours de l'expérience avec une diminution significative du nombre des mycobactéries *M. bovis* BCG exprimant la porine MspA à partir du 4ème jour ($p \leq 0,05$) (Figure 5A). Au 12ème jour, le nombre

de colonies de *M. bovis* BCG/pVVMspA était de10 ± 3 et de 95 ± 9 pour *M. bovis* BCG/pVV16.

En utilisant des macrophages BMDM comme cellules eucaryotes, nous avons observé que les deux souches *M. bovis* BCG/pVV16 et *M. bovis* BCG/pVVMspA ont survécu tout au long de l'expérience (14 jours). Nous avons observé qu'entre le 2ème et le 14ème jour la souche contrôle *M. bovis* BCG/pVV16 a cru significativement plus que la souche exprimant la porine MspA ($p \leq 0,05$) (Figure 5B). Au 14ème jour, le nombre de colonies de *M. bovis* BCG/pVV16 était de $3 \times 10^8 \pm 7 \times 10^7$ et de $1 \times 10^7 \pm 6 \times 10^6$ pour *M. bovis* BCG/pVVMspA.

En utilisant des macrophages hMdM comme cellules eucaryotes, nous avons observé que la survie de *M. bovis* BCG/pVV16 était significativement plus élevée que celle de *M. bovis* BCG/pVVMspA ($p \leq 0,05$) jusqu'à 8 jours après la coculture. Par la suite, le nombre des colonies des deux souches a diminué au cours du temps de l'expérience (Figure 5C). Au 14ème jour, le nombre des colonies était de $5 \times 10^6 \pm 3 \times 10^5$ chez *M. bovis* BCG/pVV16 et de $1 \times 10^6 \pm 1 \times 10^5$ chez *M. bovis* BCG/pVVMspA.

Dans les expériences contrôles en utilisant le milieu RPMI sans BMDMs ou hMdMs et le milieu PAS sans amibe, les mycobactéries ont survécu et leur nombre n'a pas augmenté tout au long des expériences et il n'y avait aucune différence statistiquement significative entre les taux de survie des souches contrôles et des souches transformées.

En conclusion, ces résultats indiquent que l'expression de la porine MspA chez *M. tuberculosis* et *M. bovis* BCG entraine une difficulté pour la bactérie à se multiplier dans les 3 types des cellules eucaryotes testées par rapport aux souches contrôles.

Clinique des souris inoculées avec *M. tuberculosis* H37Rv qui exprime la porine MspA.

Toutes les souris utilisées dans cette étude ont survécu tout au long de l'expérience (Figure 6). A partir du 39ème jour après l'inoculation, nous avons observé que le poids des souris inoculées avec 1×10^7 UFC/mL de *M. tuberculosis* H37Rv/pVVMspA était significativement plus important ($p \leq 0,05$) que chez les souris inoculées avec *M. tuberculosis* H37Rv (Figure 6A). Aucune différence claire et significative n'a été observée en utilisant un inoculum de 1×10^5 UFC/mL (Figure 6B). À partir du 60ème jour après inoculation, nous avons constaté que 2/10 souris inoculées avec 1×10^7 UFC/mL de *M. tuberculosis* H37Rv montrent une fistule abdominale contrairement aux souris inoculées avec la souche transformée *M. tuberculosis* H37Rv/pVVMspA (Figure 7).

Nous avons examiné les organes des souris inoculées au 60ème et 90ème jour après l'inoculation. Nous avons observé qu'après inoculation avec 1×10^7 UFC/mL, le poids de la rate des souris inoculées par la souche parentale *M. tuberculosis* H37Rv était significativement plus élevée ($p \leq 0,05$) par rapport au poids de la rate des souris inoculées avec *M. tuberculosis* H37Rv/pVVMspA (Figure

8A). Aucune différence n'a été observée dans le poids du foie et des poumons (Figure 8A). Après inoculation avec 1×10^5 UFC/mL, nous avons observé que le poids du foie des souris inoculées par la souche parentale *M. tuberculosis* H37Rv était significativement plus élevée ($p \leq 0,05$) par rapport à celui des souris inoculées par *M. tuberculosis* H37Rv/pVVMspA (Figure 8B). En utilisant cette dose, aucune différence n'a été observée dans le poids de la rate et des poumons (Figure 8B).

Au 60ème et 90ème jour après l'inoculation, nous avons observé que les poumons des souris inoculées par la souche parentale présentent un nombre de nodules significativement plus élevée ($p \leq 0,05$) que les poumons des souris inoculées avec la souche transformée *M. tuberculosis* H37Rv/pVVMspA (Figure 9) (Tableau 2). Après inoculation avec 1×10^5 UFC/mL et quelque soit la souche utilisée, aucun nodule n'a été observé dans les poumons des souris infectées après 60 jour d'inoculation, tandis qu'après 90 jour le nombre des nodules était de 3 ± 6 dans les poumons des souris inoculées avec la souche parentale et d'aucun nodule dans les poumons des souris inoculées avec la souche transformée *M. tuberculosis* H37Rv/pVVMspA (Tableau 2). Au 60ème jour après inoculation avec 1×10^7 UFC/mL, le nombre des nodules était de 28 ± 23 dans les poumons des souris inoculées avec la souche parentale et d'aucun nodule dans les poumons des souris inoculées avec la souche transformée *M. tuberculosis* H37Rv/pVVMspA (Tableau 2). Au 90ème jour après l'inoculation, le nombre des nodules était de 52 ± 30

chez les poumons des souris inoculées avec la souche parentale et de 2 ± 1 chez les poumons des souris inoculées avec la souche transformée *M. tuberculosis* H37Rv/pVVMspA (Tableau 2). En outre, nous avons observé que, contrairement aux poumons des souris inoculées avec la souche transformée, les poumons des souris infectées par *M. tuberculosis* H37Rv présentent des lésions larges de couleur blanche/verte par opposition à la couleur rose claire des poumons sains des souris (Figure 9).

A 60 et 90 jours après l'inoculation, la charge bactérienne dans le foie, la rate et les poumons des souris inoculées avec *M. tuberculosis* H37Rv était significativement plus élevée ($p \leq 0,05$) par rapport aux mêmes organes prélevés chez les souris inoculées avec *M. tuberculosis* H37Rv/pVVMspA et ceci quelque soit l'inoculum (Figure 10).

Tableau 1. Amorces utilisées dans ce travail.

Amorces	**Séquence (5'-->3')**	**Fonction**
MSPA-pVV16F	ccccccc<u>catatg</u>aaggcaatcagtc	Amplification de gène *mspA* à partir de génome de *M. smegmatis*
MSPA-pVV16ndeI	ccc<u>catatg</u>tcagttcatgttccaggg	
pVV16FI	agcgtaagtagcggggttg	Vérification de l'insertion du gène *mspA* dans le plasmide pVV16
pVV16FR	tctttcgactgagcctttcg	
MspA-InterF	aaccgtcttacccgtgagtg	
MspA-InterR	ggggtggtgtagctgaagtt	
AT-poR-FW	tggaccgcaaccgtcttacc	RT-PCR
AT-poR-R	gggtgatgaccgagttcaggc	

Tableau 2. Nombre des nodules dans le poumons des souris après inoculation avec *M. tuberculosis* exprimant MspA ou la souche parentale.

Jours après inoculation	Inoculum (UFC/mL)	Nombre* des nodules dans les poumons des souris inoculées avec	
		M. tuberculosis H37Rv	*M. tuberculosis* H37Rv/pVVmspA
60	$1.\ 10^5$	0	0
	$1.\ 10^7$	28 ± 23	0
90	$1.\ 10^5$	3 ± 6	0
	$1.\ 10^7$	52 ± 30	2 ± 1

* Moyenne de 4 souris par inoculum.

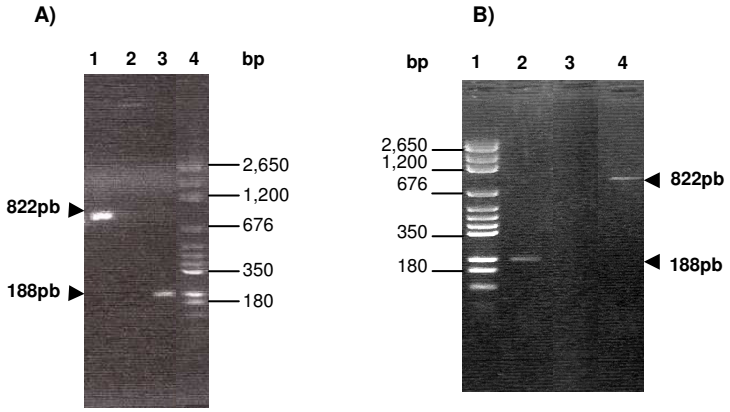


Figure 1. Vérification par PCR standard de la présence du gène *mspA* chez *M. bovis* BCG et *M. tuberculosis* H37Rv. La présence ou l'absence du gène *mspA* dans les deux souches est montrée respectivement par les bandes de 822bp ou 188bp. Les amorces utilisées sont représentées dans le tableau 1. (A) Présence du gène *mspA* chez *M. bovis* BCG. Piste 1: Extrait d'ADN total de *M. bovis* BCG/pVVMspA; Piste 2: Absence d'ADN (contrôle négatif); Piste 3: Extrait d'ADN total de *M. bovis* BCG/pVV16; Piste 4: Marqueur de taille pGEM®. (B) Présence du gène *mspA* chez *M. tuberculosis* H37Rv. Piste 1: Marqueur de taille pGEM®; Piste 2: Extrait d'ADN total de *M. tuberculosis* H37Rv/pVV16; Piste 3: Absence d'ADN (contrôle négatif); Piste 4: Extrait d'ADN total de *M. tuberculosis* H37Rv/pVVMspA.

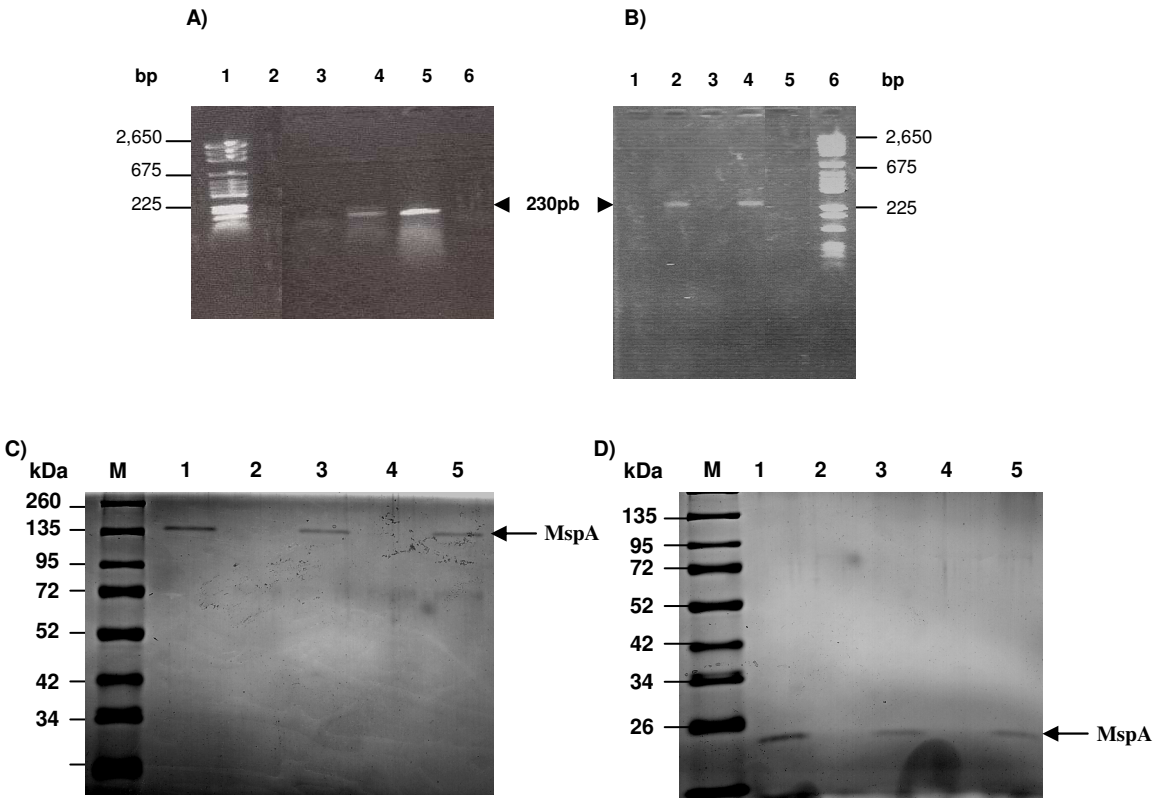


Figure 2. Vérification par RT-PCR et SDS-PAGE de l'expression de la porine MspA chez *M. bovis* BCG et *M. tuberculosis* H37Rv. Les amorces utilisées pour la RT-PCR sont représentées dans le tableau 1. (A) Transcription du gène *mspA* (230pb) chez *M. bovis* BCG vérifié par RT-PCR. Piste 1: Marqueur de taille pGEM®; Piste 2: Contrôle négatif; Pistes 3 et 4: Extrait d'ARN total respectivement de *M. bovis* BCG/pVV16 et de *M. bovis* BCG/pVVMspA; Pistes 5 et 6: Extrait d'ARN total respectivement de *M. smegmatis* et de *M. bovis* BCG. (B) Transcription du gène *mspA* chez *M. tuberculosis* H37Rv vérifié par RT-PCR. Pistes 1 et 2: Extrait d'ARN total respectivement de *M. tuberculosis* H37Rv et de *M. smegmatis*; Pistes 3 et 4: Extrait d'ARN total respectivement de *M. tuberculosis* H37Rv/pVV16 et de *M. tuberculosis* H37Rv/pVVMspA; Piste 5: Contrôle négatif; Piste 6: Marqueur de taille pGEM®. (C) Forme oligomérique et (D) forme monomérique de la porine MspA. Piste M: marqueur de masse moléculaire; Piste 1: Extrait protéique de *M. smegmatis*; Pistes 2 et 3: Extraits protéiques respectivement de *M. bovis* BCG/pVV16 et de *M. bovis* BCG/pVVMspA; Pistes 4 et 5: Extraits protéiques respectivement de *M. tuberculosis* H37Rv/pVV16 et de *M. tuberculosis* H37Rv/pVVMspA.

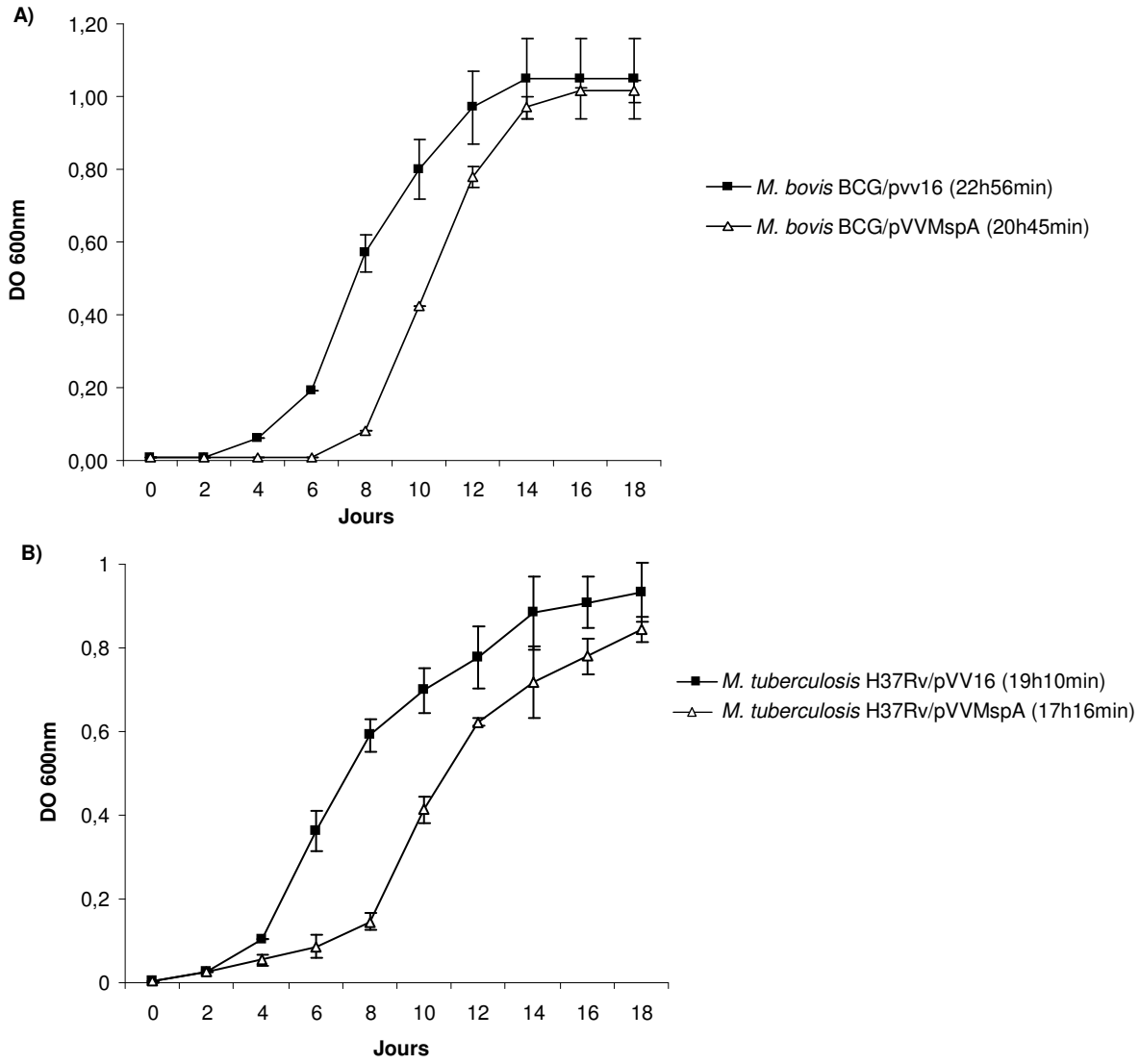


Figure 3. Influence de la porine MspA sur la croissance de (A) *M. bovis* BCG et de (B) *M. tuberculosis* H37Rv. *M. bovis* BCG/pVV16, *M. bovis* BCG/pVVMspA, *M. tuberculosis* H37Rv/pVV16 et *M. tuberculosis* H37Rv/pVVMspA ont été cultivées dans de Middlebrook 7H10 supplémenté avec 0,05% de Tween 80, 0,2% de glycérol et ADC comme enrichissement. Les temps de génération sont indiqués entre parenthèses pour chaque souche. Chaque point correspond à la moyenne de trois cultures différentes.

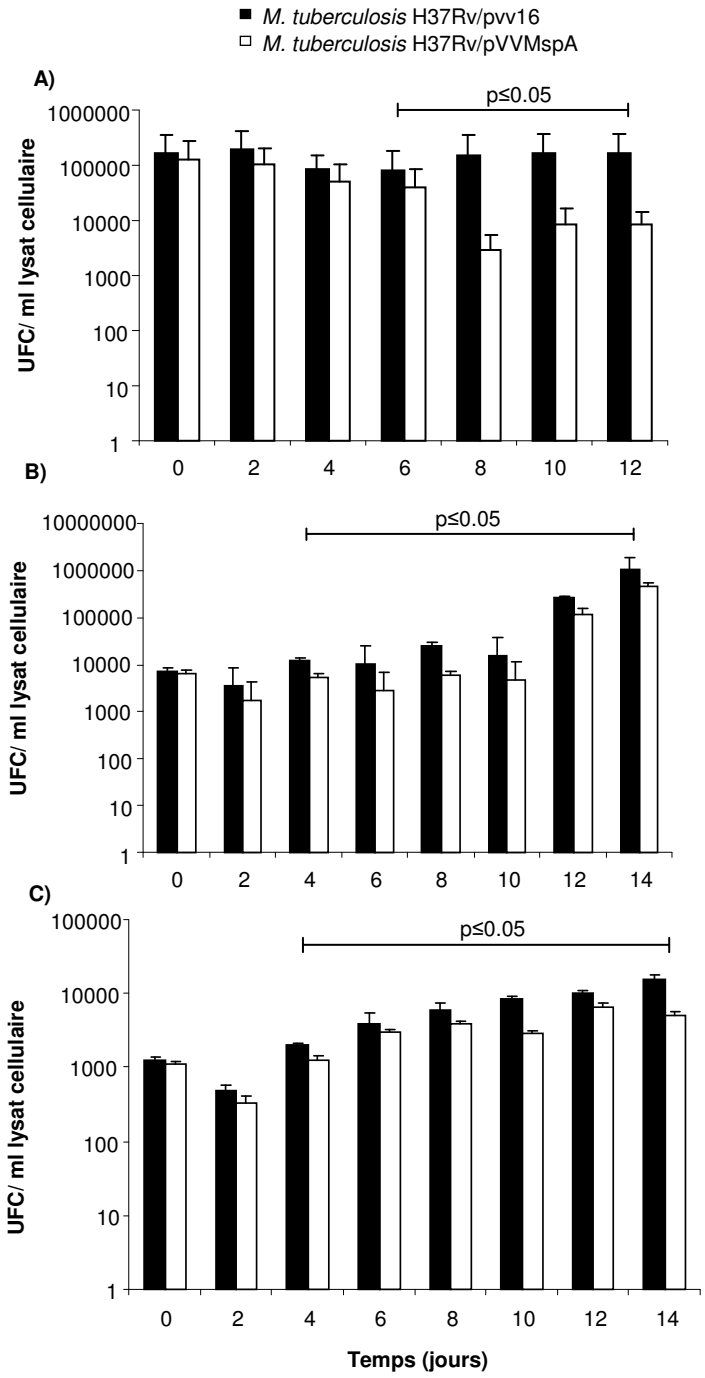


Figure 4. Croissance intracellulaire de *M. tuberculosis* H37Rv dans différentes cellules eucaryotes. Le nombre de colonies des mycobactéries (UFC) après coculture de *M. tuberculosis* H37Rv/pVV16 ou *M. tuberculosis* H37Rv/pVVMspA avec (A) *A. polyphaga*, (B) BMDM et (C) hMdM. Chaque point correspond à la moyenne de trois cultures différentes.

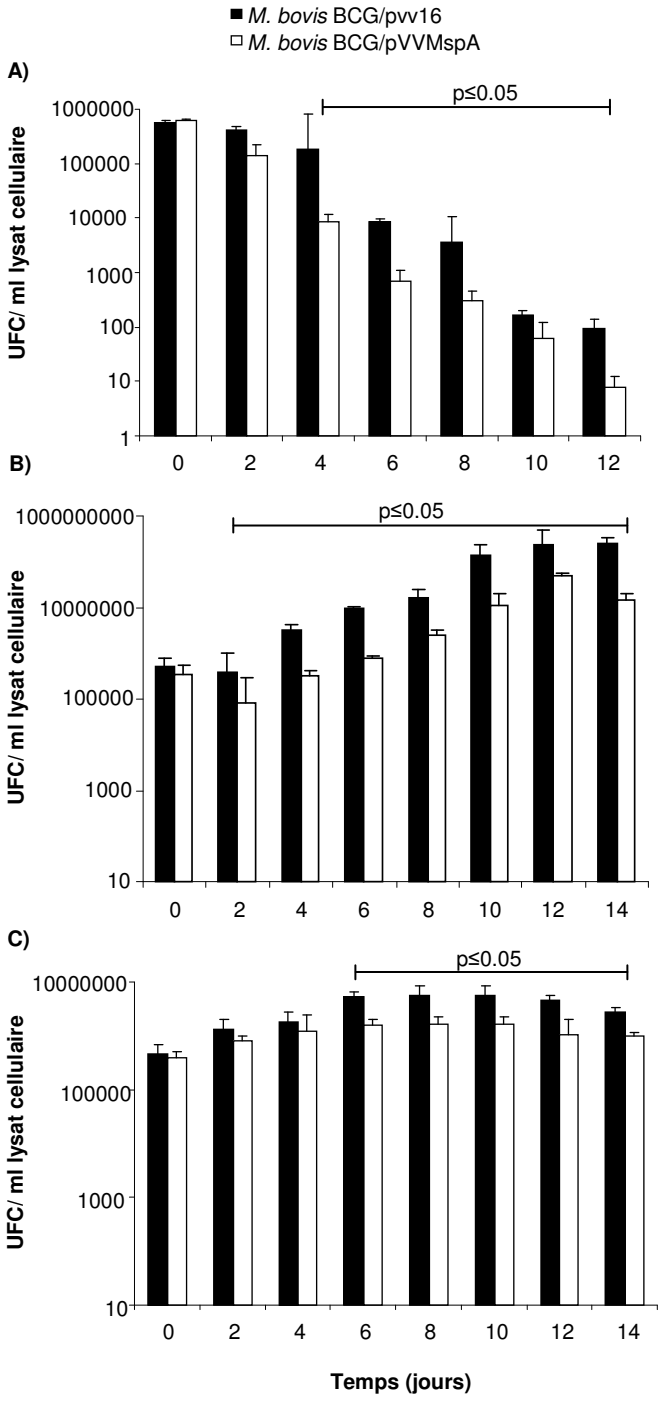


Figure 5. Croissance intracellulaire de *M. bovis* BCG dans différentes cellules eucaryotes. Le nombre de colonies des mycobactéries (UFC) après coculture de *M. bovis* BCG/pVV16 ou *M. bovis* BCG/pVVMspA avec (A) *A. polyphaga*, (B) BMDM et (C) hMdM. Chaque point correspond à la moyenne de trois cultures différentes.

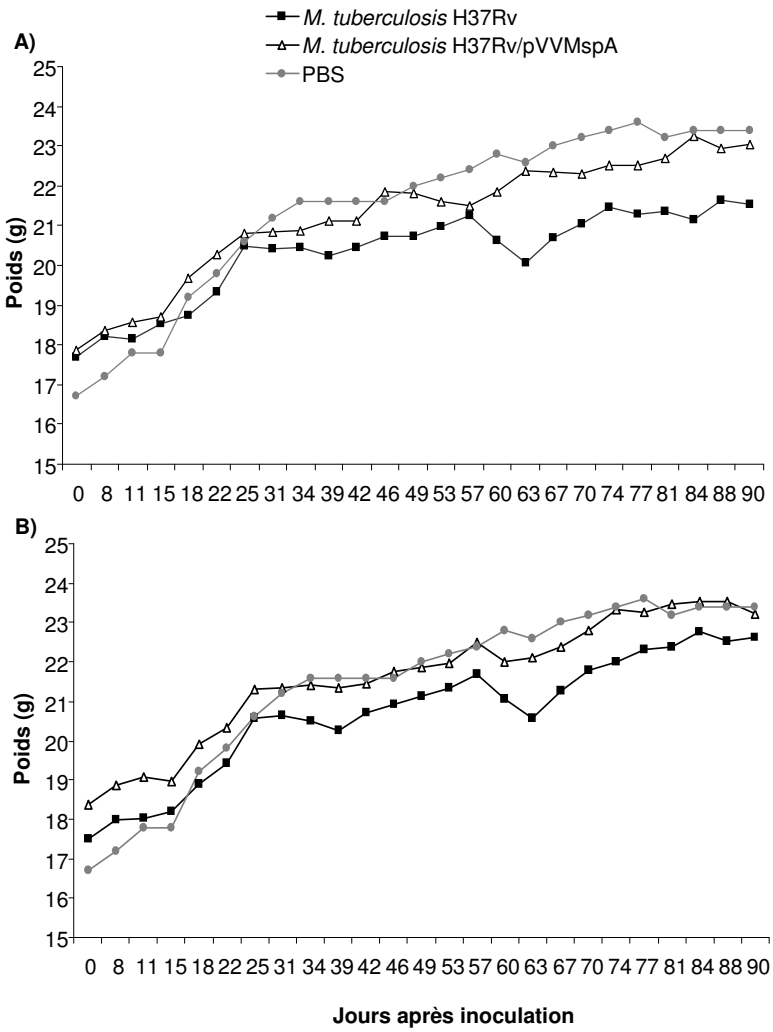


Figure 6. **Evolution du poids des souris BALB/c inoculées avec *M. tuberculosis* exprimant MspA**. Les souris BALB/c ont été inoculées par voie intrapéritonéale avec (A) 10000000 UFC/ml ou (B) 100000 UFC/ml de *M. tuberculosis* H37Rv ou *M. tuberculosis* H37Rv/pVVMspA. Les souris contrôles ont été inoculées avec PBS. Chaque point correspond à une moyenne de 10 souris sauf dans le cas des souris contrôles qui correspond à une moyenne de cinq souris.

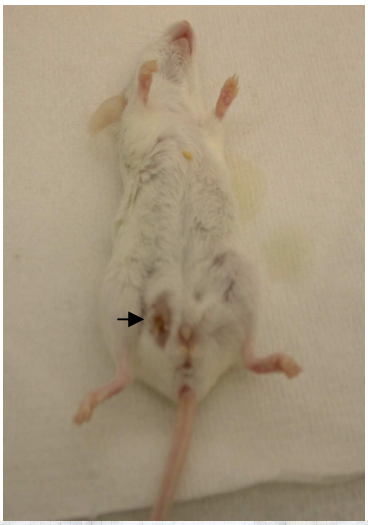


Figure 7. **Fistule abdominale des souris BALB/c inoculées avec *M. tuberculosis***. Les souris BALB/c ont été inoculées par voie intrapéritonéale avec 10000000 UFC/ml de M. tuberculosis H37Rv ou *M. tuberculosis* H37Rv/pVVMspA. La fistule abdominale (montrée par une flèche) est observée que dans le cas d'inoculation avec *M. tuberculosis* H37Rv. Les souris inoculées par la souche transformée *M. tuberculosis* H37Rv/pVVMspA ne montrent aucune anomalie.

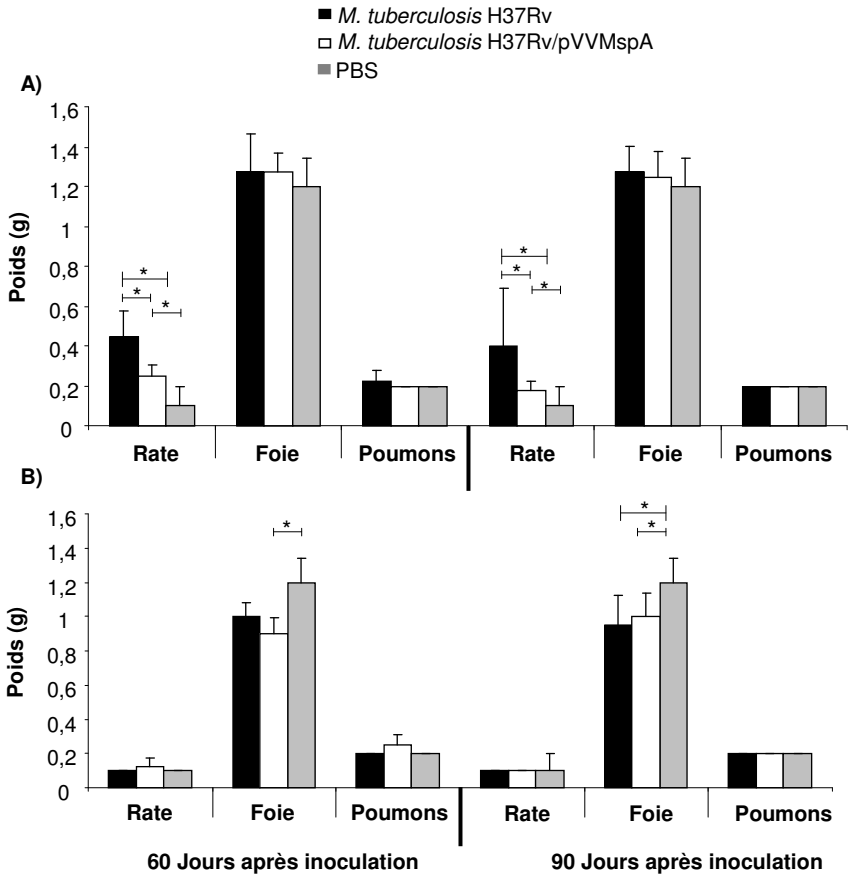


Figure 8. Poids de la rate, le foie et les poumons de souris BALB/c inoculées avec *M. tuberculosis* exprimant MspA. Les souris BALB/c ont été inoculées par voie intrapéritonéale avec (A) 10000000 UFC/ml et (B) 100000 UFC/ml de *M. tuberculosis* H37Rv ou *M. tuberculosis* H37Rv/pVVMspA. Les souris contrôles ont été inoculées avec PBS. Pour chaque organes, les résultats correspond à la moyenne de quatre souris.

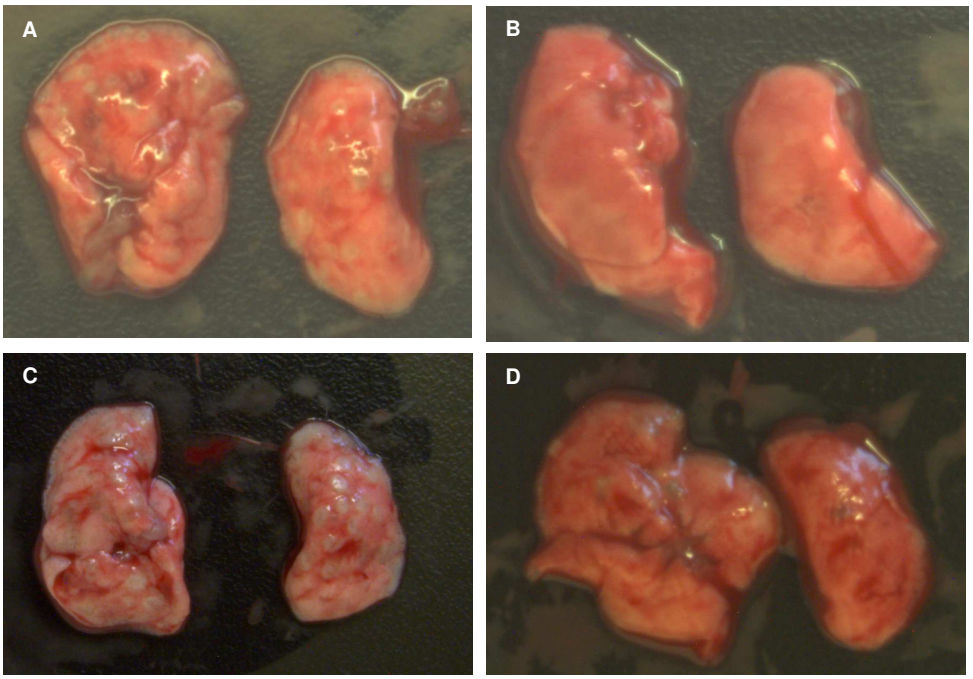


Figure 9. Examen macroscopique des poumons de souris BALB/c inoculées par *M. tuberculosis* H37Rv ou *M. tuberculosis* H37Rv/pVVMspA. Les poumons des souris ont été examinés 60 jours après l'inoculation avec (A) la souche parentale ou (B) celle exprimant la porine MspA. Les poumons des souris ont été examinés 90 jours après l'inoculation avec (C) la souche parentale ou (D) celle exprimant la porine MspA. Les souris BALB/c ont été inoculées par voie intrapéritonéale avec 10000000 UFC/ml de *M. tuberculosis* H37Rv ou *M. tuberculosis* H37Rv/pVVMspA

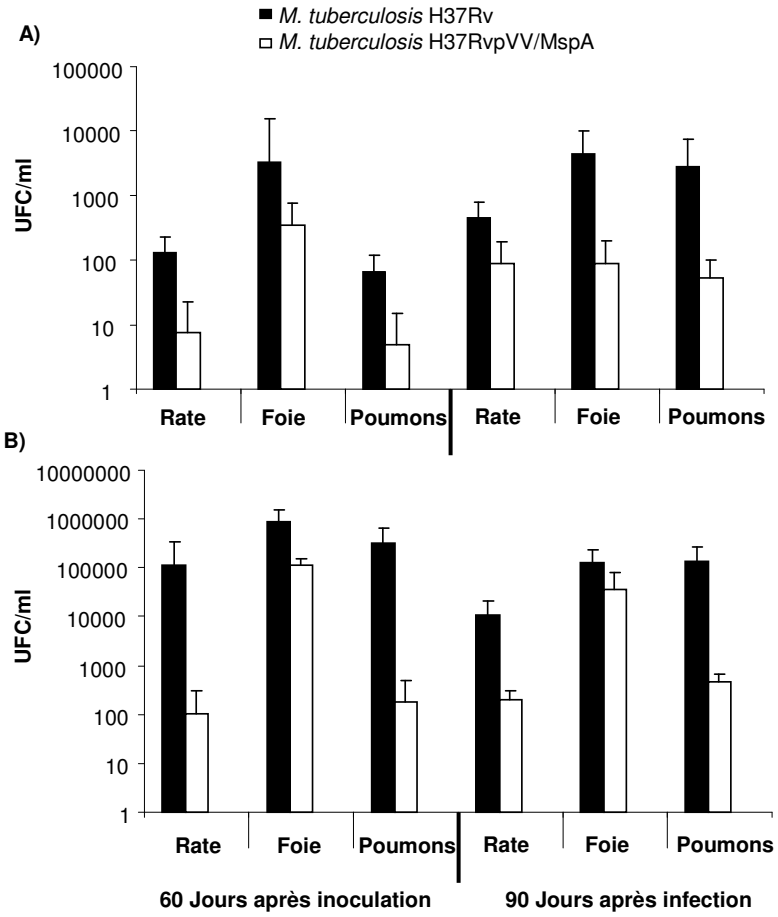


Figure 10. **Multiplication *in vivo* de *M. tuberculosis* H37Rv et de *M. tuberculosis* H37Rv/pVVMspA après leur inoculation aux souris BALB/c par voie intrapéritonéale**. Le nombre de colonies des mycobactéries intracellulaires (UFC) a été compté dans la rate, le foie et les poumons des souris à 60 et 90 jours après inoculation. Les souris ont été inoculées avec (A) 10000000 UFC/ml et (B) 100000 UFC/ml de *M. tuberculosis* H37Rv et *M. tuberculosis* H37Rv/pVVMspA. Pour chaque organes, les résultats correspond à la moyenne de quatre souris.

Chapitre V

B) Procédé d'atténuation d'une bactérie du complexe *Mycobacterium tuberculosis* pour la fabrication d'un vaccin contre la tuberculose

Otmane Lamrabet, Didier Raoult, Michel Drancourt

Brevet déposé le 22-mai-2012 (H52 888 cas 12 FR)

Chapitre V-B) – Avant propos

Résumé du brevet :

La présente invention concerne une méthode pour diminuer la croissance d'une souche d'une mycobactérie du complexe *Mycobacterium tuberculosis* dans des cellules eucaryotes, par ajout et expression dans ladite mycobactérie d'un gène *mspA* codant pour une porine A de *Mycobacterium smegmatis*, ainsi qu'une souche ainsi transformée utile pour la fabrication d'un vaccin pour la prévention d'une infection par une bactérie du complexe *M. tuberculosis* chez un hôte comprenant des cellules eucaryotes, de préférence des macrophages.

CONCLUSIONS GÉNÉRALES ET PERSPECTIVES

Conclusions générales et perspectives

La tuberculose latente reste un problème majeur de la santé publique puisqu'un tiers de la population mondiale est actuellement infectée par *M. tuberculosis* (WHO, 2011). L'infection latente est associée à un état de dormance non-réplicative due à un ralentissement de l'activité métabolique du pathogène (Wiker *et al*., 2010). La manipulation génétique des mycobactéries en général et des mycobactéries du complexe *M. tuberculosis* en particulier est compliquée (Lamrabet et Drancourt, 2012). Aucune étude de modification génétique des mycobactéries n'avait été réalisée dans notre laboratoire avant ce travail de thèse.

Les objectifs de notre travail de thèse étaient d'étudier principalement les interactions entre les mycobactéries et les différents organismes hôtes. Nous avons d'abord montré que les mycobactéries avaient la capacité d'échanger des gènes naturellement par transfert horizontal avec d'autres micro-organismes vivant dans le même hôte ou avec leurs propre hôtes et que les protistes en général sont des sources de ces transferts (Lamrabet *et al*., 2012a). Nos analyses phylogénétiques ont identifié huit gènes de mycobactéries présentant des relations phylogénétiques étroites avec des bactéries autres que les Actinobactéries. Étant donné que la plupart de ces bactéries sont résistantes aux amibes, les phylogénies trouvées sont très évocatrices d'un possible transfert horizontal de ces gènes à l'intérieur des amibes. Néanmoins, le manque d'information sur la direction du

transfert empêche la clarification de l'histoire de ces transferts. En outre, l'analyse du gène codant pour une pyr-redox nous a donné un aperçu sur l'histoire des événements du transfert horizontal des gènes en relation avec le mode de vie des mycobactéries au sein des protozoaires libres (Lamrabet *et al.*, 2012a). Pour renforcer la vraisemblance échange intra-amibien du gène pyr-redox nous avons montré pour la première fois que *Mycobacterium avium* et *Legionella pneumophila* pouvaient vivre de façon sympatrique dans l'amibe *A. polyphaga*. Les résultats de ce travail soutiennent pour la première fois l'hypothèse que les protistes peuvent servir comme une source et un lieu de transfert des gènes chez les mycobactéries. Ainsi, les mycobactéries ont suivi une stratégie évolutive similaire à celle des autres bactéries intracellulaires: elles interfèrent avec les processus cellulaires de l'hôte à travers l'expression des gènes acquis horizontalement à partir de l'hôte. Le transfert horizontal des gènes peut avoir contribué à l'adaptation des mycobactéries à un mode de vie intracellulaire.

Plusieurs études avaient montré les interactions entre les mycobactéries à croissance lente avec les amibes (Ben Salah et Drancourt, 2010; Felix *et al.*, 2011) mais les interactions des mycobactéries à croissance rapide avec les amibes restaient mal comprises (Krishna Prasad et Gupta, 1978; Cirillo *et al.,* 1997; Tenant et Bermudez, 2006). Notre étude permet d'élargir le spectre des interactions entre les mycobactéries à croissance rapide avec les amibes libres en utilisant deux systèmes de coculture : *M. smegmatis*-

A. polyphaga et *M. gilvum-A. polyphaga* (Lamrabet *et al*., 2012b ; Lamrabet et Drancourt, 2012 soumis). Les deux systèmes de coculture rapportés dans cette étude sont des systèmes standardisés qui peuvent être reproduits dans d'autres laboratoires.

La coculture *M. smegmatis*-amibe entraine une augmentation de la croissance de *M. smegmatis*, sa réplication et la lyse des amibes (Lamrabet *et al*., 2012b). En même temps, nous avons observé que le surnageant et le culot des amibes lysées entrainent une augmentation significative de la croissance des trois souches de *M. smegmatis* testées. Cette observation rappelle les observations récentes faites lors de la coculture de *Salmonella enterica* Typhi avec *Acanthamoeba castellanii* (Douesnard-Malo et Daigle, 2011) et de *Vibrio parahaemolyticus* avec *A. castellanii* (Laskowski-Arce et Orth, 2008) et suggère que les amibes contiennent un ou plusieurs facteurs non-caractérisés actuellement ou des nutriments qui favorisent la croissance de *M. smegmatis*. Il serait important de déterminer dans quelles fractions de surnageant des amibes ces facteurs sont ils présents et de déterminer ainsi leur nature.

Ce système de coculture illustre un paradigme inhabituel dans les interactions entre les mycobactéries et les amibes parce que les mycobactéries ont été essentiellement considérées comme des organismes résistants aux amibes. Il pourrait être utilisé comme un modèle simple et rapide chez les mycobactéries pour cibler les facteurs impliqués dans la croissance intracellulaire des mycobactéries.

La coculture *M. gilvum*-amibe, développée pour la première fois dans notre travail de thèse, entraine une survie de *M. gilvum* mais non sa multiplication dans les amibes (Lamrabet et Drancourt, 2012 soumis). Au cours de ce travail, nous avons observé que les mycobactéries qui mesurent moins de 2 µm telles que *M. gilvum* (1,4 ± 0,25 µm) ne se cultivent pas à l'intérieur des trophozoites d'amibes contrairement aux mycobactéries d'une taille supérieure à 2 µm telles que *M. smegmatis*. Cette corrélation entre la taille des mycobactéries à croissance rapide et leur capacité à tuer les amibes est significative ($p \leq 0.05$). Nous avons observé que la taille des mycobactéries à croissance rapide est en corrélation avec l'arbre phylogénique construit à partir de la séquence de leur gène *rpoB*. Il serait intéressant dans l'avenir de déterminer les mécanismes sous-jacents à cette observation.

Nous avons montré que le spectre des interactions entre les amibes et les mycobactéries environnementales à croissance rapide était plus large que précédemment décrit et comporte : (i) les mycobactéries à croissance rapide de taille inférieure à 2 µm, comme *Mycobacterium septicum* (Adekambi *et al.*, 2006) et *M. gilvum* (Lamrabet et Drancourt, 2012 soumis) qui survivent dans les trophozoites d'amibes mais pas dans les kystes (ii) les mycobactéries à croissance rapide de taille inférieure à 2 µm, comme *Mycobacterium fortuitum* (Adekambi *et al.*, 2006; Cirillo *et al.*, 1997) qui survivent dans les trophozoites et dans les kystes d'amibes et (iii) les mycobactéries à croissance rapide de taille supérieure à 2 µm, comme *Mycobacterium chelonae* (Pagnier

et al., 2008) et *M. smegmatis* (Lamrabet *et al*., 2012b) qui tuent les amibes.

L'observation que les mycobactéries acquièrent naturellement des gènes par transfert horizontal à partir d'autres micro-organismes depuis ou au sein du même hôte, nous a conduits à les modifier génétiquement pour observer l'effet de ces modifications sur leur pathogénicité et leur survie. De façon très intéressante nous avons constaté que l'expression du gène *mspA,* qui code pour une porine membranaire spécifique chez *M. smegmatis,* dans *M. tuberculosis* H37Rv et *M. bovis* BCG entraine (1) une augmentation significative ($p \leq 0.05$) de leur temps de croissance dans un milieu axénique mais (2) une diminution significative ($p \leq 0.05$) de leur survie dans les amibes et les macrophages (souris et humains) et (3) une diminution significative ($p \leq 0.05$) de la virulence dans un modèle souris (Lamrabet *et al*., brevet 2012).

Ces résultats contredisent le dogme selon lequel l'acquisition des gènes (ADN) étrangers augmente la virulence des bactéries (Schmidt et Hensel, 2004; Pallen et Wren, 2007). En effet, le transfert de l'ADN codant pour des toxines est clairement associé à une virulence accrue mesurée par le ratio mort de l'hôte (Schmidt et Hensel, 2004). Nos travaux montrent qu'à contrario, l'acquisition d'ADN non-toxique diminue la multiplication et la survie des bactéries. En accord avec les données précédemment publiées, le transfert de *mspA* augmente la croissance de *M. tuberculosis* dans un milieu axénique, mais aucun des travaux antérieurs n'ont suggéré que l'expression du gène *mspA*

pouvait atténuer la croissance d'une bactérie du complexe *M. tuberculosis* dans les différents types des cellules hôtes testées (amibes et cellules de mammifères) ainsi que la virulence dans un modèle souris et fortiori pour l'utilisation comme vaccin ou pour la fabrication d'un vaccin.

Par conséquent, à l'inverse des stratégies qui consistent à inactiver ou à retirer des gènes pour inactiver la mycobactérie et la rendre utilisable dans une perspective vaccinale, nous avons montré de façon surprenante qu'il était possible d'atténuer une bactérie du complexe *M. tuberculosis* par ajout et expression d'un gène en utilisant les méthodes d'ingénierie moléculaire. Cette souche atténuée déposée dans la collection ECACC (n°: 12042601) pourrait être utilisée comme vaccin, pour la prévention de la tuberculose humaine et de façon plus générale de la tuberculose chez les mammifères et les oiseaux (Lamrabet *et al*., brevet 2012).

La mise en place du système de modification génétique des mycobactéries (pathogènes ou non pathogènes) au sein de notre laboratoire a permis de commencer une étude sur l'effet de la mutation génétique du gène *folp1* chez *M. tuberculosis* H37Rv et *M. bovis* BCG sur la sensibilité aux sulfamides et sur la vitesse de croissance des clones de *M. tuberculosis*.

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Le mot de la fin sera pour ma très chère **Mimi**, merci d'avoir été là, aussi patiente et compréhensive…

RÉSUMÉ

Les mycobactéries sont classées parmi les bactéries contenant des acides mycoliques dans leur paroi et un haut GC% dans leur génome. Elles peuvent être isolées à partir du sol ou d'environnement d'eau douce où vivent aussi les protozoaires libres. Plusieurs études ont montré une possibilité de co-isolement des mycobactéries et des amibes à partir de ces sources environnementales. Il a été montré également que la plupart des mycobactéries de l'environnement ont la capacité à survivre dans les trophozoites et les kystes d'amibes et dans certaines cellules eucaryotes, y compris les macrophages. Les manipulations génétiques des mycobactéries en général et des mycobactéries du complexe *Mycobacterium tuberculosis* en particulier sont compliquées et aucune étude de modification génétique des mycobactéries (pathogènes ou non pathogènes) n'avait été réalisée dans notre laboratoire avant notre travail de thèse.

Dans notre travail de thèse, nous avons montré que les amibes ou d'autres organismes phagocytaires peuvent servir comme sources et lieu de transfert des gènes chez les mycobactéries. Ce transfert des gènes peut avoir contribué à l'adaptation des mycobactéries à un mode de vie intracellulaire. Nous avons développé ensuite deux systèmes de coculture: *Mycobacterium smegmatis-Acanthamoeba polyphaga* et *Mycobacterium gilvum-A. polyphaga* et nous avons clarifié le spectre des interactions des mycobactéries à croissance rapide avec les amibes. Ce modèle d'interaction mycobactéries-amibes a été utilisé pour tester l'hypothèse contraire au paradigme dominant que l'addition des gènes réduit la virulence des bactéries. Pour la première fois dans notre laboratoire, nous avons modifié deux espèces du complexe *M. tuberculosis*, *M. tuberculosis* H37Rv et *Mycobacterium bovis* BCG pour observer l'effet de ces changements sur leur pathogenicité et leur survie. De façon intéressante, nous avons observé que l'expression du gène *mspA* de *M. smegmatis* dans *M. tuberculosis* H37Rv et *M. bovis* BCG permet une augmentation significative de leur temps de croissance dans un milieu axénique, mais une diminution significative de leur survie dans les amibes et les macrophages, et une diminution de leur virulence dans un modèle souris.

En conclusion, l'addition des gènes plutôt que leur suppression peut être une autre façon de modifier le comportement des mycobactéries pathogènes. Ceci pourrait être utilisé comme une base pour un vaccin vivant atténué contre la tuberculose.

Mots clés: Mycobactéries, *Mycobacterium tuberculosis, Mycobacterium smegmatis, Mycobacterium gilvum*, amibes, cellules eucaryotes, transfert des gènes, modifications génétiques, porine MspA.

SUMMARY

Mycobacteria are mycolic-acid containing, high GC% bacterial organisms which can be recovered from soil and fresh water environments where free-living protozoa also live. Co-isolation of mycobacteria and amoeba collected from such environmental sources has been reported. Several experiments further demonstrated the ability of most environmental mycobacteria to survive in the amoebal trophozoites and cysts and in some eukaryotic cells including macrophages. Genetic modification of mycobacteria in general and mycobacteria belonging to *Mycobacterium tuberculosis* complex in particular are complicated and no studies using genetic modification of mycobacteria (pathogenic or non-pathogenic) had been performed in our laboratory prior to our work.

In our thesis work, we showed that amoebae or other phagocytic organisms can serve as sources and places for gene transfers in mycobacteria. Gene transfers may have contributed to the adaptation of mycobacteria to an intracellular lifestyle. In addition, we developed two co-culture systems: *Mycobacterium smegmatis-Acanthamoeba polyphaga* and *Mycobacterium gilvum-A. polyphaga* and we clarified the spectrum of rapid-growing mycobacteria and amoeba interactions. This model of mycobacteria-amoeba interactions was then used to test another hypothesis according to which unlike the prevailing paradigm, the addition of genes does not reduce the virulence of bacteria. For the first time in our laboratory we modified two species of the *M. tuberculosis* complex, *M. tuberculosis* H37Rv and *Mycobacterium* bovis BCG to observe the effect of these changes on their pathogenicity and survival. Very interestingly we found that the expression of *M. smegmatis mspA* gene in *M. tuberculosis* H37Rv and *M. bovis* BCG increased significantly their time of growth in an axenic medium but decreased significantly their survival in amoeba and macrophages and their virulence in a mouse model.

In conclusion, gene addition rather than gene suppression may be another way to change the behaviour of mycobacteria in general and especially pathogenic species. The concept of using genetic engineering to add genes in the genome of *M. tuberculosis* could be used as a basis for attenuated vaccine against tuberculosis.

Keywords: Mycobacteria, *Mycobacterium tuberculosis, Mycobacterium smegmatis, Mycobacterium gilvum*, amoeba, eukaryotic cells, gene transfer, genetic modification, MspA porin.