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A *Mycobacterium avium* subsp. *paratuberculosis* LuxR regulates cell envelope and virulence

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Mycobacterium avium subsp. *paratuberculosis* adapts to the environment via the regulation of genes affecting its envelope's composition. Bacteria grown in milk (*in vivo* conditions) presented differences in the cell wall-associated lipids and in the expression of genes involved in lipid metabolism (FadE8, FadE6 and MAP1420) and host cell invasion (MAP1203, LprL). A different lipid profile was also observed in the envelope of intracellular bacteria after 1 h of infection. Intracellular bacteria showed up-regulation of a LuxR regulator which controls the envelope's composition by up-regulation of FadE8, MAP1420, MAP1203 and LprL and by down-regulation of pks12, mmpL2 and MAP2594. A LuxR-overexpressing strain with a lipid-deficient envelope phenotype, infected epithelial cells more efficiently than the wild-type bacteria; however, it was not more resistant than the wild-type strain to the action of bactericidal proteins. Here we show that LuxR regulates virulence determinants and is involved in mycobacteria adaptation to the host.

Keywords: Mycobacterium avium, LuxR, virulence, cell envelope

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) encounters many different environmental conditions and barriers during infection of ruminants. After being ingested, the bacteria must survive the acidity of the abomasum to reach the low-oxygen, hyperosmotic environment of the small intestine. To cause infection, the bacteria cross the intestinal epithelium and then grow inside of submucosal macrophages, where they overcome exposure to cationic antimicrobial peptides and nutrient deprivation. Although the complex cell wall of mycobacteria has long been thought to play a major role in virulence,[1] there is a poor understanding of the mechanisms controlling the cell envelope's composition when pathogenic mycobacteria experience sudden changes in their environment. Recent data indicated that some enteric bacteria use quorum sensing mechanisms including the LuxR–LuxI quorum sensing system, the LuxS/AI-2 system, and the AI-3 epinephrine/norepinephrine system to recognize signals present in the environment.[2] The common theme in all the different LuxR–LuxI systems is that the activated LuxR protein regulates the transcription of different traits that enable bacteria to establish infection in their host, including motility, biofilm formation and virulence-specific genes.[3,4] *Mycobacterium avium* subsp. *paratuberculosis* significantly up-regulates a *luxR* homologue gene when exposed to cow's milk, suggesting the possibility that the bacterium responds to environmental cues with consequent change in phenotype. Because many other genes up-regulated in the presence of milk encode for enzymes involved in lipid degradation and lipid synthesis,[5] we hypothesized that environments in the host might trigger a bacterial adaptation by altering

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the cell wall. In the present study, we characterize the role of a *Mycobacterium avium* subsp. *paratuberculosis luxR* gene, its effects on gene transcription and the consequences of the expression of the regulated genes.

Materials and Methods

Cell lines

A bovine epithelial cell line, Madin-Darby bovine kidney (MDBK), was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained on Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-products, Woodland, CA, USA) as described before.[5] A bovine mammary gland epithelial cell line (MAC-T) was kindly provided by Lewis Sheffield (Department of Dairy Science, University of Wisconsin, WI, USA). MAC-T cells were grown in DMEM with 10% FBS, 5 μg/ml insulin, and 1 μg/ml hydrocortisone. A bovine macrophage cell line (BOMAC) was obtained from Judith Stabel (USDA, Ames, IA, USA) and maintained as described previously in Stabel and Stabel.[6]

Bacteria

Mycobacterium avium subsp. *paratuberculosis* strain K-10 is a bovine isolate from Nebraska that was provided by Vivek Kapur (University of Minnesota, MN, USA). The bacteria were grown at 37°C on either modified Middlebrook 7H9 broth or Middlebrook 7H11 agar (Difco Laboratories, Sparks, MD, USA), supplemented with 2 mg/l Mycobactin J (Allied Monitor, Fayette, MO, USA), 10% (v/v) oleic acid–albumin–dextrose–catalase (OADC; Hardy Diagnostics, Santa Maria, CA, USA), and 0.05% Tween 80 (Sigma, St Louis, MO, USA).

Exposure of M. avium *subsp.* paratuberculosis *to raw milk*

Approximately 10^7 colony forming units (CFUs) of *Mycobacterium avium* subsp. *paratuberculosis* cells were cultured in Middlebrook 7H9 broth; after the culture reached the turbidity equivalent (McFarland standard) to 5×10^8 organisms/ml, it was centrifuged, split and incubated in 25 ml of raw milk or Middlebrook 7H9 broth containing polymyxin B (5.5 mg/l), amphotericin (11 mg/l), carbenicillin (25 mg/l) and trimethoprim (2.5 mg/l). After 48 h at 37°C, the bacteria were recovered by centrifugation at 3500 *g* for 30 min at 4°C.

Invasion assay

Inocula for invasion assays were prepared as follows. *Mycobacterium avium* subsp. *paratuberculosis* cells were recovered from Middlebrook 7H11 agar and suspended in HBSS, diluted to match McFarland standard 1 (approximately 10^8 CFUs/ml). The suspension was passed 10 times through a 26-gauge needle, and large aggregates were allowed to settle. After 5 min, an aliquot was taken from the top half of the bacterial suspension and diluted in HBSS to 10^7 CFUs/ml. An aliquot of this suspension (100 μl) was used to infect 10^5 MDBK cells/ml growing in 24-well tissue culture plates (Costar, Pleasanton, CA, USA) and the plate was incubated at 37°C in a 5% CO_2 incubator for 4 h. The cell monolayers were then washed three times with HBSS, and then treated with 1 ml of tissue culture medium supplemented with 200 μg/ml of amikacin for 2 h at 37°C to kill extracellular bacteria. Following treatment, the monolayers were washed twice with HBSS, and the viable intracellular bacteria were released by incubation with 0.5 ml of 0.1% Triton X-100 (Sigma) in sterile water for 10 min. Subsequently, 0.5 ml of Middlebrook 7H9 broth was added to each well, and the cells were disrupted by vigorous pipetting. Lysates were collected and the number of viable intracellular bacteria was determined by plating for CFUs onto Middlebrook 7H11 agar containing Mycobactin J. The percentage of invasion was calculated as the percentage of the inoculated bacteria that was recovered from the cell lysate.

For the lipid extraction assays, confluent monolayers of MDBK, MAC-T and BOMAC cells grown in 125-cm^2 cell culture flasks (Corning Costar, New York, NY, USA) were infected with the wild-type strain at 37°C (multiplicity of infection (MOI) = 100). At 4-h post-infection, monolayers were washed twice with Hank's balanced salt solution (HBSS) and then treated with 200 μg/ml amikacin (Sigma) for 2 h at 37°C to kill extracellular bacteria. The supernatant was removed and fresh culture medium was added to the monolayers. Intracellular bacteria were recovered at different times after the infection by lysing the monolayers with sterile water. Cell debris and nuclear fraction were removed by low-speed centrifugation at 400 *g* for 5 min at 4°C. The bacterial pellet was recovered from the supernatant after additional centrifugation at 3000 *g* for 15 min.

Extraction of cell wall-associated lipids

Total lipids were extracted from lyophilized bacteria with chloroform/methanol (2:1; 30 ml/g dried cells) at 55°C for 3 h as previously described.[7] The cell suspension was centrifuged (2400 *g*, 5 min) and the supernatant was collected, dried under nitrogen and subjected to

Folch wash with 6 ml of chloroform/methanol (2:1) and 1 ml of water. After centrifugation (2400 *g*, 5 min) the organic layer was transferred to a new preweighed tube and dried under nitrogen. For further analysis, total lipids were resuspended in chloroform/methanol (2:1) at a concentration of 10 mg/ml and then separated by two-dimensional thin layer chromatography (2D-TLC) on aluminum-backed silica 60 F_{254} gel plates (EMD Chemicals, Gibbstown, NJ, USA) using different solvent systems spanning the whole range of polarity (Table 3). Lipids were visualized by spraying plates with 10% copper sulfate in 8% phosphoric acid followed by heating at 110°C until spots appeared.

Mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was performed at the Macromolecular Resources Facility, Colorado State University, with an Ultraflex MALDI/TOF/TOF (Bruker Daltonics, Billerica, MA, USA) using 2,5-dihydrobenzoic acid (DHB) as a matrix and NaI to ionize the lipids.

Isolation of RNA

Bacterial pellets were mixed with 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was isolated by rapid mechanical agitation in a bead beater as previously described.[8] To remove bacterial debris, cells were centrifuged at 13,000 *g* for 5 min at 4°C. The supernatant was removed and added to a 2-ml Phase Lock Gel Heavy (Eppendorf, Hamburg, Germany) containing 300 µl of chloroform–isoamyl alcohol (24:1). Samples were centrifuged at 4000 *g* for 10 min at 4°C, and the aqueous layer was collected, extracted with the same volume of phenol–chloroform and precipitated with isopropanol. The pellet was then washed with 75% ethanol and dried at 20–22°C for 10 min. RNA samples were treated with DNase I (Clontech, Palo Alto, CA, USA) for 1 h at 37°C, followed by precipitation with ethanol. Total RNA was quantified by measuring absorbance at 260 nm, and quality was determined by measuring the 260/280 nm absorbance ratio. Ratios of ≥ 1.8 were considered of acceptable quality. RNA was then electrophoresed on a 1% denaturing agarose gel to confirm quality.

Synthesis of DNA

Total RNA was reverse transcribed using the SuperScript First-Strand synthesis system for real-time (RT) PCR following the manufacturer's instructions (Invitrogen). Total RNA (4 µg) was incubated with 1 µl of a 10 mM concentration of deoxynucleotide triphosphate mix and 1 µl of random hexamers at 65°C for 5 min. Samples were then mixed with 2 µl of 10 × RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M dithiothreitol (DTT), 1 µl of RNase out Recombinant Ribonuclease Inhibitor (40 U/µl) and incubated at 25°C for 2 min. Then, 1 µl of SuperScript II reverse transcriptase (50 U/µl) was added to each tube, and the samples were incubated at 42°C for 50 min. The reaction was terminated at 70°C for 15 min. The tube was placed on ice, centrifuged briefly, and 1 µl of RNase H was added to each sample and incubated for 20 min at 37°C.

Quantitative real-time RT-PCR

Quantitative fluorogenic amplification of cDNA was performed using iCycler real-time detection system and SYBR green technology (Bio-Rad, Hercules, CA, USA) as previously described.[9] An RNA sample that had not been reverse transcribed was included in all experiments to exclude significant DNA contamination. Triplicate PCR reactions were carried out in 50 µl reactions consisting of 25 µl IQ SYBR Green Supermix, 1 µl of each primer (10 µM), 1 µl cDNA, and 23 µl water. The primers for the amplification of the FadE8 gene were 5′-CGAGGTGGGGGCGTTGTC-3′ (forward) and 5′-GCGGCAGCAGGAAGCAAG-3′ (reverse). For the amplification of the MAP0482 gene the following primers were used: 5′-GACGCGCACCTGCTGG-3′ (forward) and 5′-CAGCGTGCTCTCGATCACCTC-3′ (reverse). For the amplification of the 16S ribosomal RNA, primers 5′-CGAACGGGTGAGTAACACG-3′ (forward) and 5′-TGCACACAGGCCACAAGGGA-3′ (reverse) were used (Table 4). For all primers, the following temperature cycling profile for PCR was used: 95°C for 30 s, 62°C for 30 s, and 72°C for 2 min for 35 cycles with a final extension for 10 min at 72°C. An RNA sample that had not been reverse transcribed was included in all experiments to exclude significant DNA contamination. Real-time PCR efficiency was determined using a dilution series of cDNA template with a fixed concentration of primers. Slopes calculated by the LightCycler software were used with the following formula to calculate efficiency:

$$\text{Efficiency} = 10^{(-1/\text{slope})} \quad (1)$$

The results of the real-time RT-PCR amplifications is expressed as a threshold cycle (Ct), defined as the number of reaction cycles at which the reporter fluorescence reaches 10 times the standard deviation of the baseline. The expression level of each gene was normalized using 16S RNA as the house-keeping gene.

To determine fold change in gene expression, the following formula was used:

$$\text{Fold change} = 2^{-\Delta(\Delta Ct)} \quad (2)$$

where $\Delta Ct = Ct$ (target) – Ct (16S) and $\Delta(\Delta Ct) = \Delta Ct$ (experimental) – ΔCt (control). Standard deviations were calculated for the samples.

Microarray

A whole genomic microarray. containing 70-mer oligonucleotides representing the entire MAP strain K-10 coding sequences was provided by the National Animal Disease Center (Ames, IA, USA). Each ORF is represented in triplicate on the array, including intergenic regions. The array also includes probes for *M. avium* strain 104 that are not found in MAP strain K-10. RNA was prepared as described above, and cDNA synthesis of the experimental and control RNAs, labeling with Cy-3 and Cy-5 and hybridization was performed using the Genisphere 3DNA Array 900MPX kit following the manufacturer's instructions (Genisphere, Hatfield, PA, USA). The hybridized slides were scanned using a ScanArray 4000 (Perkin Elmer, Waltham, MA, USA) at the Center of Gene Research and Biotechnology, Oregon State University. Microarray red and green log intensities were defined and quantified using the QuantArray analysis software (Packard Bioscience, Billerica, MA, USA) and the BASE system. Six hybridization intensity values from two independent RNA preparations were used for statistical analysis. The median value from the raw data was LOWESS normalized and expressed as ± SEM, and the log induction ratios were calculated using BRB array tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>). The false discovery rate was also calculated (q value). To determine whether the differences in gene expression were biologically significant, a Student's *t*-test was also used. Values of $P < 0.05$ were considered to be significant.

Overexpression of the MAP0482 gene

The MAP0482 gene (LuxR gene) was PCR amplified from *M. avium* subsp. *paratuberculosis* using the following primers: 5′-CCCAAGCTTGTGATCGGCGGCGAGCTGCT-3′ (reverse) and 5′-TTTGAATTCCTAGCTTTCGCGG CCGGCCG-3′ (forward), containing *Hind*III and *Eco*RI restriction sites. Polymerase chain reaction amplification was carried out using the GC Rich Kit (Roche) and the PCR cycle parameters were 95°C for 30 s, 56°C for 1 min, and 72°C for 2 min for 35 cycles with a final extension of 72°C for 10 min. The PCR product was digested with *Eco*RI and *Hind*III and cloned downstream of the strong mycobacterial promoter G13 in pLDG13. The insert was sequenced and the resulting construct was electroporated into the MAP strain K-10 as previously described.[5] Briefly, the optical density at 600 nm of cultures of *M. avium* subsp. *paratuberculosis* was measured and determined to correspond to approximately 5×10^8 bacteria/ml. The bacteria were incubated on ice for 1.5 h, harvested by centrifugation, and washed three times with cold 10% glycerol plus 0.1% Tween 80. Pellets were resuspended in 1 ml 10% glycerol plus 0.1% Tween 80. For electroporation, 200 µl of cells were combined with 1 µg of plasmid DNA and transferred to a prechilled 0.2-cm cuvette. Electroporation was carried out using a Gene pulser (Bio-Rad, Hercules, CA, USA) at the following settings: 25 µF, 1000 Ω, 2.5 kV. The transformants were recovered with 1 ml of Middlebrook 7H9 broth, plated on Middlebrook 7H11 agar containing 500 µg/ml kanamycin and screened by PCR amplification of the kanamycin gene using 5′-ATGCCTCTTCCGACCATCAAG-3′ as reverse and 5′-CGCTCGTCATCAAAATCACTCG-3′ as forward primers.

Susceptibility in vitro *to antimicrobial peptides*

The MIC MAP-WT versus MAP-pLDG13-LuxR:polymyxin B sulfate salt, amikacin, lysozyme from egg white, mastoparan from *Vespula lewisii* were purchased from Sigma. Bacteria were grown in a modified Middlebrook 7H9 broth (Difco), supplemented with 2 mg/ml Mycobactin J (Allied Monitor), 10% (v/v) of oleic acid–albumin–dextrose–catalase (OADC; Hardy Diagnostics), 0.2% (v/v) glycerol (Sigma) and 0.05% (v/v) Tween 80 (Sigma). For MAP-pLDG13-LuxR cultures, the media was further supplemented with 400 µg/ml kanamycin (USB Corp).

Bacteria in mid-log growth phase were harvested by centrifugation at 3500 *g* for 30 min at 4°C. Bacterial suspensions and dilutions were prepared as described above, using modified Middlebrook 7H9 broth. For the LuxR-overexpressing strain, the medium was further supplemented with 400 µg/ml kanamycin. The final concentration of bacteria in the assay was 10^5 CFUs/ml. The minimal inhibitory concentrations (MICs) of the antimicrobial reagents were determined by serial 2-fold dilutions in 2 ml modified Middlebrook 7H9 broth, supplemented with 400 µg/ml kanamycin for the LuxR-overexpressing strain. The range for polymyxin B and mastoparan was 128 µg/ml to 1 µg/ml, and for lysozyme and kanamycin 200 µg/ml to 3.125 µg/ml. Cultures were incubated at 37°C with agitation for

3–4 weeks to determine bacterial growth. Media without antimicrobial reagent was inoculated with bacteria to serve as growth control.

luxR *expression upon exposure to antimicrobial peptides*

Approximately 10^7 CFUs of *M. avium* subsp. *paratuberculosis* cells were cultured in 20 ml of Middlebrook 7H9 broth and incubated at 37°C under continuous agitation. Bacteria in mid-log growth phase were exposed to the selected agents (16 μg/ml and 32 μg/ml of polymyxin B, 200 μg/ml of lysozyme, or 32 μg/ml of mastoparan). After 4 h at 37°C, the bacteria were recovered by centrifugation at 3500 *g* for 15 min at 4°C. Unexposed wild-type and *luxR*-overexpressing bacteria served as control. RNA extraction and cDNA conversion of 2 μg RNA for quantitative RT-PCR analysis was performed as described above. A primer set MAP 0482-1/MAP 0482-2 was used to amplify the internal segment of the ATPase binding domain containing *luxR* gene. For PCR amplification, the internal segment of the DNA finding domain containing *luxR* gene (MAP 0483) 5′-CCGGCTGGTGCAGGAAG-3′ as forward and 5′-AGACCGGTGTGCATGAACAGG–3′ as reverse primers were used. Normalization of gene expression was achieved by using the primers 16S-3 5′-cGAGTAACACGTGGGCAATCTG-3′ as forward and 16S-4 5′-GTCTGGGCCGTATCTCA-3′ as reverse. Quantitative real-time RT-PCR analysis was performed as described above.

Statistical analysis

The results of experiments (except for the DNA microarray) shown represent the mean ± SD of at least three experiments. The data were analyzed statistically by comparison with controls at the same time points using the Student's *t*-test or ANOVA. $P < 0.05$ was considered statistically significant.

Results and Discussion

Comparative lipidomics of MAP grown in Middlebrook 7H9 broth or incubated in milk

We previously showed that the exposure of *M. avium* subsp. *paratuberculosis* to conditions that mimic the host's environment, such as cow milk, enhanced its subsequent ability to infect MDBK cells.[5] To assess whether the composition of the bacterial envelope changes when the bacterium is exposed to raw milk, the lipids extracted from the envelope of the MAP K-10 strain grown in Middlebrook 7H9 broth or milk were analyzed by 2D-TLC in different solvent systems. Within the polar system A, the three major lipids identified in the strain K-10 when incubated in Middlebrook 7H9 broth (WC-A-01, WC-A-02, WC-A-03) were absent from the bacteria when incubated in milk (Fig. 1A,D). When using the non-polar system E, two lipids (Para-LP-01 and Para-LP-02) were observed in the lipidome of the wild-type strain when grown in Middlebrook 7H9 broth (Fig. 1B), whereas a new lipid (Lipid-550) was identified when grown in milk (Fig. 1E). The lipid Para-LP-01, a major cell wall-associated non-polar lipopeptide in MAP,[7] was only detected when the wild-type strain was grown in culture media but could not be detected within the lipidome of the wild-type bacteria when incubated in milk. System C showed also significant differences in the envelope of the wild-type bacteria grown in 7H9 Middlebrook broth and milk, with disappearance of WC-C-02 and appearance of WC-C-03 when incubated in milk (Fig. 1C,F). These data, together with our observation that *M. avium* subsp. *paratuberculosis* grown in milk has an increased ability to infect bovine epithelial cells, suggest that the modifications in the cell wall-associated lipids lead to changes in surface composition and might result in change in hydrophobicity, with consequent increased interaction with mucosal epithelial cells.

M. avium *subsp.* paratuberculosis *grown in milk up-regulates the expression of genes involved in lipid metabolism and host cell invasion*

As shown in Table 1, microarray analysis of gene expression of *M. avium* subsp. *paratuberculosis* grown in milk for 48 h revealed 18 genes with transcription levels at least 2.6-fold greater than in the bacteria grown in Middlebrook 7H9 broth ($p < 0.05$). Some of the up-regulated genes encoded for proteins involved in fatty acid metabolism, such as two fatty acyl-CoA dehydrogenases (MAP4133 [FadE8] and MAP0716 [FadE6]) and a non-ribosomal peptide synthetase (MAP1420 [NRPS]). The protein MAP4133 exhibits 87% of sequence identity with *M. tuberculosis* Rv0672 protein, a probable acyl-CoA dehydrogenase (FadE8) thought to be involved in fatty acid degradation by oxidation of fatty acyl-CoA esters.[10] The significant increase in the expression of the FadE8 gene, a 30-fold level of expression compared with bacteria grown in Middlebrook 7H9 broth, suggests that this gene might be responsible, at least in part, for the breakdown of some lipids within the cell wall of the milk-exposed bacteria. These changes in the envelope could affect the ability of the bacteria subsequently to infect host cells. Recently, two acyl-CoA dehydrogenases (FadE28 and FadE29)

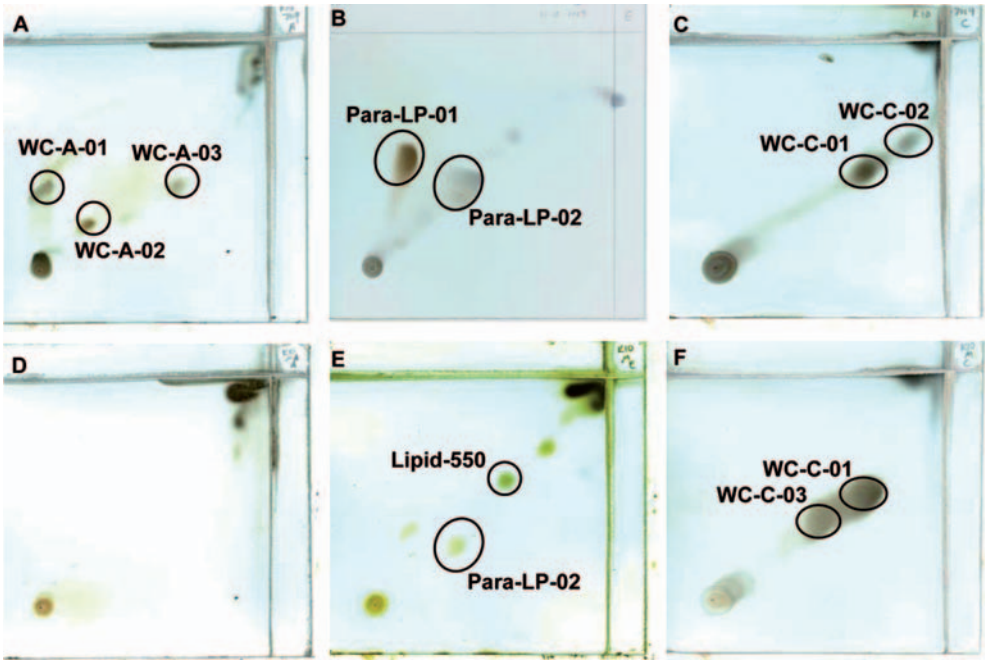

Fig. 1. Comparative lipidomics of *M. avium* subsp. *paratuberculosis* grown in Middlebrook 7H9 broth or milk. Lipidomics of MAP grown in Middlebrook 7H9 broth (A–C) or incubated in milk (D–F) in the polar system A (A,D), in the non-polar system E (B,E) and in the highly apolar system C (C,F).

Table 1. Genes up-regulated in *M. avium* subsp. *paratuberculosis* exposed to milk for 48 h

Gene name	Fold	*P*-value	Rv-homologue	Function
MAP4133 FadE8*	30.60	0.004	Rv672 – acyl-CoA-dehydrogenase	Lipid degradation
MAP1203*	28.43	0.0002	Rv1477 – hypothetical invasion protein	Involved in virulence
MAP1421 AsnB	21.21	0.004	Rv2201 – asparagine synthetase AsnB	Amino acid transport and metabolism
MAP4296c	20.15	0.031	Rv3542c – conserved hypothetical protein	
MAP1420*	17.08	0.002	Rv0101 – peptide synthetase NRPS	Lipid metabolism – conserved hypothetical protein
MAP0405c	11.24	0.015	Rv3669 – transmembrane protein	
MAP2691c	12.64	0.003	Rv1100 – conserved hypothetical protein	
MAP3311c	8.80	0.001	Rv3210c – conserved hypothetical protein	
MAP4088 LprL*	5.39	0.011	Rv0593 – lipoprotein MCE2E	Host cell invasion
MAP1721c	4	0.013	Rv3208 – transcriptional regulator (TETR)	Involved in transcriptional mechanism
MAP1620	3.19	0.031	Rv1393c – probable monooxygenase	Unknown
MAP1103c	3	0.007	Rv0263c – conserved hypothetical protein	
MAP1005	2.96	0.029	Rv0987 – ABC transporter	Transport
MAP2767c	2.85	0.008	Rv2680 – conserved hypothetical protein	
MAP0891c pgi	2.83	0.006	Rv0946c – probable glucose-6-P isomerase	Glycolysis and gluconeogenesis
MAP3500c	2.76	0.025	Rv0310c – conserved hypothetical protein	
MAP0716c FadE6*	2.68	0.008	Rv3560c – acyl-CoA-dehydrogenase	Lipid degradation

*Genes associated with lipid metabolism or invasion of host cells.

have also been shown to be involved in lipid metabolism and to be particularly important for intracellular growth in macrophages and for the early steps of *M. tuberculosis* infection.[11] Up-regulation of MAP1420 mRNA was also observed in *M. avium* subsp. *paratuberculosis* grown in milk. The MAP1420 gene encodes a non-ribosomal peptide synthase (NRPS) with an *N*-methylation and a fatty acid condensation motif, strongly indicating its involvement in the biosynthesis of *N*-methylated lipopentapeptides, such as Para-LP-01.[7] Although a

significant reduction in the cell wall-associated lipids was observed after exposing the bacteria to milk, probably as the result of FadE8-mediated degradation, the bacteria might produce compensatory changes in the synthesis of alternative lipid classes through the action of cell wall biosynthetic enzymes. In this regard, a new non-polar lipid (Lipid-550) detected in the envelope of the bacteria grown in milk, might functionally replace the missing lipids, such as Para-LP-01. Therefore, we can speculate that a cross-talk between the FADE8 and the NRPS metabolic pathways might exist, most likely controlled by a common transcriptional regulator. Other genes up-regulated within the bacteria when grown in milk encoded hypothetical proteins associated with virulence such as MAP1203 and MAP4088 (LprL). The MAP1203 gene encodes a hypothetical invasion and intracellular persistence protein (*iipA*) which contains an N-terminal signal peptide for protein secretion and a highly conserved C-terminal NLPC_p60 domain found in many bacterial pathogens. Although the function of this domain is not completely understood, some members of this family of proteins have been shown to act as murein hydrolases or peptidoglycanases involved in cell wall re-assessment by cleavage of peptide linkages within the peptidoglycan.[12] Examination of the NLPC_60 domain of MAP1203 revealed an RGD (Arg–Gly–Asp) motif that is highly conserved among homologues in all mycobacteria. The RGD sequence is well known to mediate binding of cell wall-associated proteins to cell surface integrin receptors.[13,14] This RGD motif is also required both for macrophage uptake and for intracellular growth of the iipA homologues in *M. marinum*,[15] *M. tuberculosis* (Rv1477),[16] and in *M. avium*.[17] The other invasin-like protein up-regulated in *M. avium* subsp. *paratuberculosis* grown in milk, MAP4088, encodes a protein homologous to the mammalian cell entry lipoprotein (Mce2E) of *M. tuberculosis* which contains a putative export signal sequences at the N-terminal end and is most likely located at the mycobacterial cell surface.[18] Other genes that were also up-regulated during growth in milk included those encoding for two transporters, a DGTPase, a TetR transcriptional regulator and several hypothetical proteins of unknown function.

M. avium *subsp.* paratuberculosis *lipid profile within bovine cells*

We previously demonstrated that pathogenic mycobacteria, including *M. avium* subsp. *paratuberculosis*, develop an invasive phenotype once inside the host cell which is associated with increased efficiency to invade other host cells.[5] To evaluate whether the intracellular environment influences the cell wall composition of *M. avium* subsp. *paratuberculosis*, bovine mammary gland epithelial cell line (MAC-T), MDBK and BOMAC cells were infected with the MAP K-10 strain for 4 days. Cells were lysed and the surface-lipids from the intracellular bacteria were analyzed by 2D-TLC in a variety of solvent systems (Fig. 2). Significant qualitative and quantitative differences were observed in the lipid profiles of intracellular bacteria recovered from the three cell lines when compared to the lipidome of bacteria grown in Middlebrook 7H9 broth. In the polar system A, the WC-A-01 and WC-A-03 lipids were missing in intracellular bacteria when compared with the bacteria grown in Middlebrook 7H9 broth (Fig. 2A). In the non-polar system E, the lipopeptide Para-LP-01 was barely visible in MAP grown inside of MAC-T and BOMAC cells and undetectable in the bacteria recovered from MDBK cells, while the intensity of Para-LP-02 was increased. The non-polar Lipid-550 and WC-E-03 were new major lipids only detected in the cell wall of the intracellular bacteria (Fig. 2B). System B showed minor changes in the synthesis of strong polar lipids, such as PIMs, between intracellular bacteria and bacteria grown in culture media (Fig. 2C). The observed differences of the lipids extracted from bacteria grown in Middlebrook 7H9 broth and bacteria recovered from MDBK cells after 4 days of infection were also seen in the mass spectrometry analyses performed by MALDI-TOF MS (Fig. 2D). To assess whether the changes in lipid composition occurred soon after infection, BOMAC cells were infected with the *M. avium* subsp. *paratuberculosis* K-10 strain for 1 h and the lipids of the intracellular bacteria as well as the lipids from extracellular bacteria were extracted and analyzed (Fig. 2E). No significant differences were observed between the lipids extracted from the extracellular bacteria and the bacteria grown in Middlebrook 7H9 broth, but a significant reduction of polar, highly polar and highly non-polar lipids was seen in the intracellular bacteria. In addition, two new non-polar lipids (Lipid-550 and WC-E-03) were observed upon host cell infection. Together, the results indicated that, after 1 h in the intracellular environment, the lipid composition of the bacterial envelope was significantly altered. The fact that the lipid profiles after 1 h and 4 d of infection were similar supports the idea that maintaining a particular composition of the mycobacterial cell wall might confer certain advantage in the host cell environment.

A LuxR-overexpressing strain with a lipid-deficient envelope phenotype, infected epithelial cells more efficiently than the wild-type bacteria

Previously, we had shown that the mRNA levels of MAP0482 gene were strongly up-regulated in

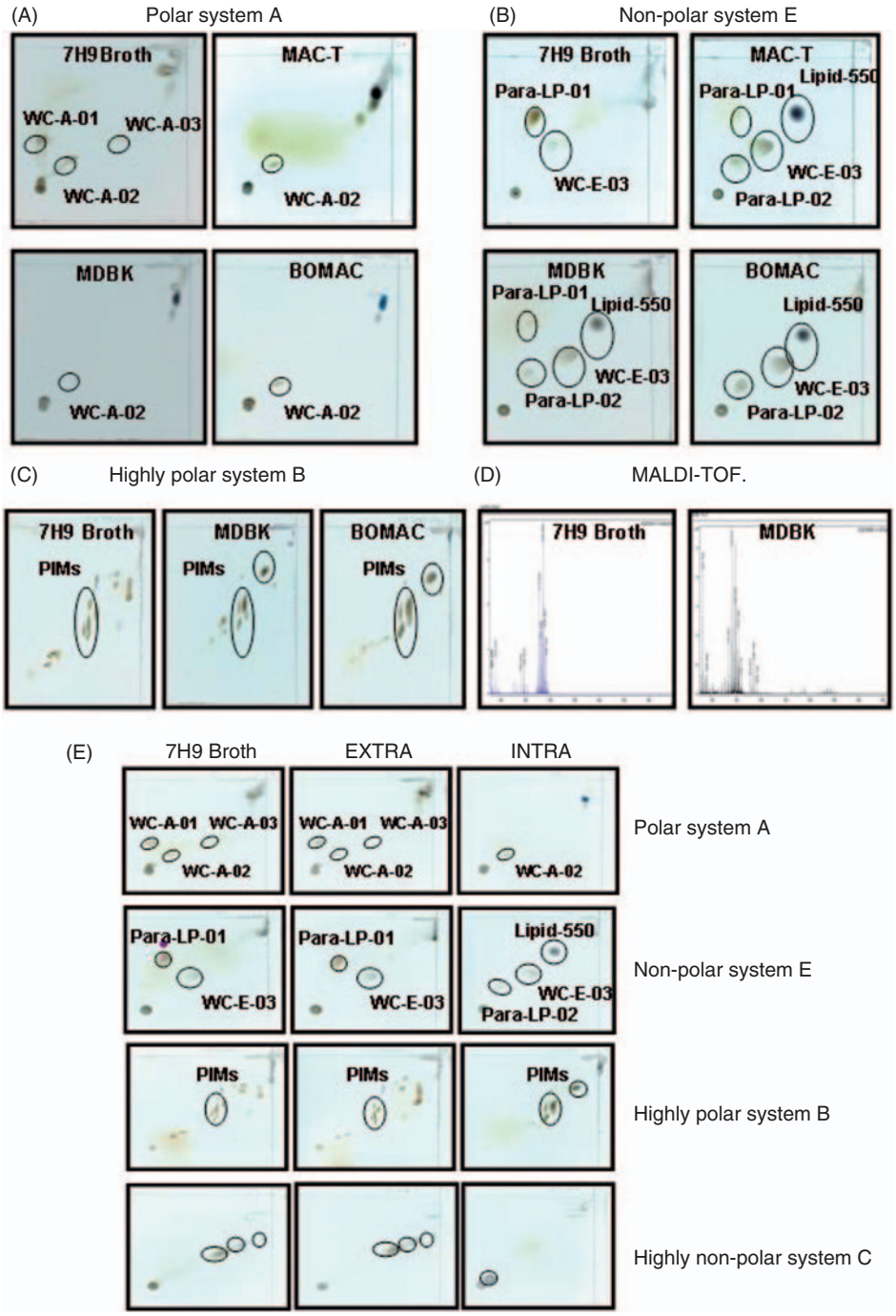

Fig. 2. Growth of *M. avium* subsp. *paratuberculosis* inside bovine cells alters the cell wall-associated lipid profile. (A) Comparative lipidomics of MAP incubated in MAC-T, MDBK and BOMAC for 4 d versus bacteria grown in Middlebrook 7H9 broth using the polar system A. (B) Cell wall-associated lipids separated within the non-polar system E. (C) Cell wall-associated lipids separated with the highly polar system B. (D) MALDI-TOF MS analysis of the total lipids extracted from bacteria recovered from MDBK cells after 4 d of infection. Bacteria grown in Middlebrook 7H9 broth were used as control. (E) Comparative lipidomics of extracellular and intracellular MAP recovered from BOMAC cells after 1 h of infection versus wild-type bacteria grown in Middlebrook 7H9 broth.

intracellular bacteria after 24 h of infection of MAC-T cells.[5] The MAP0482 gene is homologous to the LuxR transcriptional regulator of *M. avium* (99% identity) and encodes a protein of 499 amino acids with an ATPase domain. To determine whether MAP0482 was also up-regulated in *M. avium* subsp. *paratuberculosis* during the intracellular stage in MDBK and BOMAC cell lines, real-time RT-PCR at 24-h post-infection was carried out. Real-time RT-PCR amplification demonstrated that the expression of MAP0482 mRNA was

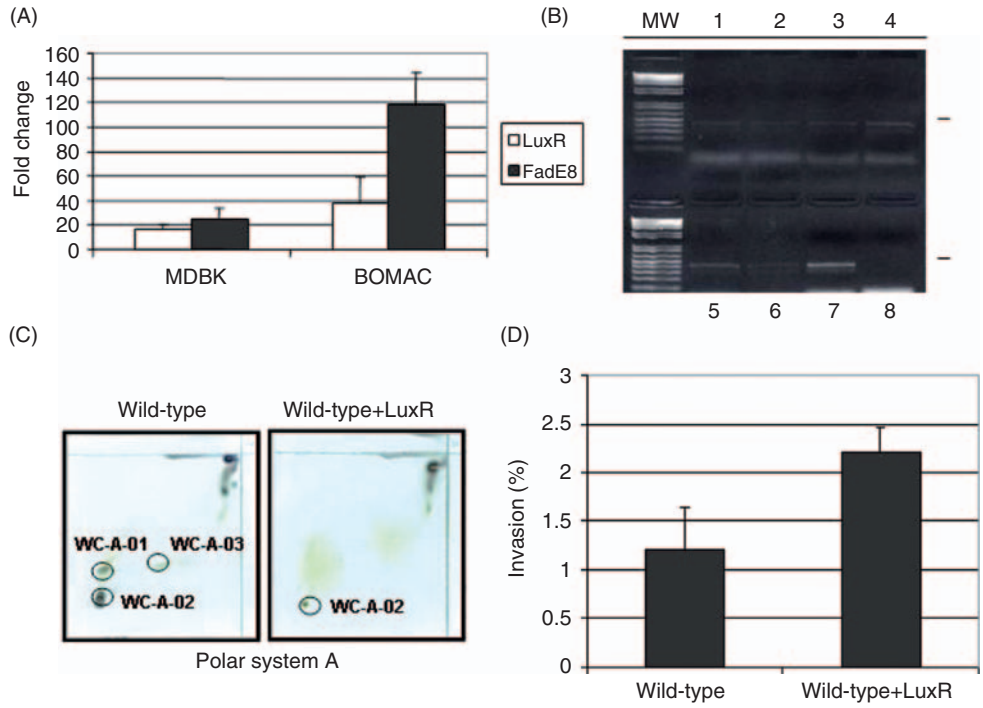

Fig. 3. MAP0482 gene regulates *M. avium* subsp. *paratuberculosis* lipid profile and is important for virulence. (A) The expression of LuxR (MAP0482) and FadE8 (MAP4133) genes in the wild-type bacterium after 24 h of infection of MDBK or BOMAC cells was examined by real-time RT-PCR. Wild-type bacteria grown in Middlebrook 7H9 broth were used as control. (B) Overexpression of the MAP0482 gene in the wild-type bacteria was carried out. Seven transformants were selected by amplification of the kanamycin gene. Lane 8 is the PCR negative control. (C) Comparative lipidomics of the MAP wild-type strain versus the LuxR-overexpressing strain using the polar system A. (D) Invasion efficiency of the wild-type and the LuxR-overexpressing strain 4 h after infection of MDBK cells. Values represent the mean of three experiments and are ± SD. $P < 0.05$ compared with the invasion percentage for the wild-type bacteria.

20- and 40-fold higher in bacteria recovered from MDBK and BOMAC cells, respectively, when compared with bacteria grown *in vitro* (Fig. 3A). A recent report found that a LuxR-type transcriptional regulator in *Streptomyces coelicolor* positively controls the transcription of the *mas–fadD1* operon encoding two acyl-CoA synthetases that are required for the formation of membrane lipids.[19] To study directly whether the *M. avium* subsp. *paratuberculosis luxR* gene regulates the cell wall lipid composition, a recombinant strain overexpressing the *luxR* gene was generated. The coding sequence of the *luxR* gene was cloned into the pLDG13 plasmid downstream of the strong mycobacterial G13 promoter.[20] *Mycobacterium avium* subsp. *paratuberculosis* competent cells were transformed with the final construct by electroporation and plated on Middlebrook 7H11 plates containing kanamycin (Fig. 3B). A recombinant strain overexpressing the *luxR* gene was selected by growth in kanamycin and subsequent amplification of the kanamycin gene. The recombinant strain was further propagated in Middlebrook 7H9 broth containing kanamycin for the analysis of its lipidome by 2D-TLC. Comparative lipidomics revealed a similar lipid profile of the recombinant strain overexpressing the *luxR* gene in comparison to that of intracellularly grown bacteria. Specifically, both lipidomes contain the newly detected major non-polar lipids, Lipid-550 and Para-LP-02. In addition, no Para-LP-01, the dominant lipid of *in vitro* grown bacteria, could be detected. Furthermore, only one lipid, WC-A-02, could be identified within the polar system A, and WC-A-01 and WC-A-03 could not be detected (Fig. 3C). To test whether the altered surface properties of the LuxR-overexpressing bacteria were visible, the morphology of the LuxR-overexpressing strain and the wild-type strain grown in Middlebrook 7H9 broth were examined in detail by transmission electron microscopy. Examination of the negative-stained bacteria failed to reveal any surface differences between the two strains. The observation that the LuxR-overexpressing strain tended to clump in liquid medium suggested that the absence of some of its cell wall-associated lipids affect the surface properties of the bacterial cells and probably their ability to interact with host cells. Western blot analysis using an antibody against LuxR confirmed the overexpression of the protein. To assess further the role of LuxR in the pathogenicity of *M. avium* subsp. *paratuberculosis*, invasion assays to determine if the constitutive expression of the LuxR operon is associated with an

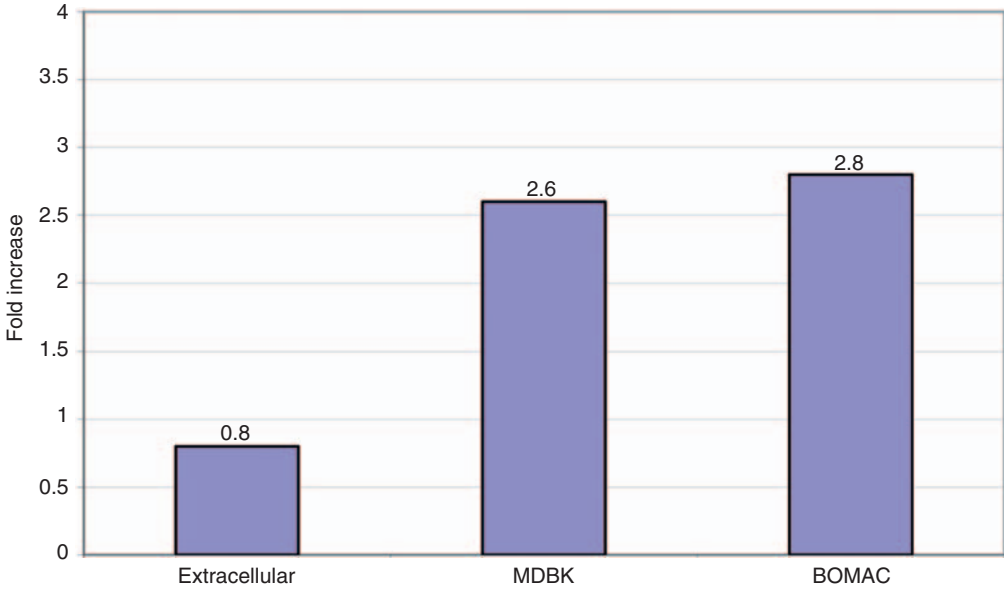

Fig. 4. Expression of luxR in intracellular *M. avium* subsp. *paratuberculosis*. MAP-infected BOMAC macrophages and MDBK cells and at 24 h cell monolayer was lysed and bacterial RNA obtained, as described in Material and Methods. As a control, extracellular bacteria had RNA obtained, as well. Real-time RT-PCR, using specific primers for MAC 0482, was performed.

increased ability to enter epithelial cells were carried out. As shown in Figure 3D, the LuxR-overexpressing strain infected MDBK cells with a 2-fold increase in efficiency compared to the wild-type bacteria containing the vector alone. This moderate, but significant, increase in the virulence of the LuxR-overexpressing strain suggests that the lack of some of its cell wall-associated lipids might account for the increased efficiency with which the LuxR-overexpressing strain binds to and enters non-phagocytic eukaryotic cells. Figure 4 shows that luxR is expressed in the intracellular environment at a level significantly greater than in the extracellular environment.

DNA-Microarray analysis of LuxR-regulated gene expression

To investigate whether the luxR gene controls cell envelope composition and virulence through transcriptional regulation, we compared the transcriptional profiles of the wild-type K-10 strain and the LuxR-overexpressing strain grown in Middlebrook 7H9 using a *M. avium* subsp. *paratuberculosis* whole-genome oligonucleotide array (Table 2). Sixteen genes that exhibited transcription levels at least 2.6-fold higher in the LuxR-overexpressing bacteria than in the wild-type bacteria were identified. We confirmed the up-regulation of some of these genes by quantitative real-time RT-PCR using the same samples as those used in the transcriptome analysis. Among the genes up-regulated in the LuxR-overexpressing strain, genes of unknown function were common (*i.e.* MAP4296c, MAP3821-3p, MAP3311c, MAP3535 and MAP1099) and are not shown in Table 2. Although some variation was observed in the rank order of the expression ratios, eight of the 16 genes that were predominantly expressed in the LuxR-overexpressing strain were also highly expressed in the wild-type strain in response to the milk environment, suggesting similar physiological adaptation. Genes involved in cell wall biosynthesis, lipid metabolism and invasion were the most commonly identified and their relevance has been described above. Interestingly, the MAP4133 (FadE8) gene encoding an acyl-CoA dehydrogenase was highly up-regulated in both arrays and also in the bacteria recovered from MDBK and BOMAC cells, suggesting that the protein might be associated with modifications of the cell wall-associated lipids (Fig. 3A). Additional genes, including a gene homologous to *M. tuberculosis* PPE31 protein, were also up-regulated by the *M. avium* subsp. *paratuberculosis* LuxR gene. It has been shown that certain PE-PGRS proteins influence the interaction of mycobacteria with the host cell but the specific functions of these proteins need to be further determined.[21,22] A significant number of genes were also repressed by the recombinant strain overexpressing the *luxR* gene, including some genes involved in lipid biosynthesis and transport such as MAP2230 (pks12, a polyketide synthetase), MAP2594 (a regulator of the polyketide synthetase) and MAP3049 (mmpL2, a fatty acid transporter). The left column of Table 2 shows the eight genes whose expression was significantly lower in the LuxR-overexpressing strain than in the wild-type strain. Our results demonstrated that, in response to an

Table 2. DNA-microarray analysis of LuxR-regulated gene expression

Gene name	Fold	*P*-value	Rv-homologue	Function
Genes up-regulated in the LuxR-overexpressing recombinant strain of *M. avium* subsp. *paratuberculosis* (MAP) relative to the wild-type MAP strain				
MAP4133 FadE8*	8.9	0.0007	Rv672 – acyl-CoA-dehydrogenase	Involved in lipid degradation
MAP1420	7.09	0.01	Rv0101 – non-ribosomal peptide synthetase	Lipid biosynthesis
MAP1469c	6.97	0.0003	Rv3121 – cytochrome P450 141	Catabolism, metabolism
MAP4088 LprL*	5.42	0.013	Rv0593 – lipoprotein MCE2E	Involved in host cell invasion
MAP1421 AsnB-1	4.05	0.017	Rv2201 – asparagine synthetase AsnB	Amino acid modification by amino transfer
MAP4029c MenA	3.44	0.027	Rv0534c – 1,4-dihydroxy-2-naphthoate octaprenyltransferase MenA	Metabolism
MAP1155	2.85	0.013	Rv1807 – PPE31 PPE family protein	Unknown
MAP2578	2.79	0.022	Rv1201c – probable transferase	Metabolism
MAP2435c	2.76	0.021	Rv1324 – possible thioredoxin	Post-translational modification, chaperone
MAP1203*	2.70	0.027	Rv1477 – hypothetical invasion protein	Unknown, supposed involvement in virulence
MAP0046c	2.66	0.034	Rv0039c – transmembrane protein	Unknown
Genes down-regulated in the LuxR-overexpressing recombinant strain vs the wild-type MAP strain				
MAP1834c prcA	0.39	0.0002	Rv2109c – pcrA proteasome	Protein degradation
MAP3049c	0.38	0.012	Rv0507 – transmembrane transport protein mmpL2	Unknown, thought to be involved in fatty acid transport
MAP0946c	0.40	0.032	Rv3328c – RNA polymerase sigma factor	SigJ, transcriptional regulator
MAP2230	0.42	0.01	Rv2048c – pks12, probable polyketide synthase	Lipid metabolism, involved in polyketide synthesis
MAP3609	0.46	0.02	Rv0174 – Mce 1F	Unknown, thought to be involved in host cell invasion
MAP2915c EfpA 2	0.49	0.013	Rv2846c – efflux protein A (cfpA)	Export
MAP1708	0.49	0.004	Rv3835 – conserved membrane protein	Unknown
MAP2594	0.51	0.04	Rv2594 – regulator of polyketide synthase	Transcriptional regulation

*Genes associated with lipid metabolism or invasion of host cells.

Table 3. The five 2D-TLC solvent systems (B, A, E, C, and D) used for lipid analyses (by volume) from polar to apolar

	First dimension	Second dimension
B	Chloroform/methanol/water (60:30:6)	Chloroform/acetic acid/methanol/water (40:25:3:6)
A	Chloroform/methanol/water (100:14:0.8)	Chloroform/acetone/methanol/water (50:60:2.5:3)
E	Chloroform/methanol (96:4)	Toluene/acetone (80:20)
C	Petroleum-ether/acetone (92:8)	Toluene/acetone (95:1)
D	Petroleum-ether/ethyl acetate (98:2)	Petroleum-ether/acetone (98:2)

unknown signal, the *luxR* gene functions as an activator of lipid degradation and lipid modification as well as a repressor of genes involved in lipid biosynthesis, thus co-ordinating fatty acid β-oxidation and synthesis in the bacterial envelope, which, in turn, modulates the lipid content and physical properties of the membrane. It also regulates the expression of genes encoding membrane-associated proteins, which increased activity, would then alter the surface characteristics of the mycobacterium and the ability of the bacteria to interact with the host cells. Based on the identity of the genes that are regulated by the *luxR* gene, and the response of this gene to the intracellular environment, our data suggest that the LuxR transcription factor plays an important role during the infection optimizing bacterial infection and survival. The LuxR regulator appeared to influence the expression of relatively few *M. avium* subsp. *paratuberculosis* genes and its effect does not appear to be mediated through control of many other regulators of transcription.

Table 4. Primers used in this study

Primer	Sequence
FadE8-1	5′-CGAGGTGGGGGCGTTGTC-3′
FadE8-2	5′-GCGGCAGCAGGAAGCAAG-3′
MAP0482-1	5′-GACGCGCACCTGCTGG-3′
MAP0482-2	5′-CAGCGTGCTCTCGATCACCTC-3′
MAP0482-3	5′-CCCAAGCTTGTGATCGGCGGCGAGCTGCT-3′
MAP0482-4	5′-TTTGAATTCCTAGCTTTCGCGGCCGGCCG-3′
16S-1	5′-CGAACGGGTGAGTAACACG-3′
16S-2	5′-TGCACACAGGCCACAAGGGA-3′

Effect of bactericidal proteins on MAP

Within the gastrointestinal tract, enteropathogenic bacteria are faced with a battery of host defense mechanisms. Bacterial tissue invasion is restricted by physical, chemical, and immunological barriers of the intestinal mucosa. The production and release of epithelial-derived antimicrobial peptides is one mode of action to combat invading pathogens at the level of innate immunity. Antimicrobial peptides target the negatively charged cell wall of bacteria leading to cytoplasm leakage and complete lysis of bacterial cells. The observed changes within the cell wall lipidome of the LuxR-overexpressing strain, compared to the wild-type strain grown in culture, might reflect a mechanism by which *M. avium* subsp. *paratuberculosis* organisms gain resistance to antimicrobial peptides by altering the cell wall composition. To test this hypothesis, we performed broth macrodilution assays to determine minimal inhibitory concentrations of polymyxin B, mastoparan, and lysozyme for the wild-type and LuxR-overexpressing bacteria. Growth of wild-type and LuxR-MAP was inhibited by polymyxin B at a concentration of 64 μg/ml growth media. However, it has to be noted that all concentrations higher than 34 μg/ml polymyxin B did not have a bactericidal effect on the bacteria, but a bacteriostatic effect. Bacterial growth of polymyxin B exposed wild-type and LuxR-MAP cultures on modified Middlebrook 7H11 could be observed for all tested polymyxin B concentrations (data not shown). In contrast to polymyxin B, all tested concentrations of mastoparan or lysozyme did not inhibit the growth of wild-type or LuxR-MAP. Results were confirmed in three independent experiments. Because the presence of antimicrobial peptides could regulate *luxR* in wild-type bacteria, we carried out an assay determining the *luxR* expression upon contact with antimicrobial peptides for 4 h. Real-time RT-PCR did not show that luxR expression increases with exposure. Future studies will evaluate the effect of a changed bacterial surface on the host immune system.

Conclusions

We have described the regulation of cell surface molecules that are likely to function in host–pathogen interactions by a mycobacterial LuxR transcriptional regulator. Moreover, our results link the LuxR regulator to changes in the *M. avium* subsp. *paratuberculosis* envelope and to an increase in the bacterium's ability to infect host epithelial cells. Our data show, however, that the changes do not confer increased resistance to antimicrobial peptides. Other possibilities, such as the interaction with other components of the immune system are being investigated.

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