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Analysing nonsynonymous mutations between two *Mycobacterium bovis* strains with contrasting pathogenic profiles

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A R T I C L E I N F O

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A B S T R A C T

Mycobacterium bovis (*M. bovis*) is the causative agent of bovine tuberculosis, a chronic infectious disease that can affect cattle, other domesticated species, wild animals and humans. This disease produces important economic losses worldwide. Two *M. bovis* strains (04-303 and 534) have been isolated in Argentina. Whereas the 04-303 strain was isolated from a wild boar, the 534 strain was obtained from cattle. In a previous study, six weeks after infection, the 04-303 strain induced 100% mortality in mice. By contrast, mice infected with the 534 strain survived, with limited tissue damage, after four months. In this study we compared all predictive proteins encoded in both *M. bovis* genomes. The comparative analysis revealed 141 polymorphic proteins between both strains. From these proteins, nine virulence proteins showed polymorphisms in 04-303, whereas five did it in the 534 strain. Remarkably, both strains contained a high level of polymorphism in proteins related to phthiocerol dimycocerosate (PDIM) synthesis or transport. Further experimental evidence indicated that only mutations in the 534 strain have an impact on PDIM synthesis. The observed reduction in PDIM content in the 534 strain, together with its low capacity to induce phagosome arrest, may be associated with the reported deficiency of this strain to replicate and survive inside bovine macrophages. The findings of this study could contribute to a better understanding of pathogenicity and virulence aspects of *M. bovis*, which is essential for further studies aiming at developing new vaccines and diagnostic techniques for bovines.

1. Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis, a chronic infectious disease disseminated worldwide that mainly affects cattle, although it can also affect other domesticated species, wild animals and humans (Wobeser, 2009; Michel et al., 2003). *M. bovis* belongs to the *Mycobacterium tuberculosis* complex (MTBC), which traditionally consists of *M. tuberculosis* (the main agent of human tuberculosis), *M. africanum, M. microti, M. canettii, M. bovis, M.caprae, M. pinnipedii* and the recently incorporated species *M. suricattae*, *M. mungi* and *M. orygis* (Alexander et al., 2010; van Ingen et al., 2012). These species are genetically very similar, and share a conserved synteny as

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well as 99.9% of the genome (Brosch et al., 2002).

Brosch et al. (Brosch et al., 2002) demonstrated that MTBC members have evolved from a common ancestor via successive DNA deletions, thus resulting in the present *Mycobacterium* speciation and in their differences in pathogenicity. Despite their high overall genetic relationship, MTBC species exhibit a wide range of phenotypes and host ranges. The deletion of genetic information, together with the presence of single nucleotide polymorphisms (SNP) across chromosomes of MTBC species, probably explains the different host ranges, virulence and immunopathology found among these species.

Garnier et al. (Garnier et al., 2003) published the complete genome sequence of *M. bovis* in June 2003, five years after the publication of the *M. tuberculosis* genome sequence (Cole et al., 1998). The genome of *M. bovis* and *M. tuberculosis* are more than 99.95% identical, but with seven deleted regions ranging from 1 kilobase (kb) to 12.7 kb. Thus, these gene deletions could account for the main evolutionary forces that have shaped the *M. bovis* genome. Indeed, many of the absent or altered genes in *M. bovis* are also missing or altered in *M. leprae*, an obligate intracellular pathogen that has undergone a significant reduction in its genome (Garnier et al., 2003).

A deeper knowledge of the mycobacterial virulence factors is essential for the development of new vaccines and drugs to help manage the disease and move towards a world free of *tuberculosis*.

The *M. bovis* 04-303 strain was isolated from a wild boar in the La Pampa province of Argentina in 2004, and characterized as spoligotype SB0140, with a Mycobacterial Interspersed Repetitive Units (MIRU) pattern of 232224263322 (Aguilar León et al., 2009; Nishibe et al., 2013). The *M. bovis* 534 strain, on the other hand was isolated from cattle in the Santa Fe province of Argentina, in 1997. This strain was characterized as spoligotype SB0140, with an MIRU pattern of 232224163322 (Aguilar León et al., 2009). In an experimental BALBc model of progressive pulmonary tuberculosis, mice infected with the 04-303 strain exhibited high mortality (Aguilar León et al., 2009). The animals developed a mild inflammatory response, followed by sudden pneumonia, with extensive necrosis. Conversely, mice infected with the 534 strain exhibited higher survival rates and limited tissue damage, with progressive expression of IFN-γ and elevated expression of IL-4, TNF-α and iNOS. The 04-303 strain induced a significantly lower expression of these immune mediators. In another study where the researchers assessed the secretion of virulence factors as well as phagocytosis of both strains by J774A.1 macrophages, the 04-303 strain secreted more ESAT-6 and CFP-10 than the genetically related strain 444, and was more phagocytized than BCG and even more than 444 strain (Vargas-Romero et al., 2016). In experimentally infected cattle, the 04-303 strain produced more severe lesions, in the evaluated organs, with granuloma and pneumonic areas, than the *M. bovis* strain, NCTC 10772 (Meikle et al., 2011). In a guinea pig model of *M. bovis* infection, the 04-303 strain was significantly more virulent than the NCTC 10772 strain (Meikle et al., 2011). Based on these results, the researchers characterized *M. bovis* 04-303 as a highly virulent strain.

Considering the extreme pathogenic phenotype of the 04-303 strain and the attenuated phenotype of the 534 strain, we hypothesized that the differences in virulence could be attributable to nonsynonymous mutations in the coding sequences. With this in mind, we performed a comparative genomic analysis to identify nonsynonymous single nucleotide polymorphisms (NS-SNP) and insertions/deletions in the coding sequences of the *M. bovis* 04-303 and 534 strains. We used the complete genome of *M. bovis* AF2122/97 (Garnier et al., 2003) as reference.

2. Material and methods

2.1. Bacterial strains and culture media

M. bovis strains were grown in Middlebrook 7H9 medium (Difco Laboratories, 271310) supplemented with albumin (A) 0.5%, dextrose (D) 0.4%, and pyruvate (P) 0.5% (M7H9-AD-P) or Middlebrook 7H10 (Difco Laboratories, 262,710) supplemented with AD-P. When necessary, 50 µg/ml hygromycin, µg/ml 20 kanamycin or Tween 80 (T80) 0.05% was added to the media.

The *M. bovis* 04-303 and *M. bovis* 534 strains were isolated from a wild boar in a free-ranging field and from dairy cattle in the humid Pampas region, respectively, both in Argentina. The DNA from the isolates were extracted and purified as described by van Soolingen et al. (van Soolingen et al., 1991).

2.2. Genome sequencing and annotation

M. bovis 04-303 (Nishibe et al., 2013) and *M. bovis* 534 were deposited in the

DDBJ/EMBL/GenBank under the accession numbers GenBank: AVSW01000000 and JQEM00000000, respectively. The genome assembly showed 169 (average coverage of 27.3 x) and 72 contigs (average coverage of 50.5 x) for the 04-303 and 534 strains, respectively. Whole genome sequencing of 04-303 and 534 strains was performed on starter cultures from frozen seed stocks (Dr Zumárraga, personal communication).

The quality of the genome assemblies was assessed following the CheckM automated method (Parks et al., 2015). This method provides a broader set of marker genes that are specific to the position of a genome within a reference genome tree, as well as information about the collocation of these genes. According to the CheckM default thresholds, both assemblies exhibited a satisfactory quality. The coding densities of both genomes were within the expected range for bacterial genomes.

2.3. Single nucleotide polymorphisms

The open reading frames and protein sequences of the reference strain, AF2122/97 (LT708304.1), were compared with the whole genome of both strains. NS-SNPs were found using an in-house script written in Python and Translated BLAST: tblastn.

The algorithm tbBLASTn was used to retrieve NS-SNPs in the *M. bovis* genomes deposited in NCBI. The Query was a multifasta of the 30 selected sequences (S1) and subject was *Mycobacterium bovis* (taxid 1765), excluding *M. bovis* BCG (taxid 33892). The expected threshold was set up to 1e-6.

2.4. Virulence-related genes analysed

This analysis was performed with all genes from both genomes, as published by Forrellad et al. (Forrellad et al., 2013) and Sassetti et al. (Sassetti et al., 2003) and/or with genes listed in the TubercuList database server (http://genolist.pasteur.fr/TubercuList). The analysis of conserved domains was done with ScanProsite (http://prosite.expasy.org/scanprosite/.) and CD search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi).

2.5. Analysis of protein stability changes due to NS-mutations

The STRUM method was used for predicting the fold stability change ($\Delta\Delta G$) of virulence factors upon single-point NS-SNP mutations (http://zhanglab.ccmb.med.umich.edu/STRUM/).

2.6. Lipid analyses

Total lipids from bacterial cells were extracted following procedures described previously (Stadthagen et al., 2005). Briefly, cultures were grown to late exponential phase (optical density (OD) 600 nm 0.8–1) and total cell wall lipids extracted with chloroform:methanol. Fatty acid and mycolic acid methyl esters (FAMES and MAMEs, respectively) were derived from extractable lipids as described by Stadthagen et al. (Stadthagen et al., 2005). Lipids were analysed by TLC on silica gel

60F254 by loading the same lipid quantities per lane (100 µg). TLCs were developed in three different solvent systems and revealed by spraying with a CuSO4-phosphoric acid solution and heating. These experiments were repeated with five independent lipid samples of each *M. bovis* strain.

2.7. Liquid chromatography-mass spectrometry (LC–MS) analysis

Total lipids were analysed by LC/MS as described by Sartain et al. (Sartain et al., 2011). High-resolution Agilent 6220 TOF and Brucker micrOTOF-Q II mass spectrometers interfaced to a LC were used for the analysis. Data files were analysed with Agilent's Mass hunter workstation software (Version B.02.00, build 2.0.197.0) and software MZmine2 (Pluskal et al., 2010) to identify compounds using 'molecular feature extractor'.

The Agilent mass profiler program was used to compare all the lipids present in the samples. Most compounds were identified using the lipid database developed by Sartain et al. Compounds of interest were semi-quantified by comparing their relative abundance in the samples.

2.8. Peripheral blood mononuclear cell (PBMC) isolation and monocyte-derived macrophages (BMDM) differentiation

Peripheral blood samples were obtained from cattle selected from the experimental herd of INTA. All these animals tested negative for bovine tuberculosis infection according to the single intradermal tuberculin test and Interferon-γ release test. A sample of blood (50 mL) was taken from each animal in sterile conditions and PBMCs were separated from heparinized blood by centrifugation over histopaque 1077 following the manufacturer's protocol. Monocytes were differentiated by culturing PBMCs on 24-well plates with coverslips (or not) containing RPMI 1640 complete medium supplemented with 10% of autologous plasma at 37 °C and 5% CO_2. Adherent cells were maintained in culture for 5 days to obtain BMDMs. The cell viability was confirmed by trypan blue exclusion assay (data not shown). Sample collection and animal handling were done in compliance with the regulations of the Ethical Committee of INTA (CICUAE).

2.9. BMDM infections

M. bovis strains were cultured to exponential growth phase (OD 600 nm 0.4-0.6), harvested, washed and resuspended in RPMI medium. The bacterial suspensions were centrifuged at 900 g for 10 min to eliminate bacterial clumps in the pellets. The optical densities of the bacterial suspensions were adjusted to a multiplicity of infection (MOI) of 2. BMDMs were infected for 2 h at 37 °C and 5% CO_2 (uptake) and then washed three times to eliminate extracellular bacteria. Subsequently, BMDMs were incubated in RPMI medium supplemented with 10% of autologous plasma for 3, 24, 48 and 72 h accordingly to the assay. Three and seven independent infections were performed for intracellular trafficking and for bacterial persistence assays, respectively.

Indirect immunofluorescence and confocal microscopy

Bovine BMDMs (4×10^5) were seeded in 24 well-plate with slides and then infected with the *M. bovis* strains at a MOI of 2, as described before. After 72 h of infection, cells were fixed with 4% paraformaldehyde solution in PBS (PFA) for 30 min and quenched by incubation with 50 mM glycine solution for 30 min. The cells were then permeabilized with 0.05% saponin in PBS containing 1% bovine serum albumin (BSA) for 15 min, followed by overnight incubation with rabbit anti-Mycobacterium primary antibody (Origene, USA) diluted 1:50 in PBS. Next, a secondary antibody anti-Rabbit conjugated to FITC (Jackson Immuno Research Labs, Inc.) diluted 1:600 in PBS was added and incubated for 1 h. Subsequently, the cells were incubated with goat anti-LAMP-3 (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody diluted 1:50 in PBS for 2 h. Secondary anti-goat antibody conjugated to cy3 (Jackson Immuno Research Labs, Inc.) was used diluted 1:600 in PBS. The cells were mounted with mounting medium (Dako, Denmark) and analysed by confocal microscopy using an SP5 AOBS confocal microscope (Leica Microsystems, Germany) at the *Laboratorio Integral de Microscopía*, CICVyA, INTA. Mycobacterial internalization was monitored using the fluorescence of FITC (green). LAMP-3 association with mycobacterial phagosomes was analysed in at least 500 cells using Fiji software (U.S. National Institutes of Health, Bethesda, MD) as described previously (Vázquez et al., 2017). The experiments were performed in duplicates in three independent experiments. The statistical analysis was performed using two-tailed Student's *t*-test.

2.10. Recombinant M. bovis strains

The *esat-6* gene was PCR amplified from genomic DNA of the 04-303 and 534 strains with specific primers, and cloned in NdeI and HindIII sites of pVV16 vector, which is a derivative of pMV261 (Stover et al., 1991) that allows His-tag fusion for expression in *M. smegmatis*. The inserts in the recombinant plasmids were sequenced; the results showed that the polymorphism of *esat-6* of 04-303 and 534 deposited in the DDBJ/EMBL/GenBank were maintained in the recombinant plasmids. The recombinant plasmids PVV16-ESAT6 04-303 and PVV16-ESAT6 534 were used to transform the 534 strain. The recombinant 534 strains were selected in 7H10-AD-P medium containing 50 µg/ml of hygromycin.

2.11. Western blot analysis

After three weeks of incubation, bacterial cells were harvested by centrifugation, broken by disruption with glass beads in a homogenizer, and resuspended in PBS 1X for protein quantification. Secreted proteins were obtained from culture supernatants of bacteria grown in medium with 0.4% glucose but without albumin. The supernatants containing secreted proteins were precipitated with 10% trichloroacetic acid overnight and centrifuged at 7000 g for 30 min at 4 °C. The proteins were neutralized with Tris 1 M and resuspended in loading buffer.

The samples were subjected to electrophoresis in 12–15 % SDS-PAGE gels, subsequently transferred to nitrocellulose membranes and stained with Rouge Ponceau solution. The membranes were blocked with TBS (10 mM tris − HCl pH 7.5, 150 mM NaCl) supplemented with 5% skim milk for 30 min before performing an overnight incubation with primary antibodies (monoclonal anti-ESAT-6 (1/100) or polyclonal GroEL (1/5000). The nitrocellulose membranes were then incubated with a secondary antibody alkaline phosphatase-conjugated at a 1:10.000 dilution for 2 h. Western blots were revealed by incubation with BCIP/NTP solution.

3. Results and discussion

3.1. Comparative analysis of predicted proteins in M. bovis strains

The comparison of SNPs of the 04-303 and 534 strains, through the evaluation of their genomes in relation to that of the *M. bovis* AF2122/97 reference strain, retrieved 193 genes with at least NS-SNP in one of the strains or in both (Supplementary material 1). In total, 57 genes carried NS-SNPs in 04-303, whereas 84 did it in 534. Interestingly, 53 of the NS-SNPs were common to both strains. From the exclusive NS-mutations, nine produced a frameshift, whereas two resulted in premature stops in the proteins (Supplementary material 1).

A functional categorization (tuberculist.epfl.ch) of the polymorphic proteins between 04-303 and 534 showed that the most represented categories in mutant proteins of 534 are *PE/PPE* and *intermediary metabolism* and *respiration*. On the other hand, 04-303 carries NS-SNPs mainly in genes of the *lipid metabolism* category (Supplementary material 1).

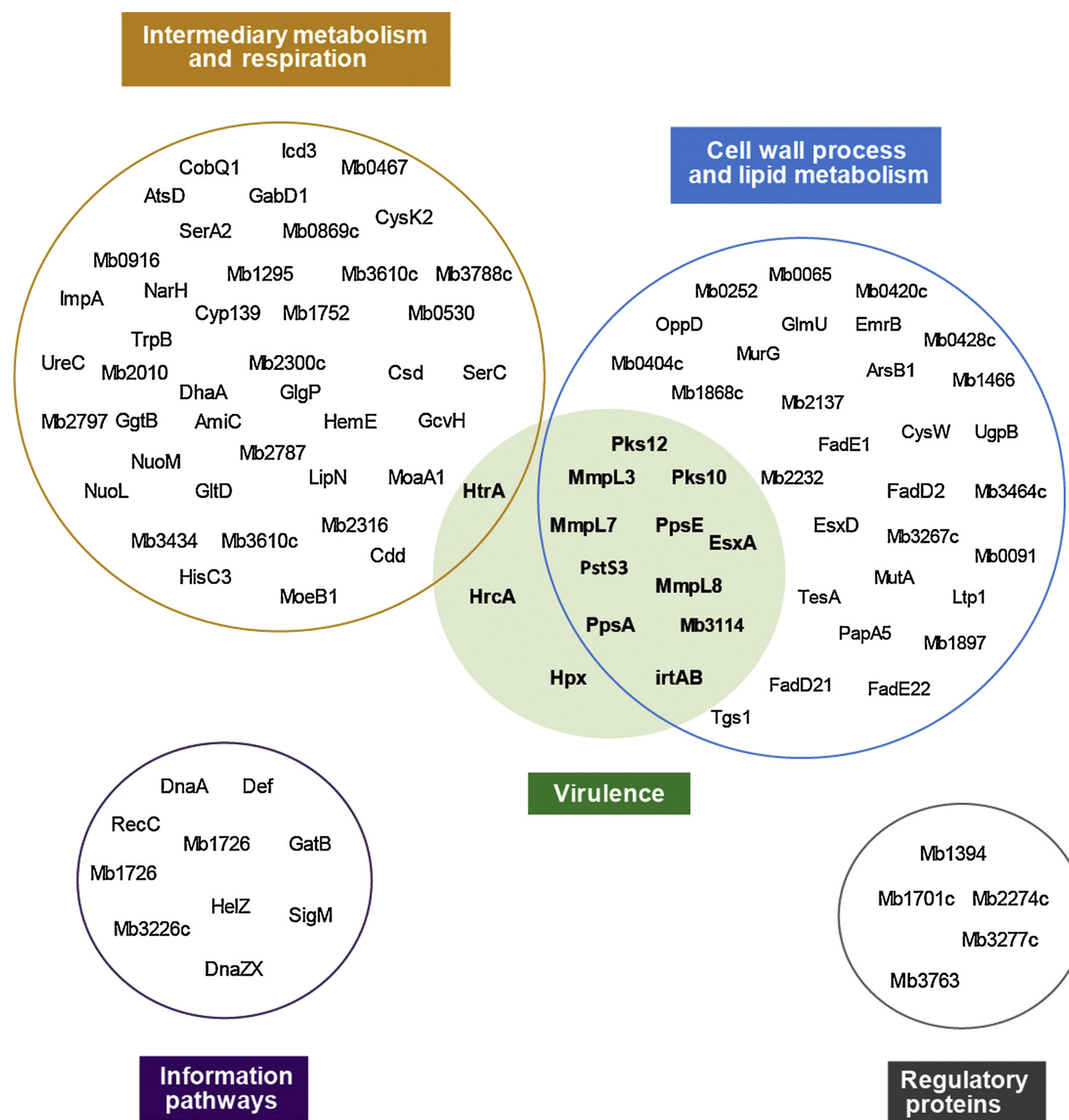


Fig. 1. Venn diagram illustrating polymorphic virulence genes of both 04-303 and 534 strains grouped as functional categories.

3.2. Polymorphic virulence genes

A search for virulence genes in the polymorphic gene dataset, based on the bibliography and on the Tuberculist functional categories, gave nine virulence genes with nonsynonymous polymorphisms in 04-303 and five in 534. These 14 polymorphic genes with a putative or demonstrated role in mycobacteria virulence grouped in five functional categories: three genes are involved in cell wall process (*esxA*, *pstS3* and *irtAB*); eight are associated with lipid metabolism (*pks12*, *mmpL3*, *pks10*, *mmpL7*, *mmpL8*, *Mb3114*, *ppsA* and *ppsE*); one was involved in intermediary metabolism and respiration (*htrA*); one encoded a regulatory protein (*hrcA*) and one categorized as virulence gene (*hpX*) (Fig. 1). These results highlight the relevance of the cell wall and related lipids in the mechanism of virulence of pathogenic mycobacteria.

Of the eight virulence polymorphic proteins involved in lipid metabolism, four are enzymes of phthioceroldimycocerosate (PDIM) synthesis/transport pathways, three are MmpL proteins involved in lipid transport and one is a putative glycerolphosphodiesterase (Mb3114) predicted to be required for in vivo survival of *M. tuberculosis* (Sassetti et al., 2003). The genes *mmpL3*, *7* and *8* have NS-SNPs in the 04-303 strain. The proteins of the MmpL family translocate complex lipids across the cell envelope of mycobacteria. MmpL8 transfers the sulfolipid SL-1 to the cell envelope (Converse et al., 2003) and MmpL7 transports PDIM. A study demonstrated that the lack of MmpL7 in *M. tuberculosis* severely affects the virulence of the bacteria, whereas the absence of MmpL8 attenuates it (Domenech et al., 2005). MmpL 7 and 8 of 04-303 showed aa changes that predictably favours its stability (Table 1). Therefore, if mutations in MmpL proteins have an impact on the virulence of 04-303, they should favour the protein functionality. MmpL3 (Mb0212) transports trehalose momomycolate, a precursor of the mycobacterial outer membrane component trehalose dimycolate as well as mycolic acids bound to arabinogalactan. However, since this gene is essential for bacterium replication in vitro, it is difficult to speculate about the role of the *mmpL3* mutation in the virulence phenotype of 04-303. In addition, the mutation seems to not affect the lipid transportation function, since we did not detect substantial differences in the content of TDM or extractable mycolic acids between strains (Supplementary material 2A and 2B).

The PDIM lipids consist of a long-chain β-diol - phthiocerol - esterified by one type of such long-chain multiple methyl-branched fatty

Table 1
Polymorphic virulence proteins in *M. bovis* strains compared to *M. bovis* AF2122/97.

Mb number	Name	Rv number	Description	Length	Location	aa ^{Mb}	aa ^{Rv}	Change	ddG	%§
	***M. bovis* strain 04-303**									
Mb0212c	MmpL3	Rv0206c	Transport protein Mmpl3	944	820	A	A	P	ND	3.1
Mb0951	PstS3	Rv0928	Periplasmic phosphate-binding lipoprotein	370	84	N	N	S	−1.37	3.1
Mb2074c	Pks12	Rv2048c	Polyketide synthase Pks12	4151	3971	S	S	Fs	ND	9.4
Mb2395c	HrcA	Rv2374c	Probable heat shock protein transcriptional repressor	343	78	D	D	N	+1.6	3.1
Mb2967	MmpL7	Rv2942	Transport protein Mmpl7	920	407	I	I	T	+2.5	3.1
Mb3114	Rv3087	Rv3087	Possible triacylglycerol synthase	472	453	S	S	L	ND	3.1
Mb3196c	Hpx	Rv3171c	Possible non-heme haloperoxidase	299	7	D	D	G	ND	3.1
Mb3853c	MmpL8	Rv3823c	Transport protein Mmpl8	1089	451	L	L	V	+4.93	3.1
Mb3905	EsxA	Rv3875	6 kda early secretory antigenic target esxa (esat-6)	95	63	T	T	A	+0.52	3.1
	***M. bovis* strain 534**									
Mb1255	HtrA	Rv1223	Serine protease htra (degp protein)	528	272	I	I	M	−0.08	18.8
Mb1383	IrtA	Rv1348	Atp-binding protein Abc transporter	859	562	R	R	L	+2.35	9.4
Mb1688	Pks10	Rv1660	Chalcone synthase Polyketide synthase Pks10	353	330	S	S	G	ND	2.6
Mb2074c	Pks12	Rv2048c	Polyketide synthase Pks12	4151	3982	H	Q	Q	ND	6.3
Mb2074c	Pks12	Rv2048c	Polyketide synthase Pks12	4151	3985	H	E	E	ND	3.1
Mb2956	PpsA	Rv2931	Polyketide synthase PpsA	1876	1809	F	F	L	+0.17	15.6
Mb2960	PpsE	Rv2935	Polyketide synthase PpsE	1488	538	G	G	S	+4.92	18.8

§ frequency of NS-mutation in *M. bovis* isolates.
aa ^{Mb} Amino acid in *M. bovis* AF2122/97 strain.
aa ^{Rv} Amino acid in *M. tuberculosis* H37Rv strain.

acids called mycocerosic acids. PDIMs constitute major virulence factors of *M. tuberculosis*, particularly, during the early step of infection when bacilli encounter their host macrophages. Two transcriptional units are involved in the synthesis and transport of PDIMs. These transcriptional units contain many genes involved in fatty acid synthesis/modification, many of them with homology to fatty acyl CoA synthases and dehydrogenases, as well as a set of genes with homology to polyketide synthases (Pks). Pks are multifunctional enzymes that, along with adjacent or neighbouring Acyl-CoA synthases and dehydrogenases could be involved in the synthesis of complex lipids such as mycocerosic acid, sulfolipids, diacyltrehalose/polyacyltrehalose (DAT/PAT). Mutants in *pks12* and *pks10* genes of *M. tuberculosis* displayed defect in PDIM production (Sirakova et al., 2003). However, later studies demonstrated that Pks12 is involved in the synthesis of Mannosyl-beta -1-phosphomycoketides (MPM) (Angala et al., 2014). Therefore, at least for Pks12, the role of this enzyme in the accumulation of PDIM seems to be secondary or indirect.

Pk10 showed polymorphisms only in the 534 strain, whereas Pks12 was polymorphic in both strains. A single nucleotide deletion in the coding sequence of Pks12 produced a frameshift in the resulting protein of the 04-303 strain, whereas a double amino-acid (aa) substitution occurred at the C terminal end (QxH and ExH) of Pks12 in 534. Moreover, PpsA and PpsE, which are also polyketide synthases encoded in one of the PDIM loci (Domenech and Reed, 2009), showed NS-mutations in the 534 strain. Although predictive analysis indicates that these mutations are supposed to produce stability to the resulting protein structures (Table 1), they map in conserved domains, according to ScanProsite (data not shown). Therefore, the enzymatic activities of PpsA and PpsE may be affected.

Altogether, this genomic analysis shows a remarkable high level of polymorphism in proteins related to PDIM synthesis or transport in both strains.

The comparative genomic analysis identified only one NS-mutation in a regulatory gene of 04-303 and none in 534. The identified polymorphic regulatory gene is *hrcA* (*Mb1255*). HrcA is a heat-shock protein that acts as a transcriptional repressor for members involved in the Hsp60 (GroEL) response to *M. tuberculosis* (Stewart et al., 2002). The mutation of HrcA in 04-303 produced an aa change from aspartate, a negatively polar side-chain aa, to asparagine, an uncharged polar side chain aa. This aa change should destabilize the protein structure as predicted by STRUM (Table 1). A western blot analysis showed no differences in the expression of the GroEL protein (Supplementary material 2D), suggesting no alteration in the expression level because of this aa change in HrcA.

In this study, we detected NS-mutations in four membrane/cell wall associated genes: *irtA*, *pstS3*, *hpX* and *htrA*. IrtA together with IrtB are proteins involved in iron acquisition. Here, we identified a change of a leucine, an uncharged polar side chain aa, to an arginine in the protein sequence of IrtA in the 534 strain. The mutation maps in a conserved domain detected by ScanProsite tool (data not shown).

PstS-3 is a protein involved in the transport of inorganic phosphate together with PhoT. Previous results indicate that PstS-3 may have a minor role in the virulence mechanisms of *M. tuberculosis* (Brodin et al., 2010). In the 04-303 strain, *pstS-3* carries a NS-SNP that is predicted to produce a negative stability change in the resulting protein (Table 1). This suggests that the mutation does not contribute to the virulence phenotype of the *M. bovis* strain.

Hpx carries a NS-mutation in the 04-303 strain. This gene encodes a putative non-heme haloperoxidase identified in the cell membrane of *M. tuberculosis* (Mawuenyega et al., 2005) that may be involved in detoxification reactions. No experimental characterization has been done so far and therefore its role in *M. bovis* virulence is still undefined.

HtrA-1 (DegP) is a member of the serine protease family. This protein is essential, as determined by saturation transposon mutagenesis and through attempts to knock-out the gene (Roberts et al., 2013) (Sassetti et al., 2003). The essentiality of *htrA-1* indicates that its NS-mutation in the 534 strain should not affect the protein functionality, although the mutation predictively affects the protein stability.

Only the 6 kDa early secretory antigenic target (ESAT-6) or EsxA, which is a secreted virulence factor, showed polymorphism between the strains. ESAT-6 is a highly conserved protein (Encinas et al., 2018) that is part of a protein family known as Esx. ESAT-6 of *M. bovis* forms a dimer with CFP10 (culture filtrate antigen or EsxB) (Mb3904), a strong stimulator of cell-mediated immunity (Jones et al., 2010). ESAT-6:CFP10 can sequester the host protein beta-2-microglobulin (b2M) to inhibit the cell surface expression of the major histocompatibility complex (MHC)-I-b2M; which may undermine the host's adaptive immune responses, thereby leading to the establishment of a successful infection (Sreejit et al., 2014).

Indeed, the 04-303 strain showed a NS-SNP in *esat-6* that improves the stability of the protein, as predicted by STRUM analysis (Table 1). Furthermore, this NS-SNP does not affect the secretion of the protein, as

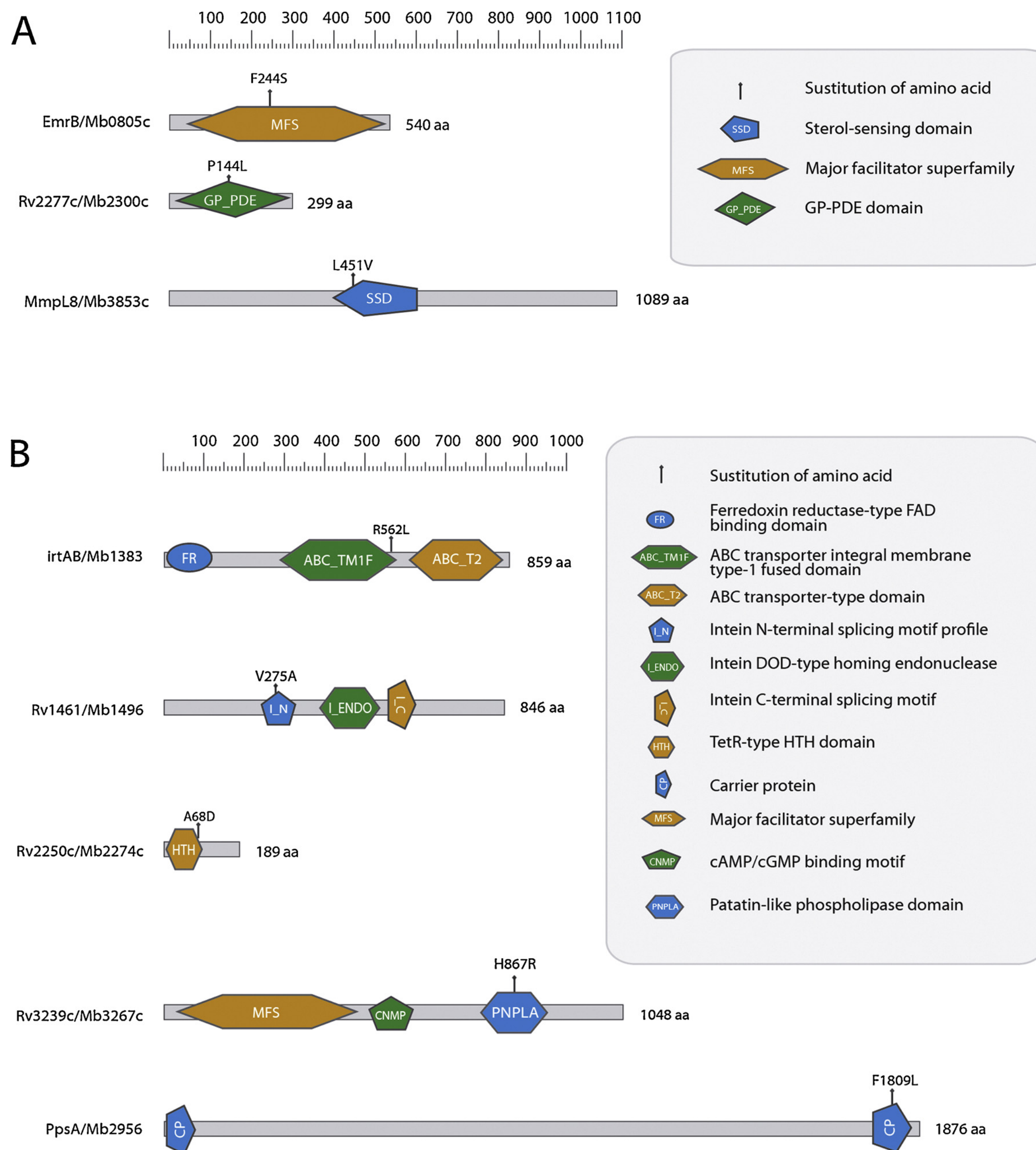


Fig. 2. Schematic representation of polymorphisms in protein conserved domains of (A) 04-303 and (B) 534 strains. Only domains identified by both ScanProsite and CD search were considered in this study.

evidenced by the equivalent amounts of ESAT-6 expression in the culture supernatants of both strains (Supplementary material 2D).

3.3. Polymorphism in conserved protein domains

Five polymorphisms map in conserved predicted domains (Fig. 2). The 04-303 strain has a polymorphism in the major facilitator superfamily (MFS) and glycerophosphodiester phosphodiesterase (GP-PDE) domain of Mb0805c and Mb2300c, respectively. Mb0805c is a putative multidrug resistance integral membrane efflux protein (Emrb), whereas Mb2300c is a putative glycerolphosphodiesterase. For both proteins, the predictive domains encompass more than 89% of the full protein length (Fig. 2A) and, therefore, it is difficult to assign an impact for the aa changes.

In the 534 strain, Mb1496c and Mb3267c are conserved hypothetical protein with an aa change in an intein N-terminal splicing and a patatin-like phospholipase (PNPLA) domains, respectively (Fig. 2B). In addition, Mb2274c is a putative transcriptional regulatory protein that carries a predictive aa change in a TetR-type HTH domain. All of these conserved domains that showed polymorphisms in the 534 strain in relation to the *M. bovis* reference strain are in proteins with no established functions in mycobacteria. However, Mb2274c has been assigned a predictive transcriptional regulation function (http://genolist.pasteur.fr/BoviList/). Thus, the mutation in its HTH domain may have an impact on the binding of the protein to the DNA, which in turn would affect the transcriptional expression of the target genes.

3.4. Frameshift mutations

Rv1264 (Mb1295) is one of the 15 adenylyl cyclases of *M. tuberculosis* that produce cAMP (Findeisen et al., 2007). This enzyme is activated at acidic pH due to pH-dependent structural transitions of the

Table 2
Frameshift mutations in *M. bovis* strains compared to *M. bovis* AF2122/97.

Mb number	Name	Rv number	Description	Length	Location of Fs
04-303					
Mb3920c	EsxD	Rv3891c	Esat-6 like protein esxd	107	40
534					
Mb1246c	PE14	Rv1214c	Pe family protein	376	69*
Mb1295	–	Rv1264	Adenylyl cyclase	397	371
Mb2437c	–	Rv2414c	Conserved hypothetical protein	523	58
Mb2813c	Ltp1	Rv2790c	Probable lipid	401	6
Mb3370	–	Rv3338	conserved hypothetical protein	297	83
Mb3610c	–	Rv3579c	Possible trna/rrna methyltransferase	403	317

§**frequency of NS-**mutation **in *M. bovis* isolates.**
*insertion **of** 5′**ACAAG.**3′

Rv1264 dimer. In the 534 strain, a frameshift mutation in *Mb1295* produced a predictive larger protein than that of the wild type (410 vs 397 aa). However, the mutated region is located at the end of the carboxyl terminal domain of the protein and does not encompass conserved domains or a conserved aa.

A single nucleotide deletion in *Mb2813* of 534 results in a predictable protein of only 17 aa. *Mb2813* encodes a putative lipid-transfer protein Ltp1 (Table 2). The role of this protein during tuberculosis infection remains unknown. Based on the relevance of the mycobacterial lipids for the interaction with their host, it is tempting to speculate that the lack of this putative lipid transfer protein may impact on the virulence of the strain 534.

Other frameshift mutations in the 534 strain are those located in *Mb2437c* and *Mb3370*, which produced truncated proteins of 133 and 150 aa, respectively (Table 2). These aberrant proteins would conserve only 56 and 83 aa of the amino terminal portion of the wild type proteins, respectively. These genes encode hypothetical proteins not previously described in the literature.

PE family protein PE14 (*Mb1246c*) of 534 carries a five nucleotides insertion that results in a shorter protein of 69 aa in relation to the reference strain. Although the function of this protein is still unknown, this five nucleotides insertion is also present in *M. tuberculosis* H37Rv reference strain but in different position. The insertion localizes at nucleotide position 205 from 5′ end of *Mb1246c* and nucleotide position 204 from 5′ end of *Rv1214c*.

A mutation that maps at the end of *Mb3610* in the 534 strain produces a predictable protein 81 aa shorter than that of the wild type (Table 2). Mb3610, a putative tRNA/rRNAmethyltransferase, seems to be essential for *M. tuberculosis* growth and, thus, any mutation detected in this gene should have no impact on the functionality of the protein.

Only two frameshift mutations were exclusively identified in the 04-303 strain (Table 2). One is in the *pks12* gene, previously mentioned, and the other maps in the *Mb3920c* gene. Mb3920c (EsxD) is a member of the ESAT-6 family that, together with Mb3919c (EsxC), may be secreted by the ESX-2 system (Knudsen et al., 2014). Nevertheless, EsxD and EsxC lack the ESX signature tag. None of these proteins have been identified in the culture supernatants. The deletion of a single nucleotide from *Mb3902c* in the 04-303 strain would produce a protein of 63 aa, of which only the first 40 are identical to the wild type protein. The dimmer EsxC/EsxD formulated in adjuvant CAF01 has shown to confer a significant level of protection against *M. tuberculosis* in a mouse model (Knudsen et al., 2014). This finding may suggest that the lack of a full-length EsxD would facilitate 04-303 to evade the host immune response.

3.5. Polymorphisms in other M. bovis isolates

We extended the analysis of the selected polymorphic proteins to other 30 *M. bovis* genomes obtained from the public database (https://www.ncbi.nlm.nih.gov/gquery/). In the 04-303 strain, most of the NS-mutations in the selected genes are unique. The exceptions are in the case of *pks12* (Table 1), which encodes a protein highly polymorphic among *M. bovis* strains, and *Mb2300c* (*Rv2300c*) which showed the same polymorphism in 96.9% of the analysed *M. bovis* isolates (data not shown) This unexpected finding may explain the exacerbated virulence of 04-303 (Meikle et al., 2011).

The frequency of mutations is contrasting in the 534 strain, where most of NS-SNPs are present in other *M. bovis* isolates. Based on the premise that evolutionary forces would not select mutations that have a negative impact on the virulence of *M. bovis*, we should conclude that most of the mutations in the 534 strain are not associated with its attenuation. The exceptions are *pks10*, the adenylyl cyclases *Mb1295* (*Rv1264*) and *Mb1496* (*Rv1461*) (Table 1 and data not shown), which have unique mutations. In addition, for *hrtA*, only another strain shares this NS-mutation with *M. bovis* 534. In the case of these genes, the mutations would be effectively associated with a disadvantage for infection.

3.6. Comparative lipid analysis

Based on the high number of lipid-related proteins with polymorphisms between strains, we decided to compare the lipid content of these bacteria. Preparation and TLC analysis of total lipids from the cellular extractable lipids showed that some lipids were exclusively present in the 04-303 strain (Fig. 3A). When total lipids prepared from cell extracts were solved in a solvent system that resolved PDIM, we detected the accumulation of multiple bands in 04-303, which were absent from the 534 strain (Fig. 3B). To determine whether these differences in PDIM profiles between strains were a consequence of bacterial sub-culturing, we analysed the PDIM content of bacterial cells obtained from seed stocks (original cultures with minimal passages). The lipid profiles were similar to those of Fig. 3B (Supplementary material 2C). This result indicates that the lower content of PDIM in the 534 strain was not due to the mutations in PDIM-related genes accumulated during in vitro bacterial culturing. These phenotypes, which were reproducible throughout five independent experiments, suggest that the NS-mutations either in *pk10*, *ppsA*, *ppsE* or altogether dramatically impact on the PDIM production of 534.

In addition, the differences in lipid content were confirmed by an LC–MS analysis of the cellular extractable lipids from both strains (Fig. 3C, D and E). Mass spectra clearly showed an increase in the peaks eluting between 22.5 and 26.5 min that matched with mycoside B, a lipid that shares much of the biosynthetic pathways of PDIM, PDIMA and PDIMB in samples from the 04-303 strain. The higher accumulation of PDIMs in the 04-303 strain than in the 534 strain was confirmed in a second LC-MS analysis with a set of lipid samples obtained from seed stocks (data not shown). Altogether, the production of PDIMs showed to be altered in 534 strain.

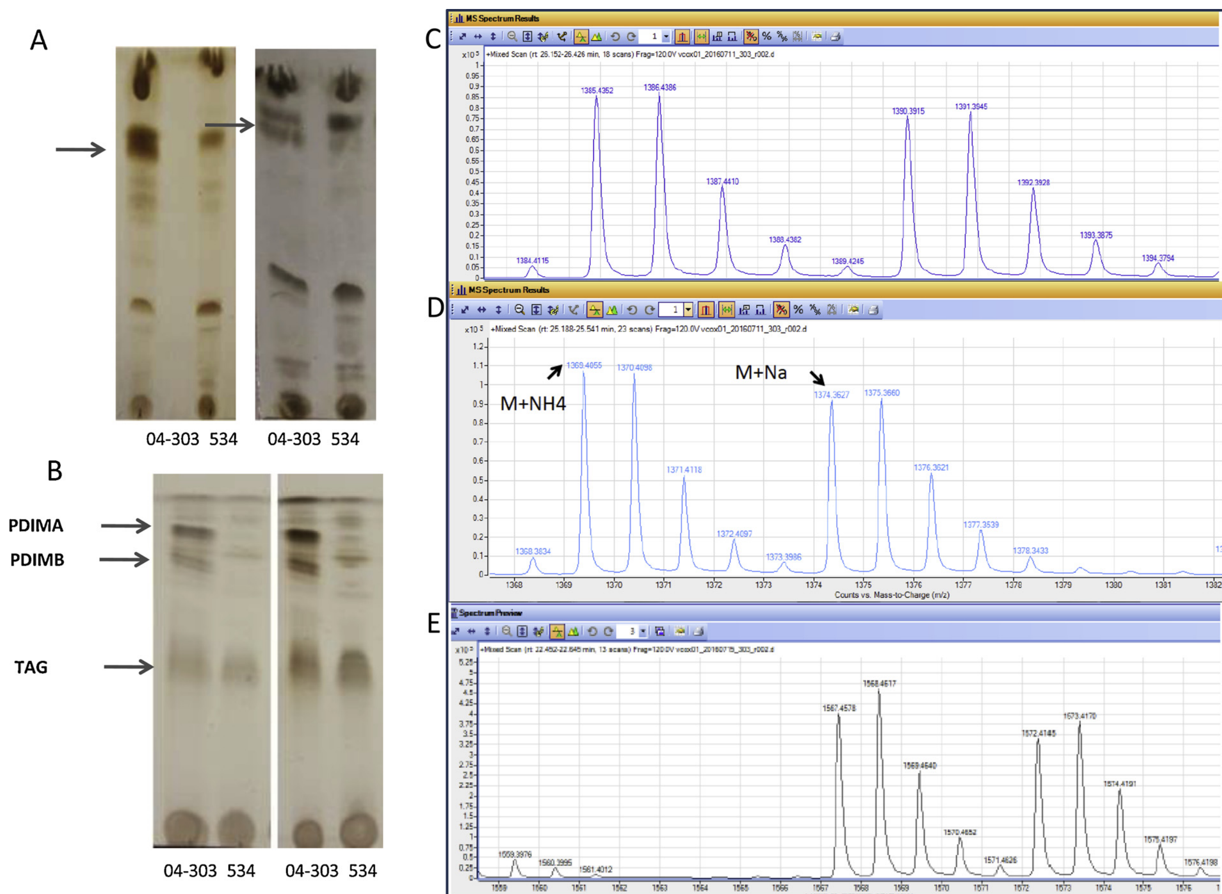


Fig. 3. Lipidomic analysis. (A, B) Thin layer chromatography analysis of total lipid from *M. bovis* strains. Total lipids were extracted from *M. bovis* strains developed by TLC in the solvent systems (A) chloroform methanol water (90:10:1) (B) petroleum ether/diethyl ether (98:2) and revealed with CuSO4 and heating. The arrows indicate increased lipids in 04-303. The lipid patterns were repeated in five independent experiments. (C, D, E) LC−MS analysis of total lipids from *M. bovis* strains. (C) The TIC and the selection ion chromatograms at *m/z* 1322.314 (M + Na + for DIMB) and at *m/z* 1530.382 (M + Na + for a DIMA based mycoside B) for total lipid extracts from 04-303 and 534 strains. Consistent with the TLC analysis, both strains show large amounts of various forms of triglycerides (labelled on the TLC of Fig. 3). Strain 04-303 also shows substantial amounts of various forms of both mycoside B and DIM (also labelled on the TLC of Fig. 3). The selected ion chromatograms show that both DIM and mycoside B are present in the 04-303 strain; integration shows the ratio at approximately 75:1 (303:534) for both lipids. DIMA and mycoside B based on DIMB were also present in the 04-303 strain in substantial amounts (data not shown). The mass spectrum of the major DIMB is shown in (D); the structural drawing, while correct in its molecular weight, is not necessarily accurate as to which CH2 groups are in the fatty acids and which are on the main chain of the phthiocerol backbone. Similarly, the mass spectrum of the major mycoside B based on DIMA is shown (E) and, although the phthiocerol backbone and the acyl groups are clearly smaller than those of the DIMs, the drawing is not necessarily accurate as to which CH2 groups are in the fatty acids and which are on the main chain of the phthiocerol backbone.

3.7. Intracellular trafficking of M. bovis strains

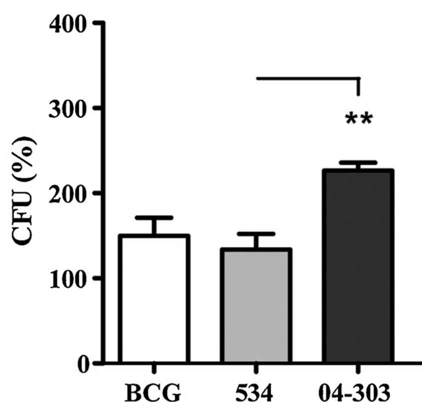
ESAT-6 in concert with other proteins and PDIM lipids contribute to the phagosomal escape of *M. tuberculosis* (Augenstreich et al., 2017). Thus, the variable abundance of PDIM between the 04-303 and 534 strains and the polymorphism in ESAT-6 may have an impact on intracellular localization of both strains. To address this point, we evaluated the maturation stage of mycobacterial phagosomes by using immunofluorescence and confocal microscopy.

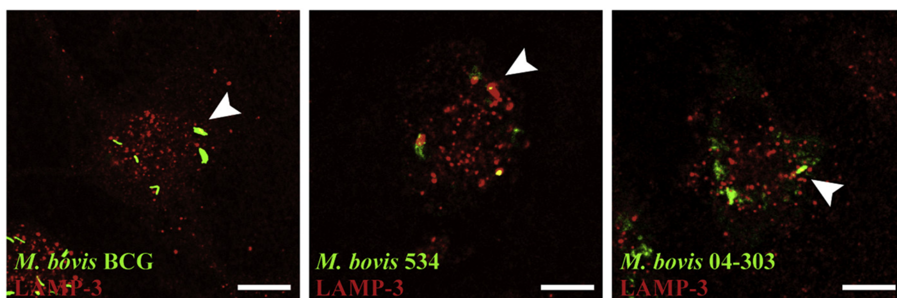
M. bovis strains were used to infect bovine macrophages for 2 h of uptake and 72 h of chase, as described in Materials and Methods. Consistent with our presumption, the LAMP-3 (the late endocytic marker lysosomal-associated membrane protein 3) association with the 04-303 was significantly lower than with the 534 strain (Fig. 4B). These results indicate that the 534 strain has a reduced ability to avoid antimicrobial mechanisms of macrophages and therefore it cannot persist inside bovine macrophages. As expected, the number of CFU in macrophages infected with strain 04-303 was significantly higher than that of macrophages infected with either strain 534 or BCG at 72 h of infection (Fig. 4A). These results are in line with our previous findings (Blanco et al., 2009).

To evaluate the impact of ESAT-6 polymorphism on the intracellular persistence of *M. bovis*, we overexpressed the mutant (ESAT-6- 04-303) and wild type (ESAT-6-534) variants of ESAT-6 in the 534 strain from plasmid pVV16. The introduction of the mutant allele of *esat-6* in 534 did not affect the intracellular localization of the strain in late endosomes (Supplementary material 2E-i). This result suggests that the mutation of ESAT-6 does not improve the phagosomal escape of pathogenic mycobacteria. In addition, we detected equivalent intracellular persistence of both recombinant 534 strains at 48 and 72 h post infection of bovine macrophages (Supplementary material 2E-ii). Altogether these results indicate that, at least with the ex vivo model here evaluated, the mutation in ESAT-6 does not seem to contribute to exacerbate the virulence phenotype of strain 04-303.

4. Conclusions

In this study, we used a genomic approach to predict the molecular bases that can explain the extreme opposite phenotypes in terms of virulence of two genetically close *M. bovis* strains. Most of the NS-mutations in virulence factors mapped in proteins related to transport or metabolism of lipids. This finding reinforces the role of these





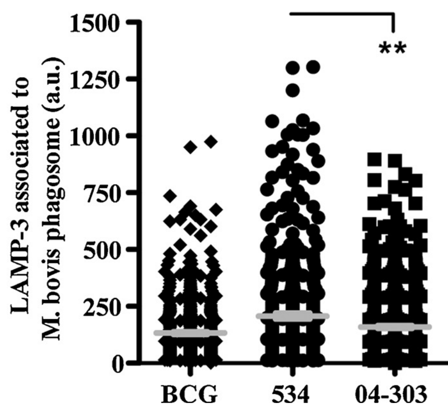


Fig. 4. Intracellular trafficking of *M. bovis* strains in bovine macrophages. The PBMC-derived bovine macrophages were infected with the different *M. bovis* strains for 2 h of uptake and 72 h of chase. (A) Intracellular survival of *M. bovis* strains in bovine macrophages. Macrophages were infected with *M. bovis* strains at MOI: 2. Colony forming units were counted 3 h post infection (to study inoculum possible variations between strains) and at 72 h (**) $p \leq 0.01$ ANOVA with Bonferroni post test. (B) Cells were fixed, subjected to indirect immunofluorescence using an antibody against *Mycobacterium* (green) and an antibody against LAMP-3 (red). Then cells were analysed by confocal microscopy (B-i). The head arrows in merge images show the mycobacterial phagosomes. Scale bars: 10 µm. (B-ii) Quantitation of LAMP-3 associated with the different *M. bovis* strain phagosomes. Data represent the Mean ± S.E.M. of three independent experiments. Asterisks show significance (***) $p \leq 0.001$, (**) $p \leq 0.01$ and ns: no significative. More than 500 phagosomes were analysed. The data were analysed using two-tailed Student's t-test and one-way ANOVA with Tukey's post hoc test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

compounds in the virulence of pathogenic mycobacteria. While most of the mutations in virulence proteins of 534 strain are conserved in a high number of *M. bovis* isolates, mutations in virulence proteins of 04-303 are mostly unique Thus, the 04-303 protein polymorphisms may contribute to the high virulence of this strain. However, experimental evidence is necessary to confirm this presumption.

Although we detected several proteins with significant changes between strains, such as premature stop, frameshift mutations and aa changes in conserved domains, the high number of NS-polymorphisms in enzymes that participate in PDIM metabolism in both strains is remarkable. This predictive analysis was then confirmed with experimental results and thus we demonstrated that the 534 strain has a dramatic defect in PDIM production. The lack of PDIM and the related lipids in the attenuated strain 534 is consistent with the role of these lipids in the virulence of *M. tuberculosis* and *M. bovis*.

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Declarations of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108482.

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