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Mycobacterium abscessus Phospholipase C Expression Is Induced during Coculture within Amoebae and Enhances *M. abscessus* Virulence in Mice

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***Mycobacterium abscessus* is a pathogenic, rapidly growing mycobacterium involved in pulmonary and cutaneo-mucous infections worldwide, to which cystic fibrosis patients are exquisitely susceptible. The analysis of the genome sequence of *M. abscessus* showed that this bacterium is endowed with the metabolic pathways typically found in environmental microorganisms that come into contact with soil, plants, and aquatic environments, where free-living amoebae are frequently present. *M. abscessus* also contains several genes that are characteristically found only in pathogenic bacteria. One of them is *MAB_0555*, encoding a putative phospholipase C (PLC) that is absent from most other rapidly growing mycobacteria, including *Mycobacterium chelonae* and *Mycobacterium smegmatis*. Here, we report that purified recombinant *M. abscessus* PLC is highly cytotoxic to mouse macrophages, presumably due to hydrolysis of membrane phospholipids. We further showed by constructing and using an *M. abscessus* PLC knockout mutant that loss of PLC activity is deleterious to *M. abscessus* intracellular survival in amoebae. The importance of PLC is further supported by the fact that *M. abscessus* PLC was found to be expressed only in amoebae. Aerosol challenge of mice with *M. abscessus* strains that were precultured in amoebae enhanced *M. abscessus* lung infectivity relative to *M. abscessus* grown in broth culture. Our study underlines the importance of PLC for the virulence of *M. abscessus*. Despite the difficulties of isolating *M. abscessus* from environmental sources, our findings suggest that *M. abscessus* has evolved in close contact with environmental protozoa, which supports the argument that amoebae may contribute to the virulence of opportunistic mycobacteria.**

The recognition of the role of *Mycobacterium abscessus* in human pathology has taken several decades, due to confusion in many studies between this mycobacterium and the very closely related species *Mycobacterium chelonae*. It was only in 1992 that these two species were distinguished and *M. abscessus* elevated to the rank of species (1).

These two phylogenetically closely related, rapidly growing mycobacteria (RGM), which have identical 16S ribosomal rRNA gene sequences, are distinguished by different pathogenicity patterns. *M. chelonae*, generally less pathogenic than *M. abscessus*, is implicated in skin and soft tissue infections and only occasionally involved in lung infections. *M. abscessus* is currently the most frequently isolated RGM in human pathology and the main RGM involved in lung infections (2, 3), with a particular link to cystic fibrosis (CF) patients (4–6). *M. abscessus* is also the main RGM responsible for iatrogenic infections in humans (postinjection abscesses, cardiac surgery infections, and plastic surgery infections) (7–9).

The environmental source of *M. abscessus* that might serve as a reservoir for human infection is currently unknown (10). Although the gene pool of *M. abscessus* (11) suggests that this bacterium has evolved in an aquatic environment at the interface with plants, as shown by the presence of genes coding for resistance to arsenic, i.e., cysteine desulfurases, which are found mainly in environmental organisms (11), some other genes of *M. abscessus* indicate that this bacterium tends to specialize in intracellular parasitism (12). The hypothesis that *M. abscessus* has evolved in an

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aquatic environment has been strengthened by a recent study showing that it can replicate and survive within zebrafish embryos at 28°C, where it can be pathogenic and capable of inducing lethal infections (13).

Amoebae are an integral part of this aquatic and telluric environment, and several reports have already shown an association of mycobacteria with free-living amoebae in water networks (14–17), although some species, including *M. abscessus*, were not recovered at all (18, 19), mainly due to aggressive methods of decontamination (20). Mycobacteria can grow in amoebae (21–25), and amoebic coculture has been successfully used to isolate *Mycobacterium massiliense* (26), a member of the *M. abscessus* complex. *M. abscessus* was also described as being able to multiply in trophozoites and to survive in amoeba cysts, the persistent stage of amoebae (12, 23), supporting the idea that factors other than rapid growth may be involved in mycobacterium-amoeba interactions.

Comparative genomic analyses of *M. abscessus*, *M. chelonae*, and *Mycobacterium smegmatis* genomes has allowed the confirmation of differences observed between these RGM in terms of pathogenicity (27) and intracellular behavior (28; A.-L. Roux, T. Deramaudt, R. Simeone, A. Viljoen, A. Bernut, A. Bah, N. Dulphy, M. Rottman, A. Toubert, J.-L. Gaillard, L. Tailleux, L. Kremer, I. Vergne, C. de Chastellier, L. Majlessi, R. Brosch, and J.-L. Herrmann, unpublished data) by highlighting several *M. abscessus* key genes encoding virulence factors (11). Interestingly, these genes seem to have been acquired by horizontal gene transfer (HGT) mainly from aquatic and telluric pathogenic bacteria, including those playing a major role in patients with CF: *Pseudomonas* spp. and *Burkholderia* spp. (11). One key determinant acquired by HGT is phospholipase C (PLC), encoded by the *plcC* gene (*MAB_0555*) (11). PLC was reported to be involved in the intracellular survival of *Mycobacterium tuberculosis* (29) and is absent from both *M. chelonae* and *M. smegmatis*. However, neither its role in the pathogenicity of *M. abscessus* nor its interaction with eukaryotic cells has been investigated yet.

Bacterial PLCs are known to play important roles in bacterial pathogenesis, increasing bacterial survival by inducing inappropriate host cellular signaling mechanisms and direct cytotoxicity or by impairing lung inflammatory responses (for reviews, see references 30, 31, and 32). In mycobacteria, PLC (and sphingomyelinase) activity seems to be associated with the most virulent species (33).

Association of PLC activity with virulent species prompted us to initiate a detailed molecular characterization of the *M. abscessus* PLC activities. Here, we describe the biological activities of the *M. abscessus* PLC. Data were obtained from experiments with purified recombinant PLC, as well as from analysis of its role in three different eukaryotic infection models, for which we employed a PLC knockout mutant of *M. abscessus* and its complemented derivative that were both constructed in this study.

MATERIALS AND METHODS

Mycobacterial and amoeba strains, reagents, and antibodies. Smooth *M. abscessus* CIP104536^{TS} (CIP-S) and the recombinant strain *M. smegmatis* mc^{2}155 groEL1ΔC (34) were grown aerobically at 37°C in Middlebrook 7H9 medium supplemented with 0.2% glycerol. *Acanthamoeba castellanii* (ATCC 30010) was grown at 28°C without CO_2 in PYG broth (35). *p*-Nitrophenylphosphorylcholine (*p*-NPPC) and the unlabeled phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were from Sigma-Aldrich. Radiolabeled 1-palmitoyl-2-[^{14}C]palmitoyl-glycerophosphocholine (DPPC) and [1-^{14}C]palmitic acid were from GE Healthcare. *Bacillus cereus* PC-PLC enzyme was from Sigma-Aldrich. Polyclonal mouse anti-PLC antibodies were obtained after three DNA immunizations of mice with a plasmid containing the PLC sequence under the control of a cytomegalovirus (CMV) promoter ((V. Le Moigne, M. Rottman, C. Goulard, B. Barteau, I. Poncin, N. Soismier, S. Canaan, B. Pitard, J.-L. Gaillard, and J.-L. Herrmann, unpublished results).

Cloning of PLC (*MAB_0555*). *MAB_0555*, encoding *M. abscessus* PLC with its predicted Tat signal sequence, was amplified by PCR (see Table S1 in the supplemental material), gel purified, and cloned after ligation into pCR2.1-TOPO (Life Technologies, France) (pTOPO-*MAB_0555*). *MAB_0555* was again amplified from pTOPO-*MAB_0555* with a second set of primers (see Table S1 in the supplemental material), which includes restriction sites for HindIII and NcoI. Amplified products were then digested, purified, and cloned into pMyC (pMyC-*MAB_0555*) as previously described (36). pMyc-*MAB_0555* was transformed into the recombinant strain *M. smegmatis* mc^{2}155 groEL1ΔC, which was further used for protein purification of PLC.

PLC purification, enzymatic activity, and cell experiments with purified recombinant *M. abscessus* PLC. PLC was purified from a single transformed colony of *M. smegmatis*::pMyc-*MAB_0555* as described previously for *M. tuberculosis* PLCs (36). Recombinant *M. abscessus* PLC (rPLC$_{Ma}$) was concentrated to 1 mg/ml, analyzed by MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry and N-terminal sequencing, and stored at −80°C. Phospholipase C activity was measured with *p*-NPPC, PC, or sphingomyelin (SM) as the substrate, as previously described (36), using the fluorescent Amplex red PC-PLC kit assay or the fluorescent Amplex red sphingomyelinase kit assay (Molecular Probes, Life Technologies) as described by the supplier. Competition assays between PC and PE (or PI) (30 mg/ml and 25 mg/ml in chloroform, respectively) were performed using the Amplex red PC-PLC assay for substrate preference. Inhibition assays were performed using the D609 compound (9.38 mM final concentration in water) (Sigma), at molecular inhibitor/enzyme ratios of 200 and 600. The residual activity was measured using the Amplex red PC-PLC kit as described above. The hemolytic and cytotoxic effects of purified rPLC$_{Ma}$ were evaluated as previously described (36). Incorporation of labeled fatty acids ([1-^{14}C] palmitic acid) into macrophages and rPLC$_{Ma}$ activity on radiolabeled macrophages were evaluated as described previously (36, 37).

Construction of the PLC KO (*MAB_0555*) mutant in *M. abscessus*. The *M. abscessus* PLC knockout (KO) mutant was obtained by allelic exchange in *M. abscessus* CIP-S using the strategy previously reported (38). Briefly, the zeocin cassette (*Streptoalloteichus hindustanus ble*) was inserted into the HindIII-ClaI region spanning the 3′ end of *MAB_0554* and into *MAB_0555* (nucleotides 731 to 801 in *MAB_0554* to nucleotides 1 to 590 in *MAB_0555*). The overall fragment was cloned into pMVZ261 and further restricted by PvuII-HpaI for purification and electroporation in *M. abscessus* CIP-S bearing the recombineering plasmid pJV53 (39). Homologous recombination was checked by a first PCR screen using forward and reverse primers outside the deleted region (see Table S1 in the supplemental material) and then by Southern blotting using a zeocin probe and a 532-bp probe matching the 3′ end of *MAB_0555* and prepared by amplification using forward and reverse primers (see Table S1 in the supplemental material). To complement the *M. abscessus* PLC KO mutant, pTOPO-*MAB_0555* was digested and *MAB_0555* was cloned under the control of the *hsp60* promoter into the integrative plasmid pMVZ361-Kan-Zeo. The plasmid was then electroporated into the wild-type (WT) and PLC KO strains. *In vitro* growth of WT, PLC KO, and PLC-complemented *M. abscessus* strains was monitored at 600 nm.

TLC and mass spectrometry comparative analysis of the WT and PLC KO mutant of *M. abscessus*. Mycobacterial wet cells were sequentially extracted with $CHCl_3$-CH_3OH (1:2, vol/vol), with $CHCl_3$-CH_3OH (1:1, vol/vol) and then three times with $CHCl_3$-CH_3OH (2:1, vol/vol). The

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organic phases were pooled, extensively washed with water, and evaporated to dryness. Lipids were analyzed by thin-layer chromatography (TLC) on silica gel 60-precoated plates (0.25-mm thickness; Merck) developed with $CHCl_3$-CH_3OH (90:10 [vol/vol]) for glycolipids or $CHCl_3$-CH_3OH-H_2O (60:35:8) for phospholipids. Sugar-containing compounds were visualized by spraying plates with 0.2% anthrone in concentrated sulfuric acid, followed by heating, whereas the Dittmer-Lester reagent and ninhydrin were used to detect phosphorus- and amino group-containing substances, respectively.

For mass spectrometry (MS) experiments, total lipids were extracted from bacterial cell pellets with methanol (MeOH)-$CHCl_3$ (2:1, vol/vol) overnight at room temperature (RT). Supernatants were filtered and then poured into new tubes for evaporation under nitrogen flow. $CHCl_3$-MeOH (2:1, vol/vol) was then added to cell pellets and incubated at RT for 24 h. After incubation, the contents of the glass tubes were filtered on glass pipettes and poured into the corresponding tube containing the previously evaporated materials. After solvent evaporation, H_2O-$CHCl_3$ (1:1, vol/vol) was added, and the tube was incubated for 24 h. After water-lipid separation, water was removed until the organic phase was limpid. Lipids extracts were evaporated and dissolved in isopropanol-methanol (70:30, vol/vol), 0.02% (mass/vol) formic acid, 0.01% (mass/vol) ammonium hydroxide. Electrospray ionization quadrupole time-of-flight mass spectrometry was performed as previously described (40). Briefly, lipid extracts were injected by infusion into the MS. Ionization was maintained at 325°C with a 5-liter/min drying gas flow, a 200,000-Pa nebulizer pressure, and 5,500 V. Spectra were collected in positive-ion mode from *m/z* 200 to 3,000 at 1 spectrum/s. Spectrometer was calibrated in positive-ion mode with a sodium iodide solution (NaI at 2 μg/ml in 50% isopropanol). Collision-induced dissociation (CID) MS was performed with energy of 30 V. Data were collected and processed through Analyst QS 1.1 software from AB-MDS-Sciex.

Coculture of *M. abscessus* strains and murine macrophages. Bone marrow-derived murine macrophages (BMDMs) were prepared as previously described (41). BMDMs were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) at 37°C with 5% CO_2. Coculture experiments were performed as previously described (36, 41, 42) at a multiplicity of infection (MOI) of one bacterium per macrophage. After 3 h of incubation, the cells were washed 3 times with RPMI to remove extracellular bacteria and incubated with amikacin (250 μg/ml) to kill the remaining extracellular bacteria. Fresh medium containing 50 μg/ml of amikacin was then added. The number of CFU/ml was determinate at days 0, 1, 3, and 6 of the culture after cold-water lysis of macrophages.

Coculture of *M. abscessus* strains and *A. castellanii*. For amoeba infection assays, *M. abscessus* cultures were washed 3 times in 30 ml of Page's modified Neff's amoeba saline (PAS), which contains no source of carbon or azote (35). The mycobacterial inoculum was thoroughly mixed, and mycobacteria were then dispersed by 10 passages of the bacterial suspension through a 25-gauge needle attached to a 5-ml syringe followed by 10 passages through a 29.5-gauge needle attached to a 1-ml syringe. Mycobacterial suspensions were then adjusted in PAS buffer to a concentration of 2.5×10^7 bacteria per ml by measuring the optical density at 600 nm (OD_{600}). CFU counts were also confirmed on the inocula. Five hundred microliters of the *A. castellanii* suspension was washed three times in PAS buffer and dispatched into a 48-well plate. Following 1 h of incubation at 32°C, the amoeba monolayer was inoculated with 200 μl of a bacterial suspension (MOI, 25 bacteria/amoeba). After 3 h of incubation at 32°C, extracellular mycobacteria were removed by three thorough washings in PAS buffer, followed by one supplementary 2-h incubation in the presence of 100 μg/ml of amikacin, in order to kill all extracellular mycobacteria. PAS (500 μl) was the added. Every 24 h, 50 μl (10^7) heat-inactivated *Escherichia coli* (70°C for 60 min) was added to each well to slow the transition from trophozoite to cyst. The number of CFU/ml was determined for each *M. abscessus* strain after lysis of the *A. castellanii* monolayer with 1% SDS for 30 min at 32°C, at 1, 2, 3, and 5 days of coculture.

Western blotting for PLC expression. *M. abscessus* strains, either grown in 7H9 or cocultured 1 to 2 days with amoebae, were lysed by sonication on ice (three times for 30 s each) with proteases inhibitors (Complete Mini; Roche) plus E64 (20 μM final concentration) and leupeptin (20 μM final concentration). Thirty micrograms of cell lysates was separated on SDS-PAGE and transferred onto nitrocellulose membranes, which were then incubated with murine anti-PLC antibodies diluted 1/300. After addition of a rabbit anti-mouse antibody linked to peroxidase, the signal was revealed using the Sirius chemiluminescent substrate (Advansta, USA).

RT-PCR for PLC mRNA expression. For total mRNA extraction of mycobacteria from macrophage cocultures, macrophages were infected for 5 days with *M. abscessus* (MOI = 1) in F75 flasks. Each day, the culture medium was discarded, and the infected monolayer was washed with 1× phosphate-buffered saline (PBS) and then resuspended in 10 ml of guanidine thiocyanate solution (4 M) to lyse macrophages. The lysates were then centrifuged at 2,500 × *g* for 30 min to concentrate intracellular bacteria. The pellet of intracellular mycobacteria or pellet of mycobacteria cultivated at 30°C or 37°C was then resuspended in TRIzol, and total-mRNA extraction was performed using TRIzol in the presence of zirconia/silica beads and after bead beating at maximum speed for 30 s twice. After a chloroform-and-isopropanol precipitation, RNA samples were treated twice with DNase I Amp Grade (Invitrogen) (1 U/μg of RNA). Total RNA integrity and concentration were assessed with the Experion automated electrophoresis system (Bio-Rad). One microgram of total RNA was used for a reverse transcription reaction with oligo$(dT)_{12–18}$ primers and SuperScript II reverse transcriptase (SuperScript first-strand synthesis system for reverse transcription-PCR [RT-PCR]; Invitrogen, Carlsbad, CA). Negative controls were made by replacing the reverse transcriptase with diethyl pyrocarbonate-treated water. Diluted cDNA was combined with primer/probe sets (see Table S1 in the supplemental material) and SYBR green I master mix (Roche) according to the manufacturer's recommendations. Samples were normalized internally using the average cycle threshold (C_T) of *sigA* as the reference (42). *sigA* was used as the constitutive gene as previously described (43). The concentration ratio (target/*sigA* mRNA) was calculated using ReLQuant Roche software and expressed in arbitrary units.

Mouse model of *M. abscessus* aerosol infection. BALB/c mice were challenged with aerosolized *M. abscessus* using an aerosol generator under agreement number B92-033-01. This apparatus used a Micro Mist small-volume nebulizer (Hudson RCI-Teleflex Medical, Research Triangle Park, NC, USA) containing 6 ml of mycobacterial solution at various concentrations. Presleeping mice (isoflurane; Abbott, Rungis, France) were anesthetized with 200 μl of Hypnomidate (etomidate; Janssen-Cilag, France) and placed in an open 50-ml syringe fixed on top of a closed compartment containing the nebulizer. The nebulization in this device lasted 15 min, the time necessary to vaporize all the bacterial solution. Aerosol infections were performed with fresh aliquots of *M. abscessus* strains grown on 7H9 as described previously (41), to achieve an inoculum of 1×10^8 mycobacteria. When mice were infected by aerosolized *M. abscessus* strains cocultured with amoebae, the infected amoebae were prepared by rapping the flasks vigorously and centrifuging at 1,000 × *g* for 5 min. The resulting pellet was suspended in 10 ml of PBS and adjusted to a concentration of 2×10^6 CFU/ml after SDS lysis of amoebae. Lungs, livers, and spleens were collected in sterile distilled water and homogenized, and 10-fold serial dilutions were then plated on VCA3 plates (vancomycin, colimycin, and amphotericin B; bioMérieux, France) for CFU enumeration. Plates were incubated at 37°C up to 5 days. Results were expressed as the mean $\log_{10}$ CFU per organ. The minimum detection limit per organ was 20 CFU (or 1.3 $\log_{10}$ CFU) per lung, spleen, or liver. A two-way analysis of variance (ANOVA) with a Tukey posttest were performed using GraphPad prism program version 5 for statistical comparison.

Statistical analysis. Fisher's exact test and Student's *t* test were used. A *P* value of <0.05 was considered significant.

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TABLE 1 Maximal percentage of identity between the different PLC amino acid sequences from *M. abscessus* and *M. tuberculosis* or nonmycobacterial microorganisms[a]

Organism	Uniprot no.	Protein name	Size (aa)	Maximal % identity	Length of homology region (aa)
Nonmycobacterial bacteria					
Pseudomonas aeruginosa	P15713*	Nonhemolytic PLC	692	45	503
Burkholderia cenocepacia	A0KBL6	PLC	723	43	538
Ralstonia pickettii	B2UDZ8	PLC, phosphocholine specific	700	42	521
Burkholderia cenocepacia	A0B2U2	PLC	704	42	519
Pseudomonas aeruginosa	P06200	Hemolytic PLC	730	42	515
Bordetella avium	Q2KUR2	Nonhemolytic PLC	693	42	509
Stenotrophomonas maltophilia	B2FL44	Putative nonhemolytic PLC	706	41	525
Burkholderia cenocepacia	A0K5Z1	PLC	714	41	516
Burkholderia multivorans	B3CZ34	PLC	718	41	515
Burkholderia multivorans	A9ADA7	PLC, phosphocholine specific	752	41	496
Bordetella avium	Q2L0W2	Nonhemolytic PLC	723	40	530
Ralstonia pickettii	B2UCT8	PLC, phosphocholine specific	719	40	520
Burkholderia multivorans	A9ALB9	PLC	771	39	519
Bordetella bronchiseptica	Q7WH05	Putative phospholipase	627	39	425
Burkholderia cenocepacia	A0B3P1	PLC	777	38	559
Pseudomonas fluorescens	Q4KC01	PLC, phosphocholine specific	715	38	546
Ralstonia pickettii	B2UJE7	PLC	474	30	456
Burkholderia multivorans	B3CZQ1	PLC	528	30	214
Burkholderia multivorans	B3D7M3	PLC	554	30	204
Burkholderia multivorans	A9AKI0	PLC	556	29	182
Burkholderia cenocepacia	A0B0A3	PLC	557	29	180
Mycobacteria					
Mycobacterium tuberculosis	P9WIA8*	PLC 4	514	42	465
Mycobacterium tuberculosis	P9WIB0*	PLC 3	517	40	496
Mycobacterium tuberculosis	P9WIB2*	PLC 2	521	39	504
Mycobacterium tuberculosis	P9WIB4*	PLC 1	520	38	503
Fungi					
Aspergillus fumigatus	B0XWP7	Phosphatidylglycerol specific phospholipase, putative	492	29	392
Aspergillus fumigatus	B0YCK0	Phosphatidylglycerol specific PLC, putative	509	26	406
Aspergillus fumigatus	B0XPD6	Phosphoesterase superfamily protein	456	26	396

[a] Alignments were performed with Basic Local Alignment Search Tool (BLAST) program at http://blast.ncbi.nlm.nih.gov, using the protein BLAST algorithm. aa, amino acids. *, PLC was biochemically characterized.

RESULTS

***M. abscessus* PLC resembles the PLC from CF pathogens.** Ripoll et al. (11) reported the presence of a PLC-encoding gene (*MAB_0555*) in the *M. abscessus* genome. *MAB_0555* has a length of 1,440 bp and encodes a 479-amino-acid protein (11). Inspection of the syntenic genomic regions and comparison with other mycobacterial genomes suggest that *MAB_0555* was inserted just downstream of *MAB_0554*, which encodes a potential hydrolase/lipase (11) that is conserved in a wide range of fast-growing and slowly growing mycobacteria (see Fig. S1 in the supplemental material). Phylogenetic analysis demonstrated a nonmycobacterial origin of *MAB_0555*, and more recent protein comparison performed for this study confirmed this trait (Table 1). The strongest identities were observed with several recently discovered *Actinomycetes* but more importantly in several major CF pathogens (Table 1): *Burkholderia cenocepacia* PLC (43% identity), *Pseudomonas aeruginosa* nonhemolytic and hemolytic PLCs (45% and 41% identity, respectively), *Burkholderia multivorans* PLC (41% identity), and *Stenotrophomonas maltophilia* putative nonhemolytic PLC (41% identity). Of note, a 29% identity was also observed with PLC of another CF pathogen, *Aspergillus fumigatus* putative phosphatidylglycerol PLC. By comparison, the percent identity with the 4 PLCs present in *M. tuberculosis* varied between 38 and 42% (Table 1).

***M. abscessus* purified recombinant PLC hydrolyzes eukaryotic cells. (i) Expression in *M. smegmatis* and purification.** $rPLC_{Ma}$ was purified after acetamide induction. The ionic detergent Sarkosyl (1%) in the lysis buffer allowed solubilization and purification of the recombinant enzyme using immobilized metal ion affinity chromatography and fast protein liquid chromatography (IMAC-FPLC). Under these conditions, 10 to 15 mg of pure $rPLC_{Ma}$ was obtained per liter of culture. SDS-PAGE and MALDI-TOF analysis of purified PLC showed that the apparent molecular mass of recombinant protein was compatible (Fig. 1A) with the expected theoretical molecular mass (52 kDa) based on the amino acid sequence, including the full-length TAT signal peptide (noncleaved), as previously noticed for *M. tuberculosis* PLCs (36). We then used approaches similar to those described previously (36) for the evaluation of *M. abscessus* PLC.

(ii) Biochemical hydrolytic activity of $rPLC_{Ma}$. $rPLC_{Ma}$ was able to hydrolyze *p*-NPPC with turnover and specific activity (5.68 mmol min^{-1} mg^{-1}) similar to those of *B. cereus* and *C. perfringens*

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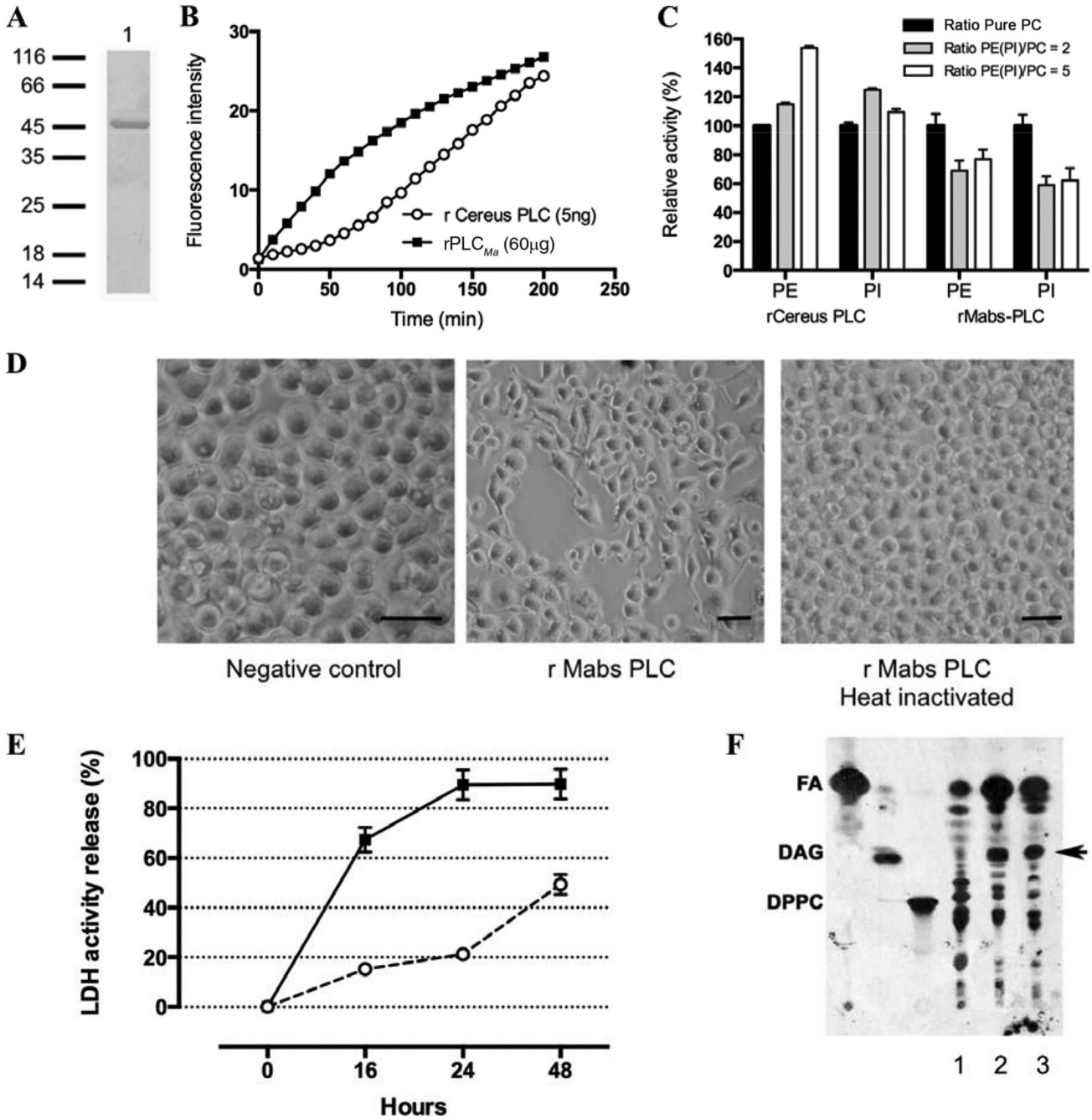

FIG 1 (A, B, and C) Biochemical characterization of rPLC$_{Ma}$. (A) Purified rPLC$_{Ma}$ was loaded on 12% SDS-PAGE. Molecular weight standards are on the left. Lane 1 contained purified rPLC$_{Ma}$ (10 µg). The sample was loaded under reducing conditions, and the gel was then stained with Coomassie brilliant blue. (B) Time course hydrolysis of phosphatidylcholine (PC) by 60 µg of rPLC$_{Ma}$ (■) or 5 ng of recombinant *B. cereus* PC-PLC (○). The release of phosphocholine was measured indirectly by the fluorescence measurement of resorufin released using the Amplex red phosphatidylcholine kit and continuously monitored at a λ_{exc} of 510 nm and λ_{em} of 590 nm, (C) Substrate preference of rPLC$_{Ma}$ and recombinant *B. cereus* PC-PLC. Competition assays between PC and phosphatidylethanolamine (PE) (or phosphatidylinositol [PI]) were carried out using different phospholipid ratios (PE [or PI]/PC = 2 or 5) and pure PC. A final PC quantity of 0.1 µmol was used in the pure-PC assay, and 0.2 µmol and 0.5 µmol of PE (or PI) were used for the other PE (PI)/PC ratios. The PC-PLC activity was continuously measured using the Amplex red PC-PLC kit. The relative activity (percent) of PC hydrolysis was calculated from the ratio of PC activity in the presence of PE (or PI) over PC activity in the absence of PE (or PI). (D, E, and F) Cytotoxic effects of rPLC$_{Ma}$ on mouse macrophages. (D) Macrophage cellular state after 24 h of incubation, with no recombinant PLC (buffer only [negative control]), with 50 µg of purified rPLC$_{Ma}$ (rPLC$_{Ma}$), and with 50 µg of heat-inactivated purified rPLC$_{Ma}$ (rPLC$_{Ma}$, heat inactivated), as shown by light microscopy (magnification, ×200; bar, 25 µm). (E) Purified rPLC$_{Ma}$ (50 µg; ■) and 15 µg of PC-PLC from *B. cereus* (○) were incubated with 1×10^6 RAW264.7 mouse macrophages. The values are shown as percent lysis, in which the amount of LDH released in wells with macrophages incubated with enzymes was compared to total LDH released in control wells in which all of the macrophages had been deliberately lysed. LDH released was quantified at 16, 24, and 48 h. The values are the means for triplicate samples. (F) Autoradiography of TLC plate showing the release of radiolabeled DAG, the product of phospholipid hydrolysis (indicated by the black arrow), after incubation of rPLC$_{Ma}$ (50 µg, lane 2) and PC-PLC from *B. cereus* (15 µg, lane 3) with radiolabeled macrophages. For the negative control (lane 1), only the buffer without any pure enzyme was added in the incubation medium. Abbreviations: DAG; diacylglycerol; FA, free fatty acid; DPPC, dipalmitoyl-glycerophosphocholine.

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PLCs (7.75 mmol min^{-1} mg^{-1} and 3.1 mmol min^{-1} mg^{-1}, respectively) (see Fig. S2A in the supplemental material). Comparatively, $rPLC_{Ma}$ specific activity (0.1 nmol min^{-1} mg^{-1}) measured using PC as the substrate (Fig. 1B) was far below the specific activity observed for *B. cereus* PC-PLC (165 nmol min^{-1} mg^{-1}). It is noteworthy that both *B. cereus* PC-PLC and $rPLC_{Ma}$ hydrolyze the same substrate; however, the catalytic mechanisms differ between the proteins, since the effect of D609 inhibitor totally abolished the activity of *B. cereus* PLC, while it had no effect on $rPLC_{Ma}$ activity (data not shown).

$rPLC_{Ma}$ was active within a large range of temperatures (25 to 55°C), its optimal temperature being 37°C, with an optimal pH of 7 to 7.5 (see Fig. S2B and C in the supplemental material). This activity was stable for the first 48 h but was completely abolished upon heat inactivation.

No sphingomyelinase activity was observed for $rPLC_{Ma}$ (data not shown). However, the $rPLC_{Ma}$ and *B. cereus* PC-PL substrate preferences and the activities of both enzymes were determined in the presence of different ratios of PI/PC or PE/PC (PE [or PI]/PC ratios = 2 and 5) (Fig. 1C). Under these experimental conditions, the hydrolysis of only the PC can be detected, and the substrate preference was deduced by the increasing or decreasing activity on PC. The PC-PLC activity of the *B. cereus* enzyme increased significantly (by a factor of 1.5) in the presence of PE, suggesting a better PC substrate presentation in the presence of PE. In contrast, the PC-PLC activity of $rPLC_{Ma}$ decreased by a factor of 1.5 in the presence of PE (Fig. 1C). Similar results were obtained in the presence of PI (Fig. 1C), suggesting no substrate preference. Finally, when a mixture of phospholipids was used as the substrate (Fig. 1C), the behavior of both enzymes can vary, again suggesting a different hydrolysis mechanism between *B. cereus* PC-PLC and $rPLC_{Ma}$.

(iii) $rPLC_{Ma}$ lyses eukaryotic cells. $rPLC_{Ma}$ did not lyse erythrocytes, unlike *B. cereus* or *C. perfringens* PLCs (data not shown). Comparatively, $rPLC_{Ma}$ exhibited a strong cytotoxic effect on mouse macrophages (Fig. 1D) and on the amount of lactate dehydrogenase (LDH) released (Fig. 1E). A level of macrophage lysis of 17% was detected after 16 h of incubation and increased over time to reach 55% after 48 h, while the cell monolayer was not affected in the presence of heat-inactivated $rPLC_{Ma}$.

We next evaluated $rPLC_{Ma}$ macrophage cytotoxicity with [1-^{14}C]palmitic acid incorporation into membrane phospholipids, and measuring the amount of radiolabeled membrane phospholipids released after membrane degradation. Purified $rPLC_{Ma}$ released radiolabeled diacylglycerol (DAG), unlike the control sample (Fig. 1F). Altogether, we demonstrated that $rPLC_{Ma}$ is active and possesses cytotoxic activity against macrophages, mainly by degrading the phospholipids of the cell membrane.

***M. abscessus* PLC as a virulence factor in eukaryotic cell infection models. (i) Construction of an *M. abscessus* PLC KO mutant.** An *M. abscessus* PLC KO mutant was obtained by allelic exchange using the recombineering system (38). For the construction of the mutant, a zeocin cassette was inserted between the 3′ end of *MAB_0554* and the 5′ end of *MAB_0555*, thereby completely disrupting the PLC-encoding gene *MAB_0555* (Fig. 2A). Southern blot analysis allowed us to confirm that double crossing-over and disruption of *MAB_0555* had occurred (Fig. 2B). We then constructed a PLC-complemented version by inserting a pMVZ361-*hsp60*pro-*MAB_0555* plasmid into the KO mutant. For control purposes, we also inserted the same plasmid into the WT *M. abscessus* strain. Measurement of the PLC activity of the parental CIP-S strain, the PLC KO mutant, the complemented mutant, and the WT strain carrying the additional copy of PLC supplied by pMVZ361-*hsp60*pro-*MAB_0555* revealed low and no PLC activity for the WT strain and the PLC *M. abscessus* KO mutant, respectively. These findings argue for low expression of PLC under *in vitro* conditions and confirmed the disruption of *MAB_0555* in the KO mutant (Fig. 2C). In contrast, expression of PLC in either of the pMVZ361-*hsp60*pro-*MAB_0555*-carrying strains was associated with high and comparable activity (Fig. 2C). Of note, *in vitro* growth characteristics of the WT, the PLC *M. abscessus* KO mutant, and its complemented version were similar (Fig. 2D). In addition, lipid extract comparison by TLC (Fig. 2E) and MS (data not shown) between the WT strain and PLC *M. abscessus* KO mutant did not highlight qualitative or quantitative differences in parietal lipid composition between both strains.

(ii) Survival of the PLC *M. abscessus* KO mutant in BMDMs. To investigate the role of PLC in intracellular survival of *M. abscessus*, BMDMs were cocultivated with the WT strain, the PLC *M. abscessus* KO mutant, and the complemented PLC *M. abscessus* KO mutant. The absence of *MAB_0555* (PLC) failed to alter the growth of the WT strain in BMDMs, as the WT, PLC mutant, and complemented strains exhibited similar growth throughout the experiment (Fig. 3A). The comparable intracellular growth rate strongly suggests that the PLC activity does not confer any supplementary advantage to *M. abscessus* within murine macrophages.

(iii) Survival of the PLC *M. abscessus* KO mutant in *A. castellanii*. *A. castellanii* and *M. abscessus* strains were cocultured in order to evaluate the PLC contribution to *M. abscessus* survival in amoebae (23). As shown in Fig. 3B, WT *M. abscessus* was able to replicate and survive inside amoebae, although it was unable to grow in the PAS amoeba medium used during the coculture (data not shown). By comparison, the *M. abscessus* PLC KO mutant was greatly impaired and unable to survive throughout the course of the experiment (Fig. 3B). The lack of PLC seems to have been directly involved in this result, since the complemented PLC *M. abscessus* KO mutant strain was able to grow (although less than the WT) and to survive inside amoebae (Fig. 3B). Of note, the PLC *M. abscessus* KO mutant strain complemented only with the *MAB_0554* gene behaved like the PLC *M. abscessus* KO mutant strain, confirming that the observed defect was not due to the 3′ loss of *MAB_0554* when the PLC KO mutant was being constructed (data not shown). Finally, we could not evaluate if growth in amoebae affects the production of the mycobacterial glycolipids, as the number of mycobacteria obtained after coculturing is insufficient to carry out a glycolipid analysis by TLC. In addition, the smooth morphotype was very stable after coculturing in amoebae, as previously demonstrated (13, 41, 43).

The different behavior of the mutant in amoebae or BMDMs suggested the putative occurrence of *de novo* synthesis of PLC when *M. abscessus* is cocultivated with *A. castellanii*, which is of particular importance given the very low PLC production in *in vitro*-grown *M. abscessus* (Fig. 2C). We therefore further assessed this phenomenon by analyzing and comparing PLC expression at a transcriptional and translational level.

Expression of PLC is induced when *M. abscessus* is cocultured in *A. castellanii*. *MAB_0555* mRNA expression was quantified by RT-PCR after intramacrophage coculture (Fig. 3C) and *in vitro* growth (30°C and 37°C) of the different strains (Fig. 3D). Under both *ex vivo* and *in vitro* conditions, specific *MAB_0555*

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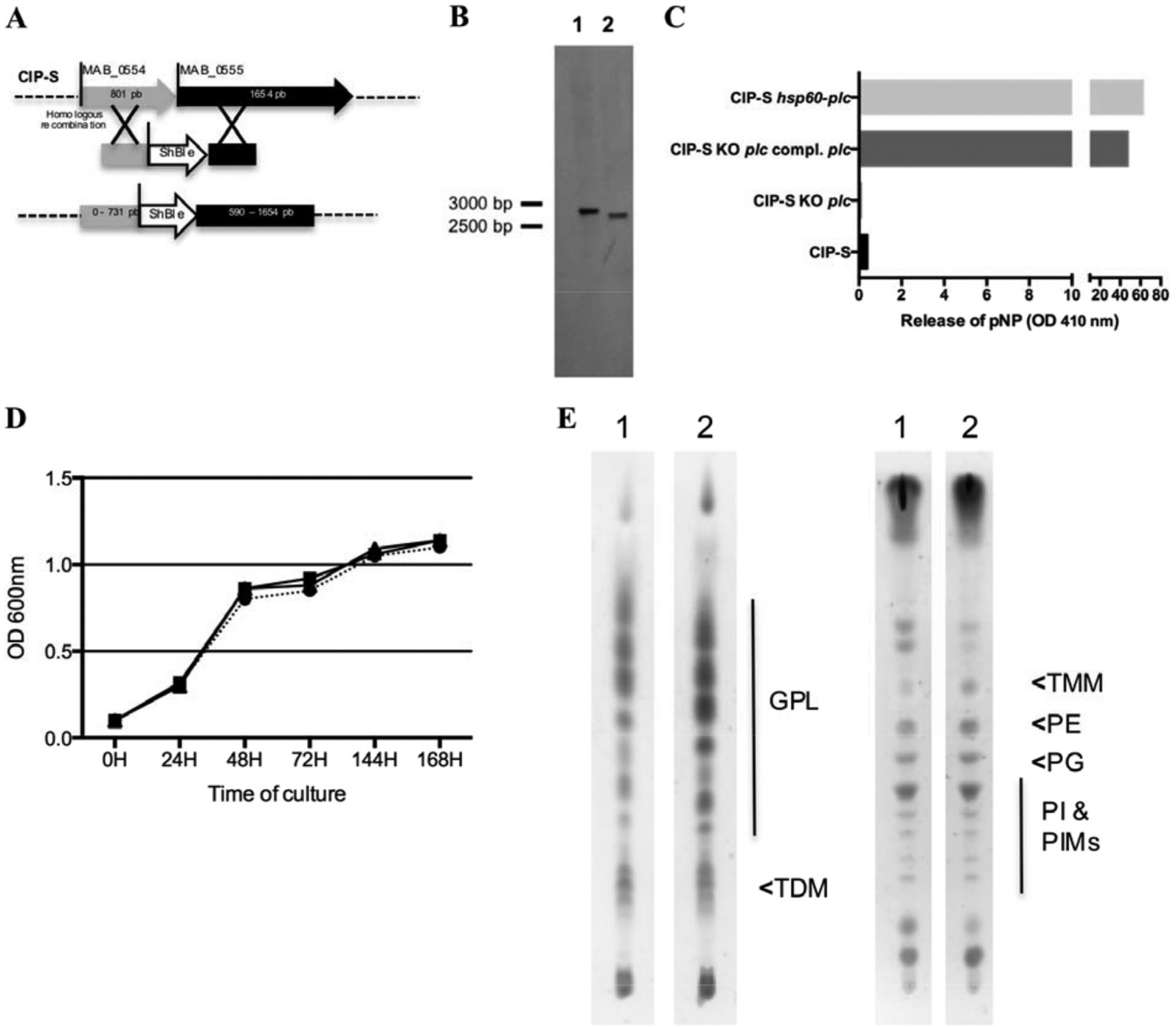

FIG 2 Construction of the *M. abscessus plc* KO mutant by homologous recombination (HR). (A) *MAB_0554* and *MAB_0555* are separated by 4 bp. The *M. abscessus plc* KO mutant was thus constructed by amplifying a nearly 2-kbp *M. abscessus* fragment encompassing this region and cloning the zeocin resistance gene (*S. hindustanus ble*) into the HindIII (position 731 in *MAB_0554*)-ClaI (position 590 in *MAB_0555*) sites. The entire fragment was then electroporated into *M. abscessus* CIP-S containing the pJV53 plasmid inserted by HR into the *M. abscessus* chromosome. (B) Southern blotting analysis was performed after genomic DNA restriction by KpnI and gel electrophoresis. A 532-bp probe targeting the 3′ conserved region of *MAB_0555* was used for hybridization with DNA fragments: a 2,704-bp band is observed with the WT strain (lane 1), and a 2,635-bp band is observed with the *M. abscessus* KO mutant (lane 2). (C) Respective PLC activity of the different constructed *M. abscessus* strains. Phospholipase C activity was measured with *p*-NPPC for the CIP-S, CIP-S *plc* KO, CIP-S *plc* KO *plc*-complemented, and CIP-S-*hsp60-plc* (*M. abscessus* pMVZ361-*hsp60*pro-*MAB_0555*, used as a control for PLC activity) strains. (D) *In vitro* growth curves estimated by spectrophotometry (OD_{600}) of CIP-S (circles), CIP-S *plc* KO (squares), and CIP-S *plc* KO *plc*-complemented (triangles) strains. (E) Glycolipid and phospholipid patterns of the different constructed *M. abscessus* strains. Total lipid contents of the WT *M. abscessus* strain (lanes 1) and the *plc* KO *M. abscessus* mutant (lanes 2) were analyzed by TLC using $CHCl_3$-CH_3OH (90:10, vol/vol) (left) and $CHCl_3$-CH_3OH-H_2O (60:35:8, vol/vol/vol) (right) as the solvent systems and anthrone revelation (GPLs, glycopeptidolipids; TDM, trehalose dimycolate; TMM, trehalose monomycolate; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides).

transcriptional expression was observed only in the complemented strains carrying the pMVZ361::*MAB_0555* driving *PLC* expression under the control of the constitutive *hsp60* promoter (Fig. 3C and D). Absence of specific *MAB_0555* mRNA expression was observed for the wild-type and the PLC-KO *M. abscessus* strains in macrophages (Fig. 3C) and between the two growth temperatures *in vitro* (Fig. 3D).

In order to confirm the presence of the expressed PLC by the wild-type strain in amoebae, Western blot (WB) analysis using polyclonal antibodies was preferred to RT-PCR. WB clearly revealed the presence of PLC when bacteria were cocultured in amoebae compared to mycobacteria grown in 7H9 rich medium, despite a strong amoeba proteolytic activity during preparation of the protein extract (Fig. 3E). A 47-kDa protein,

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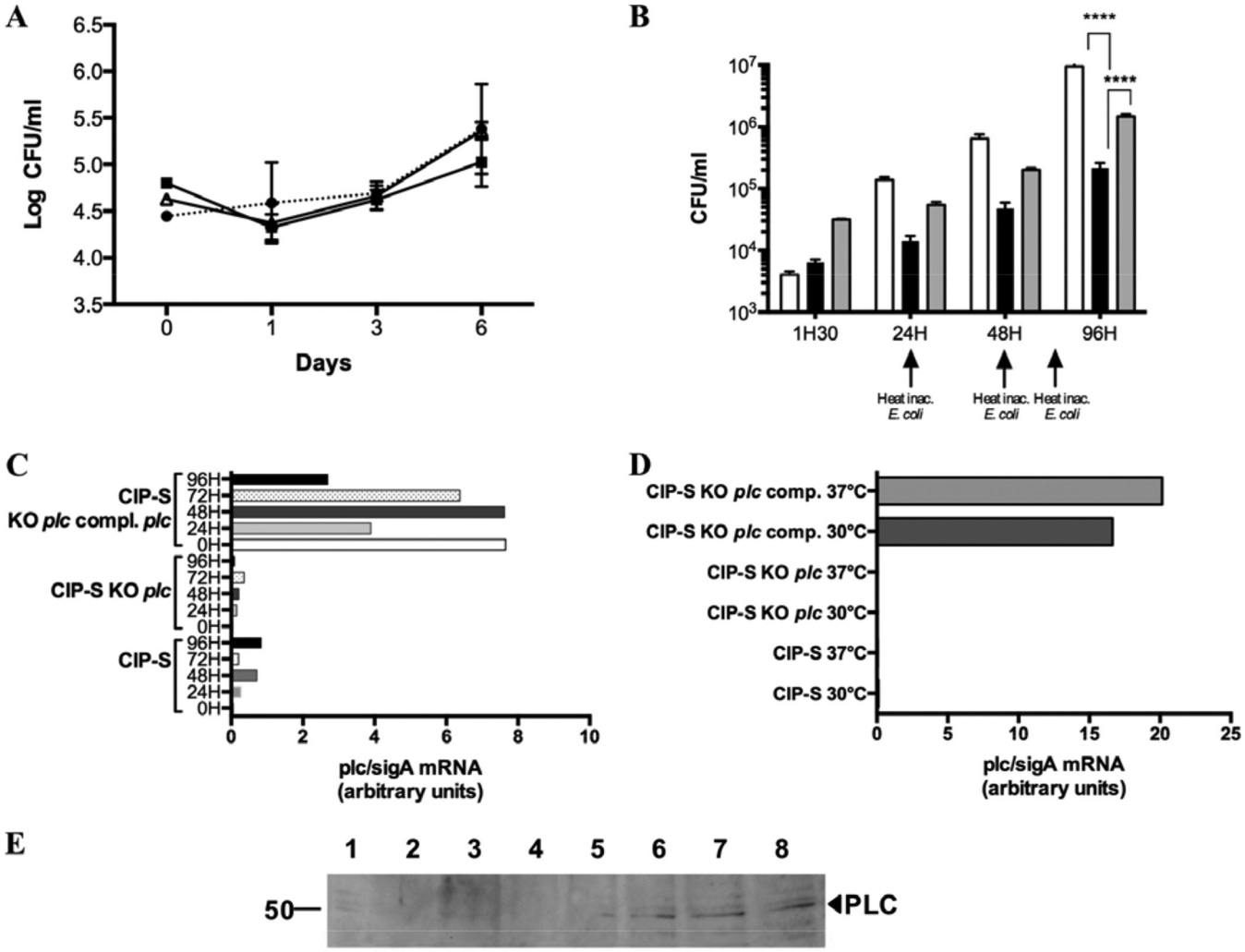

FIG 3 Survival of *plc M. abscessus* mutant in eukaryotic cells. (A) Growth of mycobacterial strains within BMDMs recorded by CFU evaluation after 1, 3, and 6 days of coculture. The CIP-S (circles), CIP-S *plc* KO (squares), and CIP-S *plc* KO *plc*-complemented (triangles) strains were used. (B) Growth of mycobacterial strains within amoebae recorded by CFU evaluation after 1.5 h and 1, 2, and 4 days of coculture. CIP-S (white bars), CIP-S *plc* KO (black bars), and CIP-S *plc* KO *plc*-complemented (gray bars) strains were used. Experiments were repeated five times in triplicate at different times for both panels A and B (***, $P < 0.001$). (C) mRNA *plc/sigA* ratio (in arbitrary units) for the CIP-S, CIP-S *plc* KO, and CIP-S *plc* KO *plc*-complemented strains cocultivated with macrophages for 5 days. (D) mRNA *plc/sigA* ratio (in arbitrary units) for the CIP-S, CIP-S *plc* KO, and CIP-S *plc* KO *plc*-complemented strains cultivated in rich medium (7H9) at 30°C or 37°C. The results are representative of two independent experiments (C and D). (E) Western blot analysis of PLC expression during coculture of mycobacterial strains with *A. castellanii*. Lane 1, total extract (30 μg) of CIP-S cultivated in 7H9 medium; lane 2, total extract (30 μg) of amoebae cultivated for 96 h in PAS buffer in the absence of mycobacteria; lanes 3 to 7, total extract (30 μg) of amoebae cocultivated for 3 h (lane 3), 24 h (lane 4), 48 h (lane 5), 72 h (lane 6), or 96 h (lane 7) in PAS buffer in the presence of CIP-S; lane 8, total extract (30 μg) of amoebae cocultivated for 48 h in PAS buffer in the presence of the CIP-S *plc* KO *plc*-complemented strain. This picture is representative of three independent experiments.

which corresponds to the native molecular mass of PLC (without the TAT signal peptide), was detected only in the presence of amoebae after 48 h until 96 h of coculture with either the WT (Fig. 3E, lanes 5 to 7) or the complemented strains (Fig. 3E, lane 8).

In vivo behavior of the PLC *M. abscessus* KO mutant in the aerosol mouse model of infection. All three *M. abscessus* strains were independently used to infect mice by aerosol for 15 min using an inoculum of 1×10^8 mycobacteria. CFU were enumerated in lungs, livers, and spleens at days 1, 7, 14, 21, and 28. A similar decline was observed for WT strain, the PLC *M. abscessus* KO mutant, and the complemented PLC *M. abscessus* KO mutant (Fig. 4A), in agreement with results from the BMDM infection studies.

Since coculture of *M. abscessus* and *A. castellanii* significantly induces the expression of PLC in *M. abscessus*, we therefore addressed the question of whether a previous coculture of *M. abscessus* with *A. castellanii* would impact the *M. abscessus* virulence phenotype in the mouse model of infection. As shown in Fig. 3B, we recovered from cocultures of *M. abscessus* in amoebae only 2×10^6 mycobacteria, which was 100 times less than the *in vitro*-prepared inoculum. At this infectious dose, CFU counts only in the lungs were performed, as no mycobacteria could be recovered from livers and spleens (Le Moigne et al., unpublished). However, after only one expansion in amoebae, we obtained significantly higher bacterial loads in the lungs at 3 and 7 days postinfection (CFU per lungs) than with the same strain grown *in vitro* in rich 7H9 medium (Fig. 4B). Amoeba lysate itself added to *M. abscessus*

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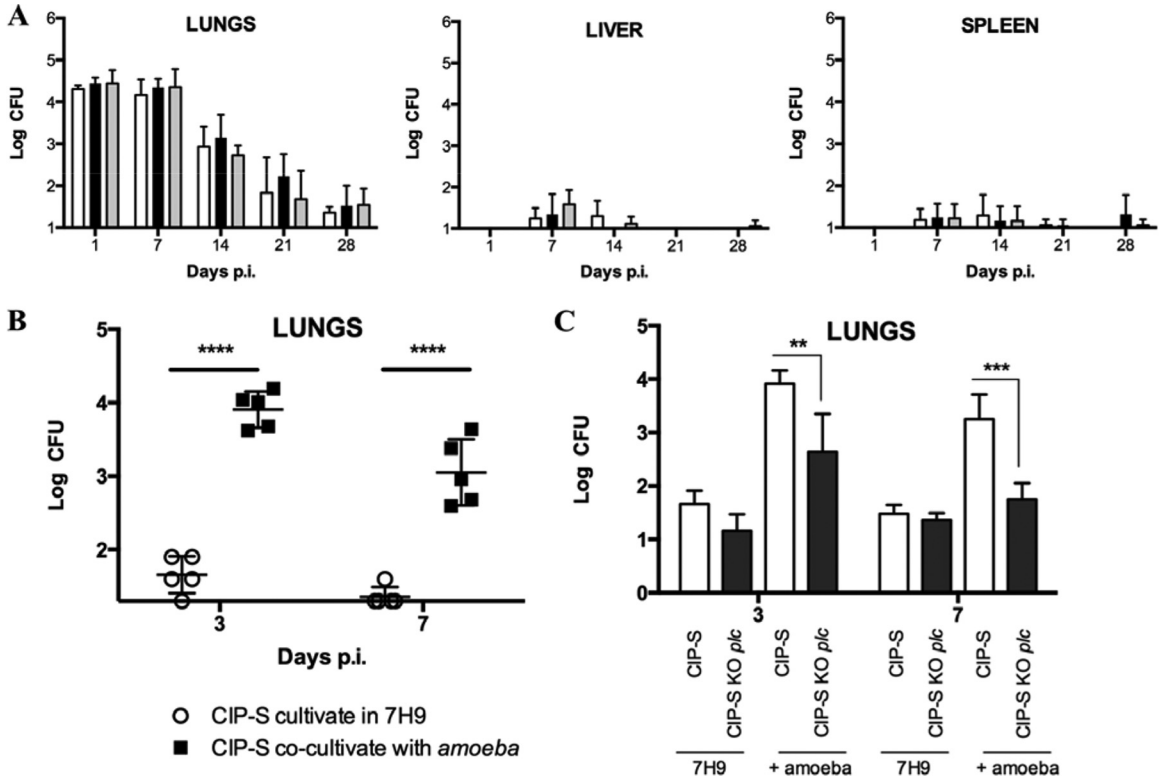

FIG 4 Effect of coculture of mycobacterial strains within amoebae on virulence in mice. (A) BALB/c mice were aerosolized with the CIP-S (white bars), CIP-S *plc* KO (black bars), and CIP-S *plc* KO *plc*-complemented (gray bars) strains cultivated in 7H9 medium. Mice were challenged with 10^8 mycobacteria and sacrificed at days 1, 7, 14, 21, and 28. Lungs, livers, and spleens were collected, homogenized, diluted (1/1, 1/5, 1/25, and 1/125), and cultured on VCA3 agar plates. CFU were counted after 5 days of growth. Twenty-five mice per group were challenged. (B) BALB/c mice were aerosolized with CIP-S cultivated in 7H9 medium (○) or obtained after 3 days of coculture in amoebae (■). Mice were sacrificed at days 3 and 7. Lungs were collected, diluted (1/1, 1/5, 1/25, and 1/125), and cultured on VCA3 agar plates. CFU were counted for the different dilutions after 5 days of growth. Ten mice per group were challenged. (****, $P < 0.0001$). (C) BALB/c mice were aerosolized with the CIP-S (empty bars) or CIP-S *plc* KO (black bars) strain cultivated in 7H9 medium or obtained after 3 days of coculture within amoebae. Mice were sacrificed at days 3 and 7. Lungs were collected, diluted (1/1, 1/5, 1/25, and 1/125), and cultured on VCA3 agar plates. CFU were counted after 5 days of growth. Ten mice per group were challenged. (**, $P < 0.01$; ***, $P < 0.001$).

before aerosol challenge was ineffective in increasing *M. abscessus* bacterial load in the lungs compared to coculturing *M. abscessus* within amoebae (data not shown).

The role of PLC expression in the enhanced survival in the lungs of immunocompetent mice was then explored by testing the phenotype of the PLC-deficient isogenic mutant cocultured with *A. castellanii*. Experiments demonstrated that the bacterial burden achieved by the *M. abscessus plc* KO mutant was significantly lower at 3 days ($P < 0.01$) and at 7 days ($P < 0.001$) postinfection than the one achieved by the WT *M. abscessus* strain (Fig. 4C). Taken together, these results indicate that growth of *M. abscessus* in *Acanthamoeba* increases virulence of *M. abscessus* in mice, most likely by inducing the expression of PLC.

DISCUSSION

Phospholipases are considered key virulence factors and are synthesized by bacterial species causing disparate infectious disease, from infection causing massive tissue destruction to food-borne diseases. Some well-studied PLCs involved in virulence are the α toxin of *Clostridium perfringens*, the PLC-H and PLC-N of *P. aeruginosa*, and the two PI-PLCs of *Listeria monocytogenes*. Cytolysis is the most common characteristic attributed to bacterial phospholipase virulence factors (31, 32). We demonstrated that *M. abscessus* synthesizes a PLC with lytic activity against eukaryotic cells, with membrane phospholipid degradation and DAG production. We were not able to demonstrate a hemolytic activity for *M. abscessus* PLC, indicating that it resembles *P. aeruginosa* PLC-N, which also lacks hemolytic activity (44). This aspect might be crucial for degradation and penetration of the mucus layer present in the lungs, as observed for *P. aeruginosa* (31, 32). As with eukaryotic PLC, the hydrolysis of phospholipids by *M. abscessus* PLC leads to the production of DAG, a well-known lipid second messenger. DAG has been shown to activate protein kinase C, which is known to modulate the activation of neutrophils and macrophages (45). This activity further emphasizes the inflammatory response observed in CF lungs and participates in the increased virulence of *M. abscessus* in CF patients compared to other mycobacteria (46).

Apart from playing a role in virulence, PLCs are thought to function in phosphate and carbon source acquisition (32). Synthesis of both *P. aeruginosa* PLC-H and PLC-N is regulated by inorganic phosphate at the transcriptional level by the positive regulator PhoB (47). Induction of PI-PLC synthesis was also shown for other bacteria in such a limited-resource environment. PI-PLC activity of *Lactobacillus rhamnosus* markedly depends on the amount of carbohydrate in the culture medium (48). Similar

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reports showed that PI-PLC-producing bacteria, including *B. cereus*, *Bacillus thuringiensis*, *L. monocytogenes*, and *Staphylococcus aureus*, required glucose-free medium for PLC activity to be detected (49–51). We demonstrated the absence of growth of *M. abscessus* in low-nutrient medium like PAS (data not shown) and correlatively a growth in the presence of amoebae, indicating that amoebae helped *M. abscessus* survive in a low-nutrient and/or eukaryotic environment. More importantly, amoeba-cocultured *M. abscessus* expressed virulence factors to be able to infect more aggressively the host as shown by the *de novo* production of PLC, as observed with the increased virulence of *M. abscessus* in mice when cocultured with *Acanthamoeba* compared to culture in 7H9 medium. Several mycobacteria were shown to survive in amoebae (12), but increased virulence after passage on amoebae was only shown for *M. avium* (21). PLC expression was stimulated when *M. abscessus* was grown intracellularly in *Acanthamoeba* but not in macrophages, thus pointing out the possible existence of regulatory networks similar to those observed in low-nutrient environments. In contrast, not much is presently known about induction of *M. tuberculosis* PLCs (29) and the role of the PhoP/PhoR regulatory network in the synthesis of PLC (52).

From the present data, we conclude that *M. abscessus* does not exhibit a clear substrate preference, as it was not possible to distinguish the phospholipids (PE, PI, or PC) preferentially hydrolyzed by *M. abscessus* PLC. An advantage conferred by the *M. abscessus* PLC activity might thus be the local degradation in the lungs of the dipalmitoyl-phosphatidylcholine, a major component of the surfactant, which would provide nutrients and/or osmoprotectants (47) necessary for *M. abscessus* survival in lung tissue. In CF patients, the thickening of the surfactant may thereby promote colonization and implementation of bacteria benefiting from PLC activity (Table 1). Colonizing CF lungs with already *de novo*-synthesized PLC would represent a major advantage for *M. abscessus* and might explain its specific tropism compared to other closely related RGM, such as *M. chelonae*. It remains to be established whether *M. abscessus* expresses PLC activity in infected patients. However, we have preliminary serological data confirming an anti-PLC antibody response in *M. abscessus*-infected CF patients (Le Moigne et al., unpublished).

Of importance was the choice of three different *ex vivo* and *in vivo* models to decipher the role of the PLC in *M. abscessus* virulence, as it helped us define the eukaryotic environment, where PLC activity was crucial for *M. abscessus* survival. Amoeba-bacterium interactions have recently been well defined (53), notably for mycobacteria (54, 55), and helped us in this study to consider the role of amoebae in shaping virulence of the bacteria toward a pathogenic phenotype (18, 53, 56, 57). Exchange and acquisition of genetic material have helped *M. abscessus* in gaining key virulence factors compared to other RGM. These *de novo*-synthesized virulence factors might explain the peculiar link between *M. abscessus* and CF lungs. Finally, it should be mentioned that identification of an environmental source where *M. abscessus* might come into contact with environmental amoebae would be very important for learning more about the potential contamination risks for CF patients. We are presently seeking such hidden sources of *M. abscessus* infection.

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