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A replicative plasmid vector allows efficient complementation of pathogenic Leptospira strains

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Key words: Leptospira, replicative vector, genetic tools, complementation, PerR

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ABSTRACT

Leptospirosis, an emerging zoonotic disease, remains poorly understood due to a lack of genetic manipulation tools available for pathogenic leptospires. Current genetic manipulation techniques include insertion of DNA by random transposon mutagenesis and homologous recombination via suicide vectors. The current study describes the construction of a shuttle vector, pMaORI, which replicates within saprophytic, intermediate, and pathogenic leptospires. The shuttle vector was constructed by insertion of a 2.9 kb DNA segment including \textit{parA}, \textit{parB}, and \textit{rep} genes into pMAT, a plasmid that cannot replicate in \textit{Leptospira} spp. that contains a backbone consisting of an \textit{aadA} cassette, \textit{ori} R6K, and \textit{oriT} RK2/RP4. The inserted DNA segment was isolated from a 52 kb region within \textit{Leptospira mayottensis} strain 200901116 that is not found in the closely related \textit{L. mayottensis} strain 200901122. Due to the size of this region and the presence of bacteriophage-like proteins it is possible this region is a result of a phage-related genomic island. Stability of the pMaORI plasmid was tested within pathogenic strains by passaging cultures 10 times without selection and confirming the presence of pMaORI. Concordantly, we report the use of trans complementation in the pathogen \textit{Leptospira interrogans}: transformation of a pMaORI vector carrying a functional copy of the \textit{perR} gene in a null mutant background restores expression of PerR and susceptibility to hydrogen peroxide comparable to that of wild-type cells. In conclusion, we demonstrate replication of a stable plasmid vector in a large panel of \textit{Leptospira} strains, including pathogens. The described shuttle vector will expand our ability to perform genetic manipulation of \textit{Leptospira} spp.
INTRODUCTION

Leptospirosis, which is caused by one of the ten pathogenic *Leptospira* spp. described to date, is a neglected zoonotic disease that has a worldwide distribution with a high incidence in tropical countries. Our current understanding of the virulence mechanisms and more generally the biology of pathogenic *Leptospira* remains largely unknown. This hindrance is partly due to a lack of efficient genetic tools available for use in pathogenic *Leptospira* spp. (8, 11). While genetic modification tools allow flexible manipulation of the genome of the saprophyte *Leptospira biflexa*, including targeted mutagenesis and cis/ trans complementation (11), genetic modification of the pathogen are limited primarily to random transposon mutagenesis.

Previously, genetic analysis of *Leptospira* was impeded by the absence of methods for the introduction of DNA into leptospiral cells. Currently, DNA can be introduced in *Leptospira* spp. by electroporation (14) or conjugation between *E. coli* and *Leptospira* spp. using RP4 derivative conjugative plasmids (10). Transformed *Leptospira* can be visualized on solid medium as subsurface colonies after one week for saprophytes and up to four weeks for pathogens. Markers for the selection of transformants include kanamycin-, spectinomycin-, and gentamicin- resistance cassettes (1, 13, 14). The replication origins of the *L. biflexa* phage LE1 (14), *L. biflexa* plasmid p74 (12) and a phage-related genomic island from *L. interrogans* (4) have previously been used to construct *L. biflexa*- *E. coli* plasmid shuttle vectors. However, a shuttle vector construct has not been reported in intermediate nor pathogenic *Leptospira* spp.

In this study, analysis of the genomes of two genetically related strains of a recently discovered pathogenic *Leptospira* species (2) revealed a prophage-like region of approximately 52 kb present in only one of the strains. Further analysis of this region allowed the identification of a putative replication origin. Cloning of a DNA fragment containing this replication origin into an *E. coli* conjugative plasmid allowed autonomous replication in the saprophyte *L. biflexa* and several intermediate and pathogenic strains. Subsequent to plasmid construction and analysis, we employed this plasmid for functional trans
complementation of a mutant in the pathogen *L. interrogans* serovar Manilae which carries an inactivation in the peroxide stress regulator-encoding gene *perR*. The PerR transcriptional regulator belongs to the Fur family and controls the expression of genes participating in the cell’s defense against oxidants. It has been previously shown that in a *Leptospira perR* mutant a catalase (*katE*) and a putative cytochrome C peroxidase (*maugG-2*) had higher expression compared to the wild type strain. Previous work has also shown that this *L. interrogans perR* mutant has enhanced survival in the presence of hydrogen peroxide compared to wild-type cells (9). We report here the successful production of PerR and concomitant restoration of reduced resistance to hydrogen peroxide when PerR is expressed in trans in the *perR* mutant using the described plasmid.
MATERIALS AND METHODS

Bacterial strains and culture conditions.

The following Leptospira spp. were used in this study: intermediate strains L. fainei serovar Hurstbridge strain BUT 6 and L. licerasiae serovar Varillal strain VAR010; pathogenic strains L. interrogans serovar Copenhageni strain Wijnberg, L. interrogans serovar Canicola strain Hond Utrecht IV, L. interrogans serovar Icterohaemorrhagiae strain Verdun, L. interrogans serovar Copenhageni strain Fiocruz L1-130, L. interrogans serovar Manilae strain L495, L. interrogans serovar Manilae perR mutant ‘M776’ (generous gift from Gerald Murray and Ben Adler; Monash University, Melbourne, Australia) (9), L. interrogans serovar Lai strain 56601, L. mayottensis strain 200901116; and the saprophyte L. biflexa serovar Patoc strain Patoc1.

Strains were grown at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (6, 7) with or without spectinomycin (40 µg/ml, Sigma Aldrich, St. Louis, MO). EMJH solid medium was prepared by adding 1% noble agar (w/v) to EMJH liquid medium containing spectinomycin (40 µg/ml), and incubated at 30°C for 7 to 30 days.

E. coli strain Π1 (ΔthyA) cells were used for plasmid transformation. E. coli strain β2163 (ΔdapA) was used for transfer of pMaORI vector into Leptospira spp. via conjugation. E. coli strains were grown in Luria-Bertani broth or agar containing spectinomycin (50 µg/ml). Strain β2163 was additionally cultured with 0.3 mM diaminopimelic acid (DAP, Sigma Aldrich), while strain Π1 was additionally cultured with 0.3 mM thymidine (dT, Sigma Aldrich) as previously described (5).

Plasmid construction and transformation of Leptospira spp.

The nucleotide sequence of the replication region of L. mayottensis strain 200901116 was amplified with primer pairs ori1 and ori2 (Table 1) and inserted into pCR2.1-TOPO by using the TOPO TA cloning kit as per manufacturer’s instructions (Life Technologies, Waltham, MA). After BamHI- Xbal digestion and gel purification, the DNA fragment containing the replication region was inserted into the corresponding restriction sites of the conjugative spectinomycin-resistant plasmid pMAT, and designated pMaORI. Plasmid constructs were
introduced into Leptospira strains by conjugation as previously described (10) with E. coli β2163 containing the pMaORI plasmid construct. Transformed Leptospira strains were recovered from solid media, grown in EMJH liquid media with spectinomycin selection (40 µg/ml), and filtered with a 0.22 µm filter into fresh EMJH liquid media with spectinomycin (40 µg/ml). Once these samples had grown to mid logarithmic growth all samples were visually verified to ensure the presence of motile leptospira cells without the presence of E. coli cells or other contaminants. Once it was determined contaminants were not present, the sample was tested for the shuttle vector pMaORI as described below.

Gene amplification of aadA and adk (an E. coli specific adenylate kinase gene) was performed via PCR. Gene specific primers are listed in table 1. Plasmid extraction of pMaORI from Leptospira spp. and E. coli strains was performed by QiaPrep Spin Miniprep Kit as per manufacturer’s instructions (Qiagen, Venlo, Netherlands). The restriction endonuclease KpnI was used to determine pMaORI restriction profile isolated from E. coli cells that were previously transformed with pMaORI from Leptospira species. 18 µl of extracted plasmid was added to 2.1 µl of 10x FastDigest buffer, and 1 µl (unit) of FastDigest KpnI (Thermo Scientific, Waltham, MA). Samples were digested for 30 minutes at 37°C followed by gel electrophoresis.

Passage experiment

Leptospira strains were passaged ten times to determine if the pMaORI construct could be conserved without selection. For each passage, a 100 µl aliquot was passaged into 9 ml of fresh EMJH liquid media without selection until it reached late logarithmic growth. Each sample was then subsequently passaged, and the remaining 8.9 ml of sample in late logarithmic growth was plasmid extracted (described above). Extracted plasmid was tested by PCR for the aadA cassette, and subsequently transformed into Π1 E. coli strain where pMaORI was verified by KpnI restriction profile.

Complementation of L. interrogans serovar Manilae perR mutant
To complement the *L. interrogans perR* 'M776' mutant (9), the wild-type *perR* allele (including its own promoter region, 310 nucleotides upstream of the *perR* start codon) was amplified from *L. interrogans* serovar Manilae strain L495 using primers PerR5 and PerR3 (Table 1).

This PCR product was cloned into pCR2.1-TOPO (Invitrogen). The *perR* DNA fragment was then released with EcoRI and inserted into the dephosphorylated EcoRI site of pMaORI to generate plasmid pMaORI-*perR* (pNB138). The pMaORI-*perR* plasmid construct was introduced by conjugation into the M776 mutant (*L. interrogans perR* mutant) as described above and spectinomycin-resistant colonies were inoculated into liquid EMJH containing 40 µg/ ml spectinomycin for further analysis.

PerR cellular content was measured by immunoblot analysis. Exponentially growing cells were lysed by sonication in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, in the presence of protease inhibitors (Roche, Basel, Switzerland). 15 µg of total cell extracts were loaded on a 15% SDS-PAGE and proteins were transferred onto nitrocellulose. PerR protein was detected by using a rabbit anti-PerR antibody at 1:2000 dilution as primary antibodies, a goat anti-rabbit HRP-conjugated IgG at a 1:150000 dilution (Sigma Aldrich) as secondary antibodies, and the SuperSignal West Pico reagent (Thermo Scientific) as HRP substrate.

Survival in the presence of H$_2$O$_2$ was assessed by incubating exponentially growing cells in the presence or in the absence of 10 mM H$_2$O$_2$ for 30 min at 30°C. Twenty microliters of cells were then incubated with the Alamar Blue cell viability reagent (Life Technologies) according to the manufacturer’s recommendations.

**Bioinformatic Analysis**

Bioinformatic analysis was completed using Microscope Microbial Genome Annotation & Analysis Platform web based software (https://www.genoscope.cns.fr/agc/mage).

**Accession number**

The annotated sequence of pMaORI has been deposited in Genbank under accession number KP784428.
RESULTS AND DISCUSSION

Genome analysis

All members of the *Leptospira* genus that have been analyzed carry at least two circular
replicons. The large circular (cI, >3.6 Mb) and the small circular (cII, 278 to 350 kb)
chromosomes encode genes involved in housekeeping and other functions (17, 18). More
recently, plasmids have also been identified by whole genome sequencing such as p74 in
the saprophyte *L. biflexa* (12), and pGui1, pGui2 in the pathogen *L. interrogans* (16). The
presence of small replicative plasmids within *Leptospira* spp. implied possible conservation
of small genetic elements for our current study.

We previously reported a group of *Leptospira* strains isolated from the blood of patients with
leptospirosis in Mayotte (Indian Ocean) which were identified as belonging to a novel
pathogenic species (2, 3). High-throughput genome sequencing of two representative
strains, 200901116 and 200901122, of this new species designated as *Leptospira*
mayottensis was performed at the J. Craig Venter Institute (http://gsc.jcvi.org/). The draft
genome sequences of strains 200901116 (4,135,276 bp, 84 contigs) and 200901122
(4,161,553 bp, 85 contigs) are deposited in GenBank under accession numbers
AKWB00000000 and AKWM00000000, respectively. The G+C content of these strains are
39.5 mol%, which is within the range 35-45 mol% reported for members of the genus
*Leptospira* (2). Comparative genome analysis was performed using the MaGe interface in

The genomes of strains 200901116 and 200901122 are highly similar as demonstrated by
their high number of common genes: they share 3,501 CDS with an average pair-wise amino
acid identity higher than 99%. In comparison, using the same criteria, strain 200901116
shares only 34 CDS with *L. interrogans* strain Fiocruz L1-130 and 194 CDS with *L.
borgpetersenii* strain L550, with >99% amino acid identity. By analyzing genome disparity,
we found a unique region of approximately 52 kb carried by 200901116 but not 200901122
at approximate position NT440186-NT492268 (Figure 1). The majority of genes in this
region encode hypothetical proteins. Genes identified in this region include phage-related gene homologues coding for Integrase, Late control protein D, Portal protein, GpA terminase, Bacteriophage resistance factor (PF05565 family), and a toxin (RelE-like family)-antitoxin (Xre-like family). Another gene (AKWB270011) in this region codes for a putative protein of 234 amino acids containing an N-terminal helix-turn-helix domain (25-76 amino acids), which is typically associated with DNA binding. Upstream of this gene, two genes, one of which, AKWB270009, exhibited similarities with homologs of the ParA partition protein. The other gene (AKWB270010) could constitute the parB of the putative partition locus. This genetic organization is similar to the parA-parB organizations identified to date in *L. biflexa* serovar Patoc strain Patoc1 and *L. interrogans* serovar Lai strain 56601 (4, 12, 14) and includes genes coding for a DNA-binding protein that could constitute a Rep protein for initiation of plasmid replication and a partitioning system (Figure 2a). Interestingly, orthologues of the *L. mayottensis* Rep-like protein were also found in *L. interrogans* pathogenic strains with a sequence identity superior to 75% (Figure 2b). Together, this suggests the 52 kb genomic island located in *L. mayottensis* strain 200901116 may represent a putative prophage. The occurrence of the *L. mayottensis* rep gene in other sequenced *Leptospira* strains suggests a widespread distribution of this prophage in *L. interrogans* strains.

### Design of a replicative vector

To construct the pMaORI replicative vector, a 2.9-kb autonomously replicating sequence (ARS) consisting of parA locus (AKWB270009), parB locus (AKWB270010), and rep (AKWB270011) located within the sequence of *L. mayottensis* strain 200901116 was amplified and inserted into the 2.2kb conjugative plasmid pMAT, derived from pSW29T (10). The pMAT vector includes the aadA spectinomycin resistant-cassette, ori R6K for replication, and oriTRK2/RP4 for conjugation. The engineered vector is 5035bp and was named pMaORI (Figure 3).

### Determination of pMaORI replication in various *Leptospira* spp.
The pMaORI construct was confirmed to be a viable shuttle vector in saprophytic, intermediate, and pathogenic Leptospira strains. Compared with original conjugative vector carrying the Himar1 transposon (10), the number of transconjugates increased from 2 to 5 fold when using our replication vector pMaORI into L. biflexa serovar Patoc strain Patoc, L. interrogans serovar Copenhageni strain Fiocruz LA-130, and L. interrogans serovar Manilae strain L495. It was possible to transform the pMaORI construct into E. coli β2163, conjugate pMaORI into leptospires, recover the plasmid via plasmid extraction, and then re-transform pMaORI into E. coli Π1 (Table 2). Transformed Leptospira strains were initially confirmed positive for pMaORI construct via aadA amplification. To ensure confirmation was not a result of E. coli contamination, positive Leptospira strains were tested for adk, an E. coli gene. Further, to ensure aadA confirmation was not a result of naked DNA contamination within the sample, pMaORI that was extracted from Leptospira strains was transformed into E. coli Π1, plasmid extracted from E. coli, and pMaORI was confirmed by KpnI restriction profile.

The pMaORI vector was introduced (via conjugation) into L. mayottensis strain 200901116 to determine if the pMaORI plasmid could replicate in its parental strain. Analysis revealed this strain was incapable of maintaining the pMaORI vector.

Following confirmation that pathogenic Leptospira strains were able to replicate and maintain the pMaORI plasmid, strains were passaged ten times in EMJH media without spectinomycin to determine if the pMaORI plasmid could be conserved in the absence of selection. It was confirmed that pathogenic strains were indeed capable of maintaining this shuttle vector through ten passages without selection (Table 2). This was confirmed via plasmid extraction of pMaORI from Leptospira cultures, followed by PCR amplification of the aadA cassette, ability to re-transform into E. coli Π1, and subsequent plasmid restriction profile analysis. A limitation in the method utilized for detecting plasmid stability is that it does not quantify the percentage of the population that retain pMaORI. Future studies should focus on quantifying the percentage of the bacterial population that retain pMaORI, and determining copy number per cell.
Plasmid-complementation of perR in Leptospira interrogans

A perR mutant was previously obtained by random transposon mutagenesis in L. interrogans serovar Manilae strain L495 (9). This perR mutant was shown to have greater survival in the presence of hydrogen peroxide compared to wild-type cells. However, repeated attempts to complement the mutation were unsuccessful (9).

In this study, complementation of the perR mutant was performed by cloning the perR wild-type allele with its native promoter into the pMaORI plasmid vector. Hundreds of transconjugants were obtained, and complemented cells showed expression of PerR (Figure 4A), indicating that perR ORF was expressed in trans from the pMaORI expression vector. Next, the ability of the complemented perR mutant cells to resist lethal concentrations of H$_2$O$_2$ were tested and compared to those of wild type and the perR mutant. Cell viability was assessed via the Alamar Blue assay, which measures the ability of cells to carry out redox reactions; reducing blue resazurin to pink resorufin. As shown in figure 4B, in the presence of H$_2$O$_2$, perR mutant cells display increased viability compared to wild type cells as shown by their ability to reduce resazurin, indicating as expected that the perR mutant had greater survival in the presence of peroxide. PerR mutant cells that had been transformed by the pMaORI vector containing the perR allele were no longer able to reduce the resazurin, which indicated that they had a decreased viability in the presence of H$_2$O$_2$ (Figure 4B). This demonstrated that expressing the perR coding sequence from the pMaORI vector could restore an H$_2$O$_2$-associated phenotype comparable to that of wild-type cells.

While replication of plasmid vectors in pathogens has not been previously described, these results demonstrate for the that plasmid complementation is possible in pathogenic Leptospira strains. The pMaORI vector will therefore be useful for the purpose of genetic complementation of mutants obtained by random transposon mutagenesis or in the generation of conditional mutants.

ACKNOWLEDGMENTS
Authors thank J. Vinetz and D. Fouts for their permission to use the draft genomes which are part of a project which was funded with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services under contract number HHSN272200900007C. This work was supported in part by National Science Foundation grant IIA-1159099 (CJP) and the Institut Pasteur (MP).

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<table>
<thead>
<tr>
<th></th>
<th>Sequence (5' -&gt; 3')</th>
<th>target</th>
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<td>spcA</td>
<td>GGGGTGAATTTGAGAATGGA</td>
<td><em>aadA</em> cassette</td>
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<tr>
<td>spcB</td>
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<td>ATTCTGCTTGGCGCTCCGGG</td>
<td><em>E. coli</em> adK</td>
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<tr>
<td>PerR5</td>
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* BamHI and XbaI restriction sites are underlined
Table 2: *Leptospira* strains are capable of replication and conservation of pMaORI

<table>
<thead>
<tr>
<th>Type</th>
<th>Species</th>
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<th>Strain</th>
<th>SpcR Colonies</th>
<th>pMaORI Conservation</th>
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<td>Intermediate</td>
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<td>Hurstbridge</td>
<td>BUT 6</td>
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<td>VAR010</td>
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<td><em>L. interrogans</em></td>
<td>Canicola</td>
<td>Hond Utrecht IV</td>
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<td>positive (^c)</td>
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<td><em>L. interrogans</em></td>
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<td>Fiocruz L1-130</td>
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<td>positive</td>
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<td>Wijnberg</td>
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<td>Verdun</td>
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<td>200901116</td>
<td>-</td>
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</tr>
</tbody>
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\(^a\): serogroup
\(^b\): -= no colony, +=1-40 colonies, +++ = 41-100 colonies, +++ = >100 colonies.
\(^c\): strain verified until passage 4
\(^d\): pMaORI conserved after 10 passages without spectinomycin selection. Results verified by KpnI restriction profile of plasmid preps, and PCR of *aadA* cassette and the *E. coli* *adk* gene.

NA: not applicable
ND: not determined
Figure legends

Figure 1: Comparative genome analysis between the draft genomes of strains 200901116 and 200901122.

Comparative genome analysis was performed using the MaGe interface (15) in the SpiroScope database (https://www.genoscope.cns.fr/agc/mage). The arrow indicates the phage-like region present in strain 200901116, but not in strain 200901122.

Figure 2: Genetic organization of replication origins of prophage and plasmids identified in Leptospira spp. A: Figure compares organization of parA and parB between saprophytic and pathogenic Leptospira spp. Sequence analysis of L. mayottensis strain 200901116 reveals a putative replication origin constituted by genes encoding a DNA-binding protein that could constitute a Rep protein for initiation of plasmid replication and ParA/ParB partitioning system. This genetic organization is similar to previously identified replication origins in L. biflexa (12, 14) and L. interrogans (4). B: Sequence alignment of putative Rep proteins from L. mayottensis serovar Mini strain 200901116, L. interrogans strain UI08452, L. interrogans serovar Medanensis strain L0448, L. interrogans strain L0996, L. interrogans serovar Zanoni strain LT2156. Identical residues are shaded in black, conserved residues are shaded in grey.

Figure 3: Schematic representation of plasmid vector pMaORI.

Plasmid pMaORI (5035 bp) was generated by cloning the replication origin of L. mayottensis strain 200901116, which carries the rep, parA, and parB genes, into the XbaI-BamHI restriction sites of conjugative plasmid pMAT, which contains the spectinomycin resistance cassette (aadA/Spc'), ori R6K, and oriT RK2/RP4. The aadA gene is expressed from the B. burgdorferi flgB promoter.

Figure 4: Complementation of a L. interrogans perR mutant

A. PerR production in the wild-type (WT), perR mutant (perR), and complemented perR mutant (perR + pMaORI-perR) cells. Western blot analysis shows recovery of expression of PerR in the perR mutant carrying the replicative plasmid pMaORI-perR.

B. Cell viability of the wild-type (WT), perR mutant (perR), and complemented perR mutant (perR + pMaORI-perR) cells during peroxide stress. Exponentially growing wild-type, perR
mutant, and complemented *perR* mutant cells were exposed to H$_2$O$_2$ and cell viability was assessed in the presence of resazurin. Cell viability is measured by the capacity of the cell to carry out the redox reaction of reducing blue resazurin into bright-pink resorufin.
Figure 2
Figure 4

A

WT  M776  pMaORI perR

15 kDa

B

-  +  \(H_2O_2\)

WT

M776

M776 pMaORI perR