Oleoyl Coenzyme A Regulates Interaction of Transcriptional Regulator RaaS (Rv1219c) with DNA in Mycobacteria

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Background: RaaS mediates mycobacterial survival in nonpermissive growth conditions by controlling expression of ATP-dependent efflux pumps.

Results: Oleoyl-CoA regulates binding of the RaaS transcription factor to DNA and thus expression of the RaaS regulon and RaaS-mediated persistence.

Conclusion: The activity of bacterial efflux is regulated by metabolites that are produced during active growth.

Significance: Dysregulation of efflux pumps results in killing of persisting mycobacteria with low metabolic activity.

Tuberculosis remains a major infectious disease, with 8.6 million new cases estimated in 2012 claiming 1.3 million lives (1). Moreover, the causative agent of tuberculosis, Mycobacterium tuberculosis, plays an important role in mycobacterial survival in prolonged stationary phase and during murine infection. Here, we demonstrate that long chain acyl-CoA derivatives (oleoyl-CoA and, to lesser extent, palmitoyl-CoA) modulate RaaS binding to DNA and expression of the downstream genes that encode ATP-dependent efflux pumps. Moreover, exogenously added oleic acid influences RaaS-mediated mycobacterial improvement of survival and expression of the RaaS regulon. Our data suggest that long chain acyl-CoA derivatives serve as biological indicators of the bacterial metabolic state. Dysregulation of efflux pumps can be used to eliminate non-growing mycobacteria.

We have recently shown that RaaS (regulator of antimicrobial-assisted survival), encoded by Rv1219c in Mycobacterium tuberculosis and by bcg_1279c in Mycobacterium bovis bacillus Calmette-Guérin, plays an important role in mycobacterial survival in prolonged stationary phase and during murine infection. Here, we demonstrate that long chain acyl-CoA derivatives (oleoyl-CoA and, to lesser extent, palmitoyl-CoA) modulate RaaS binding to DNA and expression of the downstream genes that encode ATP-dependent efflux pumps. Moreover, exogenously added oleic acid influences RaaS-mediated mycobacterial improvement of survival and expression of the RaaS regulon. Our data suggest that long chain acyl-CoA derivatives serve as biological indicators of the bacterial metabolic state. Dysregulation of efflux pumps can be used to eliminate non-growing mycobacteria.

The abbreviations used are: BCG, bacillus Calmette-Guérin; ITC, isothermal titration calorimetry.
Oleoyl-CoA Is a Ligand of RaaS

TABLE 1
Oligonucleotides used in this study

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a qRT-PCR, quantitative RT-PCR.

EXPERIMENTAL PROCEDURES

Organisms and Media—The M. bovis BCG Glaxo strain was grown in Sauton’s liquid medium supplemented with albumin-dextrose complex as described previously (5). Ethambutol and oleic acid were added, using a 1-ml syringe fitted with a 25-gauge needle, 30 days after inoculation at final concentrations of 98 and 200 μM, respectively. An equivalent volume of sterile water was added to control cultures. Bacterial viability was assayed by counting colony-forming units on 7H10 agar.

Transcriptional Profiling—Total RNA was isolated from 30 ml of early logarithmic (7 days) or stationary phase (31 days) M. bovis BCG cultures after a 24-h exposure to 200 μM oleic acid using the guanidinium thiocyanate/TRIzol method (6). DNA contamination was removed with Turbo DNA-free DNase (Ambion) before cDNA was generated using SuperScript reverse transcriptase II (Invitrogen) as described previously (7). Quantitative PCR was performed in a Corbett RotorGene 6000 real-time thermocycler (Qiagen) by application of Absolute qPCR SYBR Green mixture (Thermo Scientific) and gene-specific primers (see Table 1). Calibration curves were generated for each gene using genomic DNA (regression value of ±0.95 and efficiency of ±2.0). Copy numbers of specific transcripts per 1 μg of RNA were estimated using the Corbett RotorGene 6000 software and normalized to 16S rRNA expression (7). Relative gene expression in treated samples was calculated as the ratio of normalized gene copy number after treatment to normalized gene copy number before treatment and expressed as -fold change.

Purification of Recombinant RaaS—The M. tuberculosis raaS gene was cloned into the Ndel and NheI sites of the pET15-Tev plasmid to generate a hexahistidine-tagged recombinant protein (4). Protein expression in Escherichia coli BL21(DE3) was induced by isopropyl β-D-thiogalactopyranoside at a final concentration of 0.2 mM. Recombinant RaaS was purified using a HiTrap 1-ml IMAC HP column (Amersham Biosciences). Site-directed mutants of RaaS were generated using a GeneArt mutagenesis kit (Invitrogen) according to the manufacturer’s instructions.

Fluorescence Anisotropy—The synthetic oligonucleotides, containing the imperfect direct repeats (Pr14F and Pr14R in Table 1), were covalently labeled with ATTO 647N succinimidyl ester dye (Invitrogen). Steady-state fluorescence anisotropy binding titrations were carried out on a ‘Tecan Safire’ micro-plate reader using a 635-nm light-emitting diode for excitation and a monochromator set at 680 nm (bandwidth of 20 nm) for emission in buffer containing 50 mM Tris-HCl (pH 8.5) and 150 mM NaCl.

Isotothermal Titration Calorimetry (ITC)—RaaS protein and oleoyl-CoA (Sigma) were diluted in 50 mM Tris-HCl (pH 8.5) and 150 mM NaCl. RaaS (15 μM in a 1.4-ml cell) was then titrated at 25 °C by 5-μl injections of the ligand (250 μM in the syringe) using a VP-ITC calorimeter (MicroCal). Raw data were normalized and corrected for heats of dilution of the ligand. Binding stoichiometries, enthalpy values, and equilibrium dissociation constants were determined by fitting the corrected data to a bimolecular interaction model using Origin 7 software (OriginLab).

Small-angle X-ray Scattering Experiments and Data Analysis—Synchrontron x-ray scattering data were collected on the SWING beamline of the SOLEIL Synchrontron (Gif-sur-Yvette, France) using a PCCD-170170 detector at a wavelength of 1.03 Å. The scattering patterns were measured by merging 10–20 data recordings with 1-s exposure time each for several solute concentrations at 1 and 3 mg/ml. To check for radiation damage, all successive exposures were compared, and no changes were detected. Using a sample-detector distance of 1.8 m, a range of momentum transfer of 0.0065 < s < 0.6 Å−1 was covered (s = 4πsin(θ)/λ, where 2θ is the scattering angle, and λ = 1.5 Å is the x-ray wavelength). The data were processed using standard procedures and extrapolated to infinite dilution using the program PRIMUS (8). The forward scattering, I(0), and the radius of gyration, Rg, were evaluated using Guinier approximation, assuming that at very small angles (s < 1.3/Rg), the intensity is represented as I(s) = I(0)exp(−s2Rg2/3).

EMSA—Annealed Pr14F and Pr14R (Table 1), containing the RaaS-binding site or the raaS upstream region (174 bp), were used for EMSAs as described previously (5). Briefly, the reaction buffer (10 μl) contained 50 mM Tris HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl, 4% (v/v) glycerol, and 1 g of sheared salmon sperm DNA per reaction. 32P-Labeled annealed oligonucleotides and purified recombinant RaaS were added to the reaction at concentrations of 60 and 215 nM, respectively, except where stated otherwise. Oleoyl-CoA, palmitoyl-CoA, and dodecyl-CoA were added to samples as indicated. The samples were incubated for 10 min at room temperature before running on a polyacrylamide gel as described above. Following electrophoresis, gels were fixed in 20% (v/v) methanol and 10%
(v/v) glacial acetic acid for 10 min, dried at 65 °C under vacuum, exposed overnight, and scanned with a Canon 5600F scanner.

Native Gel Electrophoresis—Wild-type RaaS and site-directed mutants were loaded onto 10%(w/v) polyacrylamide gels and run in 0.5 \( \times \) Tris borate/EDTA buffer (pH 8.5) for 20 min at 230 V. Gels were stained using colloidal Coomassie Blue stain (Sigma). Oleoyl-CoA was added at concentrations of 30 and 60 \( \mu \)M.

RESULTS

Prediction of Putative RaaS Ligands Using Bioinformatic Approaches—RaaS is annotated as a member of the TetR transcriptional regulator family (pfam00440). It has a short N-terminal DNA-binding domain and a C-terminal domain comprising the effector-binding site. The latter is responsible for the conformational and dynamic changes that regulate DNA binding. Many TetR proteins control expression of multidrug efflux pump systems in which DNA binding of the repressor is regulated by the corresponding exported drug that interacts with the ligand-binding domain (9). However, we could not detect direct binding of RaaS to any of the antimicrobials (ethambutol, isoniazid, and cerulenin) that enhance mycobacterial survival (5); the ITT traces displayed identical spikes with increasing antimicrobial concentrations (up to 5 mM).

Extensive fold-recognition analysis was performed using the @TOME-2 server (10), and a structural alignment with proteins sharing low sequence identity (<20%) to RaaS was generated. These distantly related proteins share a strong hydrophobic ligand-binding pocket within the C-terminal domain. One of these proteins, YsiA (FadR), is a transcriptional regulator of the fatty acid degradation pathway in Bacillus subtilis, and its structure has been solved in complex with stearoyl-CoA (Protein Data Bank ID 3WHB) (11). Comparative modeling of RaaS suggested that it has features compatible with the binding of the CoA moiety of the acyl-CoA ligand, despite a low sequence identity to the regulator domain of YsiA (14%) (Fig. 1).

In particular, the C-terminal domains (involved in ligand binding) of both proteins contain conservation clusters around a short motif D\( \times \)R (Asp-138 and Arg-140 in RaaS). The conserved aspartate does not interact directly with the effector, but instead, it is involved in N-terminal cap of an \( \alpha \)-helix and in the formation of a salt bridge with the conserved arginine. Arg-140 is then well positioned to directly interact with the adenosine acyl-CoA moiety of the YsiA ligand (ID 3WHB). We examined residues in the neighborhood position of the D\( \times \)R motif. The Gln-154 lateral chain in FadR points toward the ligand in the YsiA structure. This residue is Arg-144 in RaaS. In addition, the dimeric structure motif, Tyr-174 (corresponding to Tyr-174 in RaaS). This residue in the monomer is in contact with the ligand molecule bound to the other monomer. The aromatic ring of the tyrosine is stacked with the adenine ring of the acyl-CoA ligand. Thus, according to our dimer model, Tyr-174 in RaaS was postulated to play the same role as Tyr-174 in YsiA to stabilize the bound acyl-CoA. On the basis of these observa-
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Oleoyl-CoA Regulates RaaS Binding to DNA—Mycobacterial growth media and eukaryotic cells (a natural niche for mycobacterial replication and persistence) are rich sources of oleate (12). Mycobacteria convert oleate to oleoyl-CoA by fatty acyl-CoA ligases (13), and oleoyl-CoA is an abundant fatty acid precursor for the synthesis of mycobacterial cell wall components. Therefore, we tested the effect of oleoyl-CoA on the RaaS-DNA interaction using EMSAs. As shown in Fig. 2A, oleoyl-CoA was able to completely abolish the RaaS-mediated band shift at concentrations = 15 μM. Using ITC, we demonstrated direct binding of oleoyl-CoA to RaaS and a dissociation constant ($K_d$) for oleoyl-CoA binding to RaaS of 3.65 ± 0.28 μM (Fig. 2B).

We next investigated whether other acyl-coenzyme derivatives have any effect of the RaaS-DNA complex. Oleoyl-CoA (C18:1), palmitoyl-CoA (C16:0), and palmitoleoyl-CoA (C16:1) had no effect on the band shift in the EMSA (Fig. 3, A–C). Stearoyl-CoA (C18:0) showed a dose-dependent inhibition; however, it did not eliminate RaaS-DNA binding even at the highest concentration tested, 100 μM (Fig. 3D). These results suggest that RaaS preferably binds C18 fatty acid derivatives (C18:1 oleoyl-CoA or, to some extent, C18:0 stearoyl-CoA). Longer acyl-CoA metabolites (>C18) could not be tested in our assay due to their poor solubility.

### Figure 2

**Oleoyl-CoA regulates RaaS binding to DNA.**

A, purified RaaS (215 nM) was mixed with radiolabeled annealed Pr14F/Pr14R oligonucleotides (60 nM); oleoyl-CoA was added as indicated. B, characterization of the interaction between RaaS and oleoyl-CoA by ITC. Samples were prepared as described under “Experimental Procedures.” Oleoyl-CoA bound to RaaS with a $K_d$ of 3.65 ± 0.28 μM in an enthalpy-driven reaction ($\Delta H = -6.42 ± 0.14$ kcal/mol). The band shift identifying RaaS binding to DNA is marked with an arrow.

### Figure 3

**Effect of acyl-CoA derivatives on RaaS-DNA binding.** Lauroyl-CoA (A), palmitoyl-CoA (B), palmitoleoyl-CoA (C), stearoyl-CoA (D), and oleoyl-CoA (E) were added at 0, 5, 10, 25, 50, and 100 μM, respectively.

We confirmed the structural integrity of the RaaS proteins by performing small-angle x-ray scattering experiments.
Guinier plot analysis yielded radii of gyration of 30.4 ± 1, 29.7 ± 1.8, and 30.7 ± 1.3 Å for the wild-type and mutant R144A and Y174A proteins, respectively. The R140A mutant was unstable at high concentrations, and therefore, it was not possible to obtain reliable data for this protein. The apparent molecular masses calculated from the I(0) values were 40, 44, and 40 kDa for wild-type RaaS, R144A, and Y174A, respectively, confirming that all proteins were present as folded homodimers in solution.

We next investigated their binding to DNA. All three mutations caused substantial increases in the dissociation constant measured by fluorescence anisotropy, ranging from 129 ± 70 nM for R144A to 611 ± 120 and 654 ± 300 nM for Y174A and R140A, respectively, compared with 31 ± 6 nM determined for wild-type RaaS (Fig. 4). The reduced DNA binding affinity resulting from these mutations was consistent with the results of EMSAs. As shown in Fig. 5A, the mutant proteins did not cause substantial DNA band shifts under the conditions used. The observed changes in the RaaS-DNA binding affinity in the wild-type and mutant proteins could be explained by altered RaaS conformation and/or flexibility, as observed in other TetR-like regulators (14). We therefore could not use EMSA to investigate the influence of oleyl-CoA on the interaction of mutant RaaS with its DNA-binding site. The mutants also required higher oleyl-CoA concentrations for titration in ITC experiments compared with wild-type RaaS. In fact, the RaaS-oleoyl-CoA binding affinities for the mutant proteins could not be measured by ITC because oleyl-CoA aggregates at the high concentrations required (>300 µM), reducing data quality. We thereupon employed native gel electrophoresis to investigate oleoyl-CoA binding to RaaS mutants, measuring the effect of oleoyl-CoA binding on migration of RaaS proteins. As shown in Fig. 5B, the addition of 30 or 60 µM oleoyl-CoA resulted in more...
Oleoyl-CoA is a Ligand of RaaS

Oleic Acid Influences RaaS-mediated Biological Effects—Finally, to confirm the biological relevance of our findings with oleoyl-CoA, we investigated the effect of oleic acid on expression of the RaaS regulon and the drug-mediated improvement of M. bovis BCG survival. We have recently shown that RaaS is an autorepressor protein, and its regulon includes several genes encoding efflux pumps and conserved proteins of unknown function (5). In mycobacterial cells, oleic acid is converted to oleoyl-CoA by the coordinated action of fatty acyl-AMP ligases and acyl-CoA-synthesizing fatty acyl-CoA ligases (12), resulting in an increase in intracellular acyl-CoA concentration. An increase in oleoyl-CoA concentration should prevent the transcriptional repressor RaaS from binding DNA, leading to elevated expression of the RaaS efflux regulon in nonreplicating mycobacteria, which is detrimental to long-term survival. Expression levels of selected genes belonging to the RaaS regulon were measured using quantitative RT-PCR. We compared the effect of oleic acid treatment on expression of raaS, bgc_1277c, bgc_1278c, and drrC in logarithmic and stationary growth phases. Oleic acid had no statistically significant effect on expression of these genes in logarithmic phase (Fig. 6A). However, expression of RaaS-regulated genes (bgc_1277c, bgc_1278c, and drrC) was induced by 4.8-, 2.7-, and 3.7-fold, respectively, in stationary phase M. bovis BCG cultures 24 h after treatment with oleic acid (Fig. 6A). Expression of raaS (in the same operon as bgc_1277c and bgc_1278c) in stationary phase mycobacteria was also increased after treatment with oleic acid. Exposure of log phase mycobacteria to oleic acid did not result in growth alteration (Fig. 5B). Strikingly, the addition of oleic acid to 1-month-old stationary phase cultures completely abolished the antimicrobial survival-enhanced effect (Fig. 6C) mediated by RaaS, thus implicating oleoyl-CoA in the regulation of long-term mycobacterial survival.

DISCUSSION

Expression of bacterial operons is tightly controlled by transcription factors, repressors or activators, which alter transcript levels in response to physiological bacterial state or environmental cues (15). In some cases, as pertinent here for RaaS, repressors are cotranscribed with their regulon (14). RaaS is a transcriptional regulator that plays an important role in long-term mycobacterial survival in vitro and in vivo (5). Moreover, RaaS mediates an improvement in mycobacterial survival after exposure of nonreplicating bacteria to antimicrobial agents targeting cell wall biosynthesis. Here, we have demonstrated that the RaaS-DNA complex is regulated by acyl-CoAs, specifically oleoyl-CoA and, to a lesser extent, stearoyl-CoA (Figs. 2B and 3). The addition of oleic acid, a precursor of oleoyl-CoA, completely abolished the survival-promoting effects of ethambutol and dysregulated the RaaS regulon (Fig. 5), confirming the biological relevance of oleoyl-CoA as a regulator of mycobacterial persistence.

During the preparation of this manuscript, a crystal structure of RaaS (Rv1219c) was solved (16). The structure analysis confirmed our predictions concerning the importance of Arg-140 and Arg-144, but not Tyr-174, in ligand binding. However, the structure revealed the presence of a very large hydrophobic cavity of 880 Å³ compared with the calculated volume of the aliphatic chain of oleoyl-CoA of 345 Å³ (Fig. 7B). The RaaS cavity size significantly exceeds volumes of the binding pocket of YsiA (403 Å³) (Fig. 7A) or other mycobacterial TetR regulators such as EthR (399 Å³; Protein Data Bank ID 4DW6) and KstR (190 Å³; ID 3MNL). This striking difference in the RaaS structure indicates that other longer chain acyl-CoAs or complex lipids containing fatty acids might potentially fit into the binding pocket and

![Image](image_url)
regulate the DNA-binding activity of RaaS. Mycobacteria are able to produce and export a great variety of lipids containing fatty acids from oleic acid itself (17) to acyl-trehaloses (18). It is possible that the RaaS-regulated Rv1218c/Rv1217c pump is involved in the export of these oleate-containing lipids. Moreover, DrrC (which may also be regulated by RaaS) has been implicated in the export of the complex lipid phthiocerol dimycocerosate in \textit{M. tuberculosis} (19).

Derivatives of fatty acids have been previously demonstrated to regulate the expression of mycobacterial lipid transporters (20). Our findings support the importance of acyl-CoA metabolites in the regulation of mycobacterial efflux and long-term persistence. As free acyl-CoA accumulates, RaaS is released from its DNA-binding site, and downstream genes encoding efflux pumps are expressed (Fig. 8A). Depletion of free acyl-CoA allows RaaS to bind to DNA, resulting in repression of the downstream transporter genes (Fig. 8B).

ATP-dependent efflux pumps are integral components of the energy and metabolic circuitry of growing cells, and their activity depends on the metabolic state of bacteria, which may be finely tuned by metabolites binding to transcriptional regulators. In non-growing cells, the levels of acyl-CoAs drop following the slowing down of metabolism. Under stressful conditions (for example, hypoxia or iron limitation), mycobacteria can redirect carbon flux from the tricarboxylic acid cycle to alternative metabolic pathways and to the synthesis of triacylglycerols (21, 22). This mechanism is important for long-term survival, as it generates storage compounds, removes toxic fatty acid derivatives, and depletes free fatty acids and their CoA derivatives (21, 22).

We hypothesize that under stressful conditions or to survive long-term during infection, free RaaS binds to its DNA recognition sequence, repressing the transcription of this cluster of efflux pump genes and itself in a classical feedback loop (Fig. 8).
We further propose that antimicrobial treatment potentiates this process, directly or indirectly inhibiting fatty acid synthases and rapidly depleting residual (C_{16}–C_{18} chain) fatty acid precursors (23–25).

We have shown that RaaS is a component of the complex regulatory mechanisms orchestrating a coordinated down-regulation of energy-consuming processes and the activation of regulatory mechanisms orchestrating a coordinated down-regulation of energy-consuming processes and the activation of regulatory mechanisms. Our findings indicate that dysregulation of efflux pumps can be employed to kill non-growing mycobacteria.

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induces accumulation of the FASI end products and cell lysis of *Mycobacterium smegmatis*. *J. Bacteriol.* 182, 4059–4067