Characterization of phylogenetically distant members of the adenylate cyclase family from mycobacteria: Rv1647 from *Mycobacterium tuberculosis* and its orthologue ML1399 from *M. leprae*

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Analysis of the genome sequence of *Mycobacterium tuberculosis* H37Rv has identified 16 genes that are similar to the mammalian adenylate and guanylate cyclases. Rv1647 was predicted to be an active adenylate cyclase but its position in a phylogenetically distant branch from the other enzymes characterized so far from *M. tuberculosis* makes it an interestingly divergent nucleotide cyclase to study. In agreement with its divergence at the sequence level from other nucleotide cyclases, the cloning, expression and purification of Rv1647 revealed differences in its biochemical properties from the previously characterized Rv1625c adenylate cyclase. Adenylate cyclase activity of Rv1647 was activated by detergents but was resistant to high concentrations of salt. Mutations of substrate-specifying residues to those present in guanylate cyclases failed to convert the enzyme into a guanylate cyclase, and did not alter its oligomeric status. Orthologues of Rv1647 could be found in *M. leprae*, *M. avium* and *M. smegmatis*. The orthologue from *M. leprae* (ML1399) was cloned, and the protein was expressed, purified and shown biochemically to be an adenylate cyclase, thus representing the first adenylate cyclase to be described from *M. leprae*. Importantly, Western-blot analysis of subcellular fractions from *M. tuberculosis* and *M. leprae* revealed that the Rv1647 and ML1399 gene products respectively were expressed in these bacteria. Additionally, *M. tuberculosis* was also found to express the Rv1625c adenylate cyclase, suggesting that multiple adenylate cyclase proteins may be expressed simultaneously in this organism. These results suggest that class III cyclase-like gene products probably have an important role to play in the physiology and perhaps the pathology of these medically important bacteria.

Key words: adenylate cyclase, cAMP, enzyme, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, phylogenetic analysis.

INTRODUCTION

Class III nucleotide cyclases, represented by the mammalian adenylate and guanylate cyclases, are enzymes that have been biochemically and structurally characterized from a variety of organisms [1]. In addition, genome wide analysis has identified a number of putative genes belonging to this family in a variety of bacteria and archaebacteria [1]. Interestingly, the genomes of Actinobacteria, a group containing soil bacteria of medical and industrial interest, have been found to encode a large number of class III nucleotide cyclases with apparently complex domain organization [2]. Genomes of the *Mycobacterium tuberculosis* strains CDC1551 [3] and H37Rv [4] encode 16 and 17 cyclases respectively and some of these gene products have been cloned, expressed and biochemically characterized [5–9]. In contrast, the genome of *M. leprae* has only four cyclases predicted to be functional, based on conservation of residues shown to be important for catalytic activity. An additional eight pseudogenes are identifiable. This is reflective of the massive gene loss that has occurred in *M. leprae* [10], suggesting that genes conserved in this organism are essential for mycobacteria in general, and perhaps have a role to play in the pathogenesis of these organisms [11]. Early reports have indeed implicated a role for cAMP in the protection of mycobacteria within macrophages [12]. In addition, there is also evidence to suggest that several different pathogens, e.g. *Bordetella pertussis* and *Trypanosoma*, increase intracellular cAMP to a high non-physiological level that leads to phagocyte dysfunction [13,14]. In view of this, and the fact that mycobacteria harbour several genes for putative nucleotide cyclases, it becomes important to study the role of these enzymes in the biology and pathobiology of these organisms.

The biochemical characterization of nucleotide cyclases from different organisms will reveal the conformational flexibility that the cyclase domain can adopt, as is seen from crystal structures available of adenylate cyclases. The crystal structure of the catalytic domain of a mammalian adenylate cyclase has helped in the understanding of the reaction mechanism of these enzymes as well as identified roles for residues that are important for catalysis and substrate binding [15]. Class III cyclases have their active site at the dimer interface with both subunits contributing towards the formation of the catalytic centre [16]. Metal-binding residues are contributed by the C1 domain in the mammalian 12-transmembrane adenylate cyclase, and the substrate specifying residues as well as the transition-state stabilization residues are contributed by the C2 domain. Individual C1 or C2 domains are inactive and only a mixture of the two proteins forms the active enzyme [17]. Evidence has been observed based on mutational analysis on Rv1264 and Rv1625c adenylate cyclases from *M. tuberculosis* that residues identified in mycobacterial enzymes at positions equivalent to those important for activity in the mammalian enzymes are also important for catalysis [5,7]. Interestingly, however, for Rv1625c, mutation of residues that specify binding to the adenine nucleotide to those anticipated to convert the enzyme into a guanylate cyclase severely abrogated

Abbreviations used: 2-ME, 2-mercaptoethanol; DSG, disuccinimidyl glutarate; DSS, disuccinimidyl suberate; mAb, monoclonal antibody; Ni-NTA, Ni^{2+}-nitriloacetate.

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The sequence data for the Rv1647 gene from *Mycobacterium smegmatis* will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY742224.

adenylate cyclase activity, with no gain in guanylate cyclase activity. These mutations also led to an altered oligomeric status, thus implicating the substrate-binding residues in the maintenance of the dimeric state of the protein [9], something not apparent from the crystal structures of the mammalian counterparts. Preliminary crystallographic results of a mutant of Rv1625c suggests that differences are in fact observed between the mammalian and Rv1625c enzymes, in the structure of the loops around the dimeric interface of the enzyme [18].

The putative adenylate cyclase Rv1647 gene is conserved in *M. tuberculosis*, *M. leprae* and other mycobacteria, and may therefore be an important and essential adenylate cyclase in these organisms [2]. A number of residues required for activity are present in Rv1647, suggesting that it is indeed an adenylate cyclase. However, significant differences in primary sequence are also seen that suggest that this gene is a divergent member of the adenylate cyclase family. In this paper, we describe the cloning, expression, mutational and biochemical analyses of the Rv1647 catalytic domain. In addition, we report the cloning and the expression of ML1399, the *M. leprae* orthologue of Rv1647, which is the first adenylate cyclase to be characterized from the leprosy bacillus. Importantly, using antibodies to the *M. tuberculosis* enzyme, we demonstrate that Rv1647 and ML1399 are expressed in lysates prepared from these bacteria, thereby showing for the first time that members of the class III cyclase family are expressed in mycobacteria.

EXPERIMENTAL

Sequence analysis

BLAST analysis was performed at the NCBI website and offline using the BLAST suite of programs [19]. Multiple sequence analysis was performed using hmmalign (http://hmmer.wustl.edu) and rendered using ClustalX [20] and phylogenetic analysis was performed using Molecular Evolution Genetics Analysis (MEGA) software version 2.1 [21]. A neighbour-joining tree was constructed with Poisson's correction and tested using the interior-branch test. The consensus tree retaining branches with significant bootstrap support is shown in Figure 1. Prediction of transmembrane helices was performed using TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM). The sequence of Rv1647 used was as annotated at The Institute for Genomic Research website (http://www.tigr.org) and is longer by 12 amino acids at the N-terminus than the sequence as annotated at the Sanger Centre (http://www.sanger.ac.uk). The orthologue from *M. smegmatis* was identified by a BLAST search on its partially sequenced genome at the NCBI website (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?) and using the flanking nucleotide sequence to predict a possible open reading frame (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). In addition, we have cloned and sequenced the Rv1647 gene from *M. smegmatis* and deposited the sequence in GenBank® under accession no. AY742224.

Cloning, mutagenesis, expression and protein purification

PCR was performed using 100 ng of genomic DNA from *M. tuberculosis* H37Rv (obtained from Colorado State University under the NIH/NIAID contract N01 AI-75320) using Pfu polymerase (Fermentas Life Sciences, Burlington, Ontario, Canada) in a modified touch-down method with the following primers: 5′-CGGGATCCATGCGACTTGTGCCACAAACACC-3′ and 5′-TAGGCGGCCGCGTTCTACTGTGCATCGGGACT-3′. The *Bam*HI and *Not*I sites present in the primers were used to clone the PCR product into pPROExHT vector (Invitrogen Life Technologies) to generate plasmid pPRO-1647$^{1\text{-}328}$. Sequencing revealed the cloned sequence to be identical with that in the genome. The region containing the catalytic domain of the protein (amino acids 97–328) was obtained by cloning the XhoI–NotI fragment from pPRO-1647$^{1\text{-}328}$ into SalI–NotI-digested pPROExHT to generate the plasmid pPRO-1647$^{97\text{-}328}$. Mutagenesis was performed on the pPRO-1647$^{97\text{-}328}$ plasmid to generate Rv1647$_{D147A}$, Rv1647$_{K187E}$, Rv1647$_{D241C}$ and Rv1647$_{K187E/D241C}$ mutant proteins using a single mutagenic oligonucleotide-based method described by us earlier [22]. The primers used were the following: D147A, 5′-CGTTGGTGTTCACAGCGCTGGTCGGCTTTTCCACG-3′, K187E, 5′-GGCGGGCACATCGTCGAACGCCTAGGCGACGGGATCATGG-3′; and D241C, 5′-GATGTTGACGTCCACGCCGAGCCAGCAGGCGGCCAGCCGCTG-3′. The Rv1647$_{K187E/D241C}$ double mutant was obtained by performing mutagenesis with the D241C primer using the Rv1647$_{K187E}$ mutant DNA as the template. The Deep Vent polymerase (New

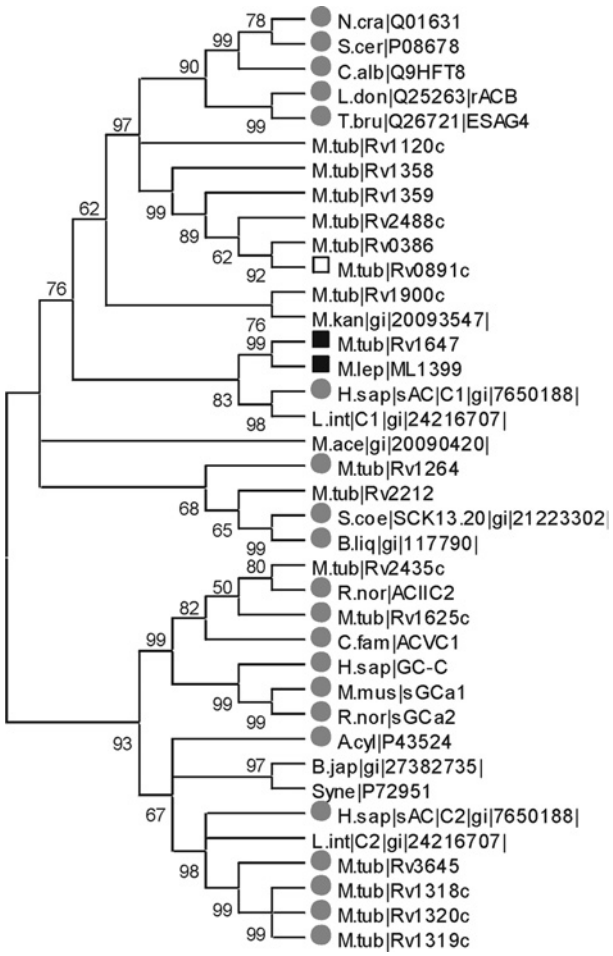

Figure 1 Phylogenetic analysis of Rv1647

(**A**) The consensus neighbour-joining tree drawn in MEGA retaining branches with >50% support in an interior-branch test of phylogeny is shown. Rv1647 and ML1399 are indicated as black squares; cyclases characterized biochemically are indicated by filled circles and the empty square indicates that the cyclase (Rv0891c) has been found to be an adenylate cyclase (A. R. Shenoy and S. S. Visweswariah, unpublished work). Abbreviations for the names of organisms are: A.cyl, *Anabaena cylindrical*; B.jap, *Bradyrhizobium japonicum*; B.liq, *Brevibacterium liquifaciens*; C.alb, *Candida albicans*; C.fam, *Canis familiaris*; H.sap, *Homo sapiens*; L.don, *Leishmania donovanii*; L.int, *Leptospira interrogans*; M.ace, *Methanosarcina acetivorans*; M.kan, *Methanopyrus kandleri*; M.lep, *M. leprae*; M.mus, *Mus musculus*; M.tub, *M. tuberculosis*; N.cra, *Neurospora crassa*; R.nor, *Rattus norvegicus*; S.cer, *Saccharomyces cerevisiae*; S.coe, *Streptomyces coelicolor*; Syne, *Synechocystis* sp.; T.bru, *Trypanosoma brucei*.

England Biolabs, Hitchin, Herts., U.K.) was used for mutagenesis and mutants were sequenced to confirm the presence of the desired mutation (Macrogen, Seoul, South Korea).

The catalytic domain protein was expressed on induction at 37 °C for 3 h in *Escherichia coli* CodonPlus RP strain transformed with the pPRO-1647$^{97-328}$ plasmid. Cells were lysed in 50 mM Tris/HCl (pH 8.5), 5 mM 2-ME (2-mercaptoethanol), 500 mM NaCl and the cytosolic fraction was loaded on to Ni-NTA (Ni^{2+}-nitriloacetate) Sepharose (Qiagen, Crawley, West Sussex, U.K.). The column was washed with the lysis buffer and then further washed with 100 mM Tris/HCl (pH 8.5), 5 mM 2-ME, 150 mM NaCl and 20 mM imidazole. Protein was eluted in 500 mM Tris/HCl (pH 8.5), 5 mM 2-ME, 500 mM imidazole and 10 % glycerol and desalted in to 50 mM Tris/HCl (pH 8.5), 5 mM 2-ME, 100 mM NaCl and 10 % glycerol using a HiTrap Desalting column on an AKTA FPLC system (Amersham Biosciences, Hong Kong, People's Republic of China). Protein was found to precipitate in the absence of NaCl and at a lower pH and was routinely stored in 5 mM CHAPS. The protein was found to bind Coomassie Blue G250 very poorly and therefore estimation of protein concentration was performed by monitoring absorbance A_{280} and using the molar absorption coefficient as calculated from the Biopolymer Calculator website (http://paris.chem.yale.edu/extinct.html). All mutant proteins were purified similarly. The full-length Rv1647 protein was purified similarly following induction for 18 h at 25 °C. All buffers during purification were sodium phosphate (pH 7.4)-based, to avoid a pH value too close to the predicted pI of the full-length protein.

The ML1399 protein from *M. leprae* was cloned using *M. leprae* genomic DNA provided by the University of Colorado as template. PCR was performed using the following primers: 5′-CGGGAATTCCCCCGTCCGCAAAATCTACC-3′ and 5′-CGGGCGGCCGCCCCAGAGCACCGAAACTAGATG-3′. The PCR product was cloned first into pGEM-T vector (Promega, Chilworth, Southampton, U.K.) and subsequently into the *Eco*RI and *Not*I sites of pPRO-ExHT vector to generate the plasmid pPRO-1399$^{1-324}$. The catalytic domain of ML1399 (amino acids 100–324) was generated by PCR using pPRO-1399$^{1-324}$ plasmid as template and a 5′-primer with sequence 5′-ACGGTCGACCTGCCGACCGGTTGCTAGGGG-3′ along with the same reverse primer as above. The product was cloned into SalI–NotI-digested pPRO-ExHT plasmid to generate pPRO-ML1399$^{100-324}$ plasmid. Sequencing confirmed the product to be identical with that seen in the genome. Full-length and catalytic domain proteins were expressed and purified in a manner similar to that used for Rv1647.

Purification of the Rv1625c protein was as described earlier [18].

Adenylate cyclase assays

Proteins were assayed at 25 °C in the presence of 100 mM Tris/HCl (pH 8.5), 5 mM 2-ME, 10 mM NaCl and 5 mM CHAPS unless otherwise described. Assays were also performed under different pH conditions (tested range 4.5–9.5) using buffers at 100 mM concentration. Activation energy was calculated from the Arrhenius plot of the initial reaction rates obtained from assays of approx. 690 nM protein at temperatures ranging from 10–45 °C (no activity was observed at 55 and 65 °C), in the presence of 1 mM MnATP and 10 mM free Mn. The assays at protein concentrations below 100 nM were performed for 2–5 min and initial reaction rates that remained constant with time have been reported. The K'_{MnATP} (app) for MnATP was estimated by assaying approx. 170 nM protein in the presence of 12 mM free Mn and different concentrations of MnATP. The concentration of free Mn varied from 10–12 mM under these conditions. As can be seen later, this concentration of free Mn is saturating. The K'_{Mn} (app) was estimated by assaying approx. 170 nM protein in the presence of a fixed concentration of 1 mM MnATP in different concentrations of the free metal. Concentrations of free metal ions in the presence of ATP were calculated using WinMaxC (http://www.stanford.edu/~cpatton/max.html).

Guanylate cyclase assays were performed with approx. 690 nM protein at 37 °C, using 1 mM MnGTP as substrate, in the presence of 12 mM free Mn. These assays were performed for 10 min. All assays were terminated with cold 50 mM sodium acetate buffer (pH 4.6) followed by incubation in boiling water for 2 min. Cyclic nucleotide concentrations were estimated using a modified RIA [23]. Rv1625c protein was assayed as described earlier in [9]. Results from enzyme kinetics experiments were fitted to the simplified Hill equation and inhibitor dose responses were fitted to the sigmoidal dose–response equation and subjected to statistical calculations using GraphPad Prism software.

Cross-linking analysis

Cross-linking was performed in 20 mM Hepes/NaOH (pH 8.5), 5 mM 2-ME and 500 mM NaCl using approx. 200 nM protein. The protein was incubated at 4 °C for 10 min in the absence or presence of 1 mM ATP and 12 mM Mn followed by the addition of 2 mM cross-linker [DSS (disuccinimidyl suberate) and DSG (disuccinimidyl glutarate) were used] and incubation continued at 25 °C for 10 min. The assay was terminated by the addition of Laemmli loading dye. Samples were subjected to SDS/PAGE followed by Western-blotting with affinity-purified antibody raised to the Rv1647 catalytic domain.

Western-blot analysis

An antibody was raised against the purified Rv1647 catalytic domain protein in rabbits. The antibody was affinity-purified using the immunogen immobilized on NHS-activated Sepharose (Amersham Biosciences) according to the manufacturer's instructions. The coupled resin was interacted with the antiserum for 2 h at 4 °C in 50 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl. The resin was washed extensively with the same buffer. Antibody was eluted using 200 mM glycine/HCl (pH 2.3) and immediately neutralized using Tris base. The antibody was dialysed against PBS and used for Western-blot analysis. The Rv1625c antibody was affinity-purified using the purified Rv1625c catalytic domain immobilized similarly. The mAb (monoclonal antibody) HBT-12 [24,25] against the phosphate transporter PstS-1 of *M. tuberculosis* was obtained from Colorado State University.

Whole-cell lysates and subcellular fractions from *M. tuberculosis* and *M. leprae* were obtained from the Colorado State University, and protein was loaded on to gels according to the manufacturer's instructions. Proteins were subjected to SDS/PAGE (12 % polyacrylamide) and transferred on to a PVDF membrane. The Rv1647 antibody was used at a concentration of 0.1–0.2 $\mu g \cdot ml^{-1}$ and the Rv1625c antibody was used at 2.5 $\mu g \cdot ml^{-1}$. The anti-rabbit horseradish peroxidase-conjugated secondary antibody was used at 1:50 000 dilution (Amersham Biosciences) or anti-mouse conjugate at 1:10 000 dilution (Amersham Biosciences). Proteins were detected using chemiluminescence (ECL® Plus reagent, Amersham Biosciences).

RESULTS

Sequence analysis of the Rv1647 gene and its neighbourhood

Using sensitive bioinformatics tools and genome sequence analysis, we have shown earlier that the genome of *M. tuberculosis*

Table 1 Distances between groups of class III cyclases

The distances (numbers of amino acid substitutions per site) calculated between group means for drawing a neighbour-joining tree (Figure 1) with Poisson's correction are shown in the lower half of the matrix. The upper half of the matrix shows the S.E.M. values calculated from a bootstrap test (1000 replicates).

	1647	Mycobacteria	Fungi	Protists	Mammals	Bacteria	Archae
1647		0.09	0.12	0.13	0.11	0.10	0.13
Mycobacteria	1.58		0.10	0.09	0.08	0.08	0.11
Fungi	1.36	1.61		0.10	0.11	0.11	0.14
Protists	1.42	1.52	1.14		0.11	0.11	0.15
Mammals	1.75	1.71	1.83	1.83		0.08	0.012
Bacteria	1.65	1.64	1.77	1.92	1.81		0.012
Archae	1.84	1.79	1.92	1.99	2.11	1.96	

H37Rv encodes 16 putative nucleotide cyclases [2]. Of these, Rv1625c, Rv1318c, Rv1319c, Rv1320c, Rv1264 and Rv3645 have been cloned and expressed and were shown to possess adenylate cyclase activity [5–9]. Comparison of the catalytic domains of all nucleotide cyclases from the *M. tuberculosis* genome reveals a great degree of variation in their primary amino acid sequence [2] that is reflected in the phylogenetic representation of these enzymes (Figure 1 and Table 1). The *M. tuberculosis* nucleotide cyclases cluster into distinct groups and representative members of some of these clusters have been biochemically characterized (Figure 1).

Rv1647 and its orthologue in *M. leprae* (ML1399) lie on a separate branch and have greater sequence similarity to the fungal and protist class III cyclases (~30% identity across 160 amino acids) when compared with other eukaryotic cyclases (~30% identity across a 100 amino acid stretch). This clustering is supported by the distance of Rv1647 and its orthologues from the different groups of nucleotide cyclases (Table 1). Thus Rv1647 is not the only protein that is more distant to the well-characterized eukaryotic nucleotide cyclases, but is also a member of a group of *M. tuberculosis* nucleotide cyclases that has not yet been biochemically characterized.

Rv1647 encodes a predicted protein of 328 amino acids with a pI of 10.2 without any obvious hydrophobic stretches, suggesting that it is probably a cytosolic protein. Approximately 120 amino acids lie N-terminal to the predicted catalytic domain, but this region does not appear to contain any known protein domains identifiable from the current protein family databases. The class III cyclase domain of Rv1647 has residues required for catalytic activity, such as the two aspartic residues (Asp^{147} and Asp^{191}) that bind the two metal ions and the critical asparagine–arginine (Asn^{248} and Arg^{252}) pair required for transition state stabilization (Figure 2A). The substrate specifying residue pair in Rv1647 is formed by lysine (Lys^{187}) and aspartic acid (Asp^{241}), identical with the pair seen in the majority of adenylate cyclases, although a number of prokaryotic adenylate cyclases show a threonine/serine substitution in place of the aspartate residue [1,26]. Therefore we predicted that Rv1647 is an adenylate cyclase. Hannenhalli and Russell [27], using their program for predicting subclasses within protein families, have also predicted this gene to be an adenylate cyclase.

The secondary structure prediction of the sequence of Rv1647 (results not shown), and a comparison with the sequences of the mammalian C1 and C2 proteins, indicate that although one can locate the essential amino acids required for catalytic function and substrate binding, certain deletions in Rv1647 are readily observed (Figure 2A). A short deletion of approx. 6 amino acids between Asp^{212} and Ala^{213} in Rv1647 (Figure 2A) shortens $\alpha 3$, which, in the mammalian adenylate cyclase C2 domain, contains important contact points for interaction between C2 and $G_{s\alpha}$ [15]. In addition, following this region, a loop between the $\beta 4$ and $\beta 5$ (as seen in the structure of the rat C2 domain [15]) is truncated in Rv1647 (between Arg^{234} and Gln^{236}). This loop contains critical interfacial residues in the mammalian enzyme, and deletions in this loop are also seen in a number of other actinobacterial adenylate cyclases [2]. In this respect, Rv1647 is similar to the class IIIc cyclases as described recently [26].

Several other important amino acid positions are conservatively substituted in Rv1647 when compared with the mammalian counterparts. A case in point is the Phe-Ala-Asp-Ile motif just preceding the P-loop in mammalian adenylate cyclase C1 domains, which is replaced by Phe^{145}-Thr-Asp-Leu^{148} in Rv1647 and related proteins (Figure 2A). A threonine residue (Thr^{401} in the canine C1 domain; Figure 2A) known to be important in binding the ribose group of ATP in the mammalian enzyme is replaced by a serine residue (Ser^{152}) in Rv1647. However, a serine residue involved in binding the ribose (Ser^{1028} in the C2 domain; Figure 2A) is replaced by an alanine residue (Ala^{251}) in Rv1647. The phosphate-binding lysine residue (Lys^{1065} in the rat C2) is conservatively replaced by an arginine residue (Arg^{286}) in Rv1647.

Genomes of closely related mycobacterial species such as *M. tuberculosis* CDC1551 [3], *M. bovis* subsp. *bovis* AF2122/97 [28], *M. avium* subsp. *paratuberculosis* str k10 (accession no. AE016958), *M. leprae* [10] and the unfinished genome of *M. smegmatis* were analysed for the presence of orthologues of Rv1647. These organisms were found to contain a nucleotide cyclase that showed high sequence similarity to Rv1647 (Figure 2A). The proteins in the *M. tuberculosis* strains and *M. bovis* are identical, whereas putative genes in *M. leprae* (ML1399), *M. avium* (MAP1357) and *M. smegmatis* show 82, 80 and 87% sequence similarity to Rv1647 respectively.

The genes flanking Rv1647 in *M. tuberculosis* H37Rv are annotated as 'conserved hypotheticals' (Figure 2B). Rv1646 protein is a member of the PE family (Pro-Glu rich proteins), whereas Rv1648 has two transmembrane helices and is therefore predicted to be a membrane protein. The *M. bovis* genome also has a PE family gene (Mb1673 or PE17) upstream of the Rv1647 orthologue (Mb1674). However, the orthologue of Rv1646 is deleted in *M. leprae* and pseudogenes corresponding to the Rv1645 and Rv1648 flank ML1399. The region immediately upstream of Rv1647 is deleted in the *M. avium* genome as well. Therefore, despite the apparent variation in genes in the neighbourhood of Rv1647 in different genomes, it is intriguing that Rv1647 is so highly conserved. The presence of orthologues of Rv1647 in related bacteria, importantly *M. leprae*, and its sequence diversity from the characterized class III cyclases prompted us to study its biochemical and enzymatic properties in detail.

Cloning and expression of the Rv1647 protein

The full-length Rv1647 protein was found to be highly susceptible to proteolysis, expressed poorly and, despite displaying high adenylate cyclase activity, did not show a band of the expected size on a Western-blot analysis using an anti-His-tag antibody (results not shown). We therefore sought to study the activity of Rv1647 by cloning and expressing a region corresponding to the predicted catalytic domain.

The region from amino acids 97 to 328 of Rv1647 was cloned and expressed to a very high level in *E. coli*. The protein was purified to apparent homogeneity by immobilized metal affinity chromatography (Figure 3) and assayed for adenylate cyclase

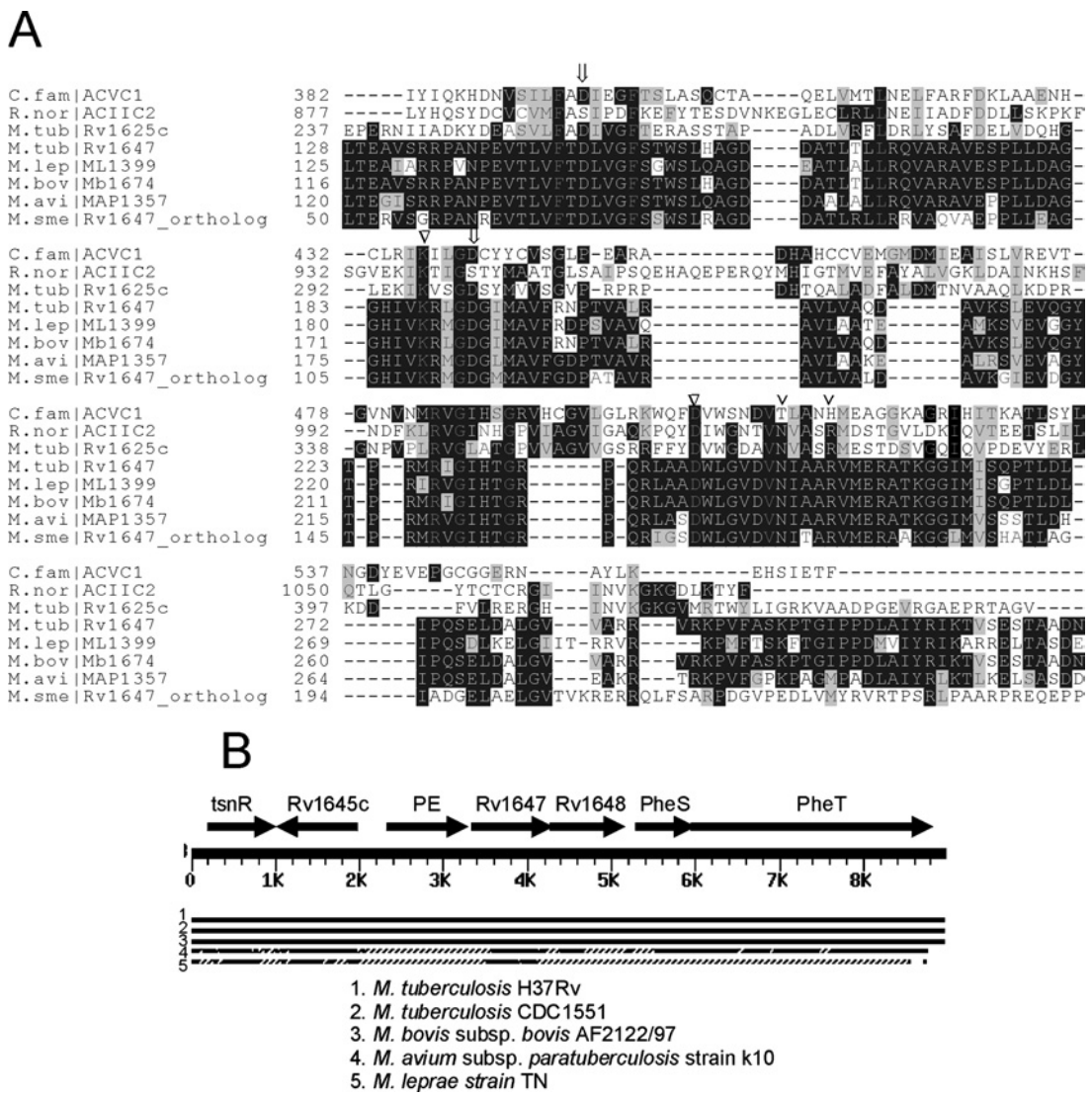

Figure 2 Sequence analysis of Rv1647, its orthologues and genome neighbours

(**A**) Multiple sequence alignment of the catalytic domains of Rv1647 and its orthologues from related mycobacteria and the mammalian adenylate cyclase C1 and C2 domains. C.fam, *C. familiaris*; R.nor, *R. norvegicus*; M.tub, *M. tuberculosis*; M.bov, *M. bovis*; M.avi, *M. avium* subsp. *paratuberculosis*; M.lep, *M. leprae*; M.sme, *M. smegmatis*. ACVC1 and ACIIC2 stand for the C1 domain of the canine type V and C2 domain of the rat type II adenylate cyclase respectively. Metal co-ordinating aspartic acids are indicated by ⇓, substrate specifying residues are indicated by ∇ and the transition state stabilizing asparagine and arginine residues are indicated by **V**. (**B**) Schematic alignment output of the nucleotide sequences of the region flanking Rv1647 from related mycobacteria whose genomes have been sequenced. Arrows indicate the genes from 5′ to 3′ direction and the *M. tuberculosis* H37Rv gene names are indicated on top. A bar with numbers in kb is given as a ruler. Solid lines indicate bit scores of > 200 and slashed lines indicate score of < 40. Notice the absence of the gene from the PE family in *M. avium* and *M. leprae*.

activity in the presence of 1 mM MgATP or MnATP as substrates with the concentration of the corresponding free metal at 12 mM. As shown in Figure 3, the activity in the presence of MnATP was nearly 200-fold greater than that observed using MgATP as substrate. The pH optimum was found to be 8.5 (results not shown). Interestingly, we found that the catalytic activity of the enzyme was enhanced in the presence of detergents (2–2.5-fold activation) and CHAPS was found to be the most effective in this case (results not shown). Therefore protein was stored and assayed routinely in the presence of 5 mM CHAPS. In addition, the presence of high concentrations of NaCl (500 mM) also led to an increase in activity in a manner similar to that seen with detergents. The optimum temperature for activity was 37 °C and the activation energy was 74.4 ± 4.8 kJ · mol^{-1} (results not shown). No guanylate cyclase activity was detected at any pH (results not shown).

The relationship between the protein concentration and activity is shown in Figure 4(A). Significant activity was observed at low concentrations of protein (< 1 μM), indicating that the affinity of the protomers of Rv1647 to form dimers is high. A slight reduction in activity was observed at very high concentrations of protein, in a manner similar to that earlier reported for Rv1625c [5].

Kinetic analysis of the purified protein, as expected, displayed some co-operativity between the two catalytic sites (Figure 4B). This is similar to the kinetics observed with homodimeric eukaryotic guanylate cyclases [29–31]. The activity of the enzyme with increasing concentrations of free Mn in the presence of 1 mM MnATP showed sigmoidal enzyme kinetics (Figure 4C). In a similar assay with increasing concentrations of Mg in the presence of a fixed concentration (1 mM) of MgATP, the K'_{Mg} (app) was found to be approx. 7 mM and the $V_{\max}$ (app) was 7.7 nmol of cAMP produced · min^{-1} · (mg of protein)$^{-1}$ (results not shown).

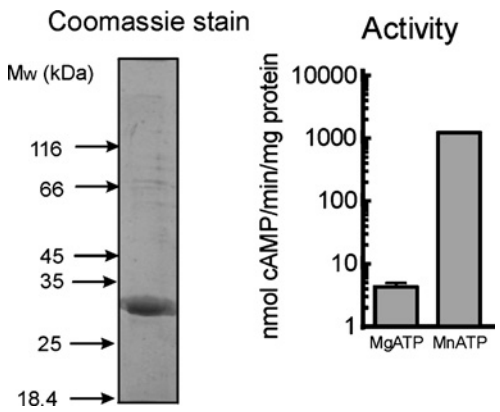

Figure 3 Purification and enzymatic activity of Rv1647

Coomassie-stained SDS/polyacrylamide gel of the purified Rv1647 catalytic domain protein after Ni-NTA chromatography. Protein was assayed in the presence of 5 mM CHAPS with 1 mM ATP and 12 mM metal (Mg or Mn) as indicated. Values represent the means for duplicate determinations of a representative assay.

Oligomeric state of Rv1647

Using chemical cross-linkers, we observed that Rv1647 can form a dimer under assay conditions in the presence of 500 mM NaCl. As shown in Figure 5, the purified protein could form cross-linked dimers in the presence of DSS and DSG that have distinct spacer arm-lengths (11.4 and 7.7 Å respectively) between their reactive groups. Cross-linking performed in the presence of MnATP did not increase the relative fraction of dimeric and higher oligomeric species. To confirm that the dimeric nature of Rv1647 was necessary for catalysis, mutagenesis and complementation experiments were performed as described below.

Mutational analyses and complementation of activity between mutants

We have earlier shown that the substrate specifying residues in the Rv1625c adenylate cyclase also play a role in maintaining the optimal oligomeric status of the protein [9]. To assess this role of the substrate specifying residues in Rv1647, we generated mutants in which either one ($Rv1647_{K187E}$ and $Rv1647_{D241C}$) or both ($Rv1647_{K187E/D241C}$) of the substrate-binding residues were changed to those present in guanylate cyclases. Neither protein, however, showed any guanylate cyclase activity (results not shown). However, a loss of adenylate cyclase activity was observed for $Rv1647_{K187E}$ and $Rv1647_{K187E/D241C}$ mutant proteins (Figure 6A). Importantly, all the mutant proteins could form dimers on cross-linking, in a manner similar to the wild-type protein (results not shown).

We proceeded to generate the C2-like $Rv1647_{D147A}$ mutant, where one of the metal-binding residues that attribute a C1-like function is mutated to an alanine residue. This mutant protein should be capable of complementing the inactive C1-like $Rv1647_{K187E/D241C}$ mutant (with mutated substrate specifying residues), if the catalytic mechanism of Rv1647 was similar to other known class III cyclases (Figure 6B). This was indeed observed, as $Rv1647_{D147A}$ protein had very low activity, but when reconstituted with $Rv1647_{K187E/D241C}$ generated a protein with robust adenylate cyclase activity (Figure 6C). These results show conclusively that Rv1647 functions as a homodimer, with residues required for catalytic activity being similar to those described in the mammalian enzymes.

Interestingly, complementation of catalytic activity was not observed using a C1-like-mutant of Rv1625c protein described earlier in [9], or the C2-like Rv1647 protein. Thus no increase in activity was observed on mixing $Rv1625c_{K296E/D365C}$ with the $Rv1647_{D147A}$ mutant, indicating that these proteins are incapable of heterodimerizing with each other (Figure 6C). These biochemical

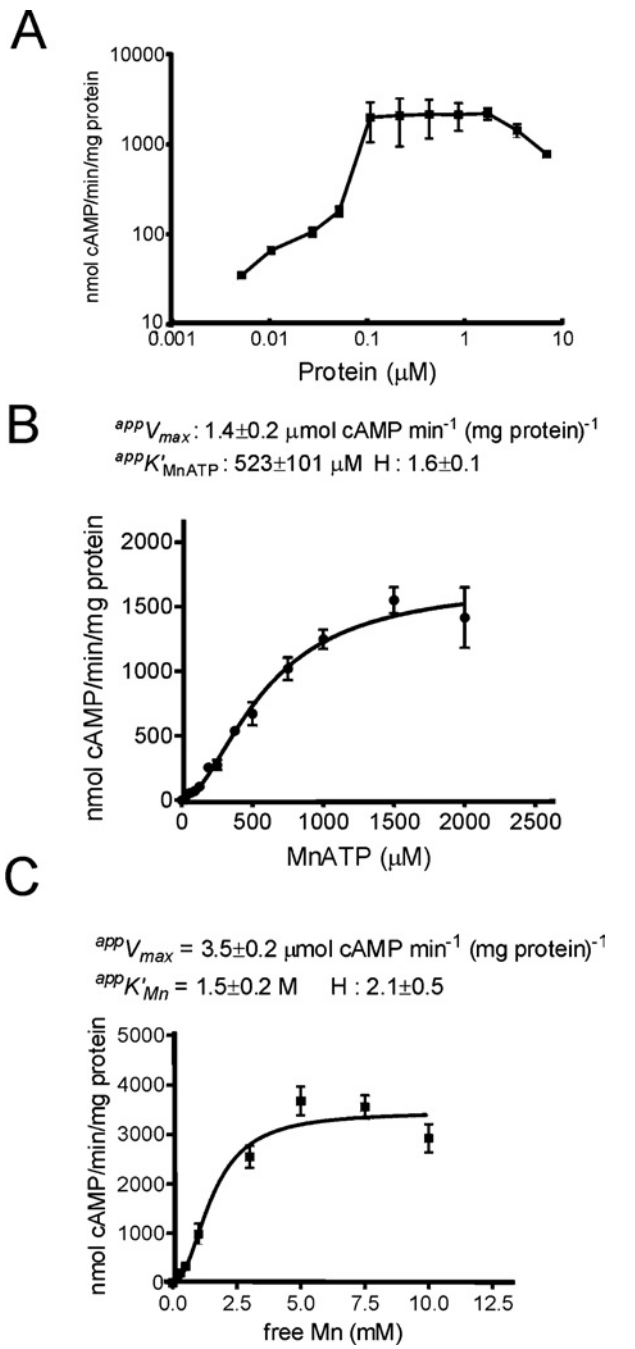

Figure 4 Biochemical characterization of the Rv1647 catalytic domain protein

(**A**) Purified protein was assayed at indicated concentrations with 1 mM MnATP and 10 mM free Mn, and initial reaction rates are plotted. Results are representative of assays performed twice in duplicate and means ± S.E.M. are shown. (**B**) Rv1647 protein (~170 nM) was assayed in the presence of different concentrations of substrate (MnATP) as indicated in the presence of a fixed amount of 10 mM free Mn. Assays were performed three times in duplicate and means ± S.E.M. ($n = 6$) are shown. (**C**) Protein (~170 nM) was assayed in the presence of a fixed concentration of 1 mM MnATP and different concentrations of free Mn as indicated. Experiments were performed twice in duplicate and means ± S.E.M. ($n = 4$) are shown.

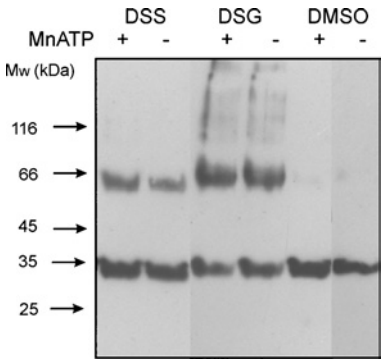

Figure 5 Oligomeric status of the catalytic domain of Rv1647

Cross-linking was performed using 2 mM DSS or DSG or in the presence of solvent alone (DMSO), and in the presence or absence of 1 mM ATP and 12 mM Mn as indicated. Samples were transferred on to PVDF and analysed by Western blotting using an affinity-purified antibody against the Rv1647 catalytic domain.

studies have shown that although Rv1647 is an adenylate cyclase, with the requirement of catalytic residues similar to those seen in other class III cyclases, the residues required for correct dimer formation are sufficiently different to disallow heterodimerization with Rv1625c.

Regulation of catalytic activity of Rv1647

The effect of known regulators of mammalian adenylate cyclases on the activity of Rv1647 was tested. Its activity in the presence of forskolin, the P-site inhibitor 2′-deoxy-3′-adenosine monophosphate, calcium and bicarbonate, using MgATP as substrate, was not altered (results not shown).

We have recently shown that tyrphostins are inhibitors of mammalian adenylate and guanylate cyclases [30]. The purified intracellular domain of receptor guanylate cyclase C and the engineered soluble construct of the mammalian adenylate cyclase were inhibited by tyrphostin A25 with IC_{50} values of approx. 13 and 16 μM respectively [30]. The structural requirement for

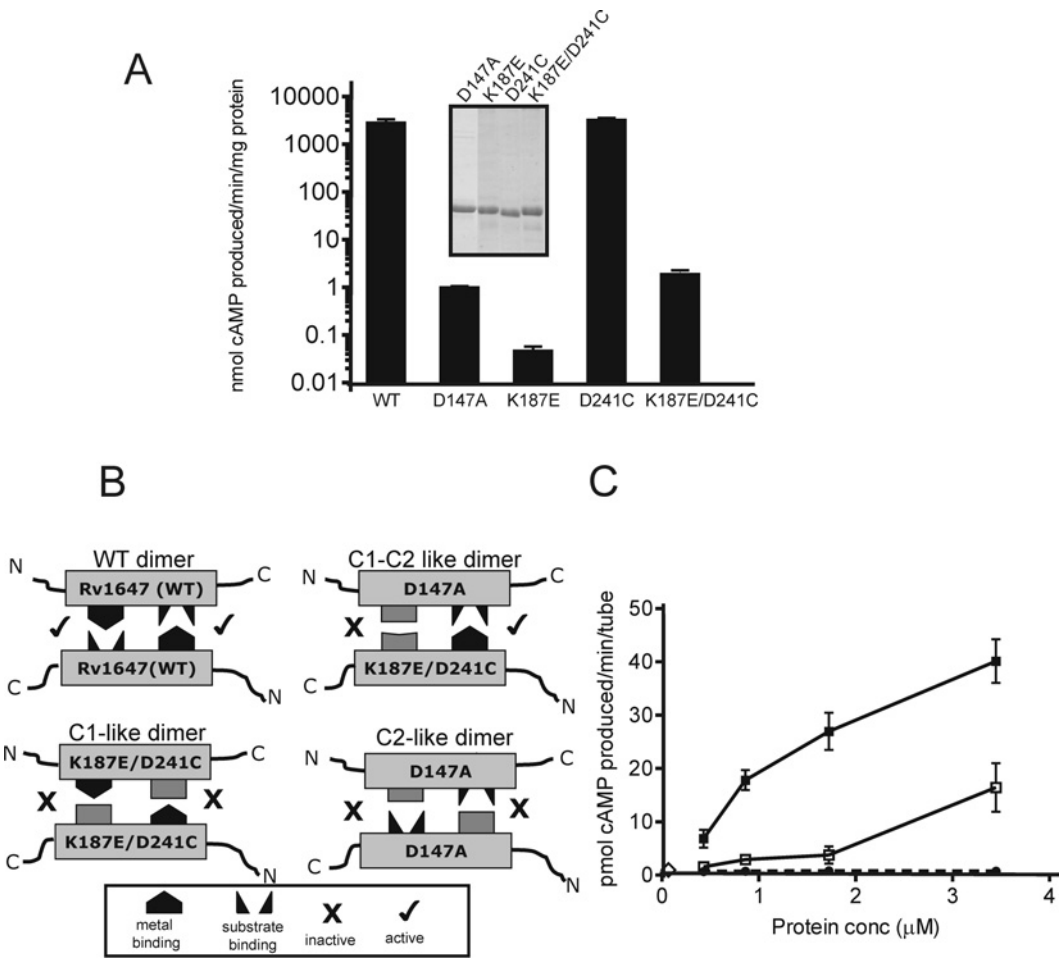

Figure 6 Mutational studies on the catalytic domain of Rv1647

(**A**) The adenylate cyclase activity of indicated proteins (~690 nM) when assayed with 1 mM MnATP and 10 mM free Mn is shown. Results shown represent means ± S.E.M. ($n = 4$). Coomassie-stained gel showing the purified mutant Rv1647 catalytic domain proteins is shown as an inset. (**B**) Schematic representation of complementation between C1- and C2-like Rv1647 proteins. An active site with the critical functional residues from both protomers will be competent catalytically, whereas other combinations (C1–C1 or C2–C2 homodimers) will not. (**C**) The activities of $Rv1647_{D147A}$ alone (~70 nM; ◇) and in the presence of different concentrations of $Rv1625c_{K296E/D365C}$ (●) are shown. Activities of $Rv1647_{K187E/D241C}$ at different concentrations in the absence (□) or presence of approx. 70 nM of $Rv1647_{D147A}$ protein (■) are shown. Assays were performed twice and means ± S.E.M. are shown ($n = 4$).

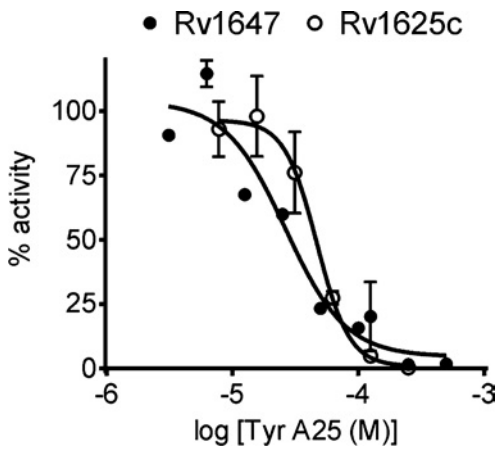

Figure 7 Effects of tyrphostin A25 on Rv1647 and Rv1625c

Proteins were assayed in the presence of indicated concentrations of tyrphostin A25 in 1 % DMSO as solvent. Values are expressed as percentage activity observed in the presence of a similar amount of solvent alone. These activities were approx. 3.5 μmol of cAMP produced · min^{-1} · (mg of protein)$^{-1}$ for Rv1647 and approx. 300 nmol of cAMP produced · min^{-1} · (mg of protein)$^{-1}$ for Rv1625c. Values are representative of assays performed at least three times and means ± S.E.M. are shown ($n = 6$).

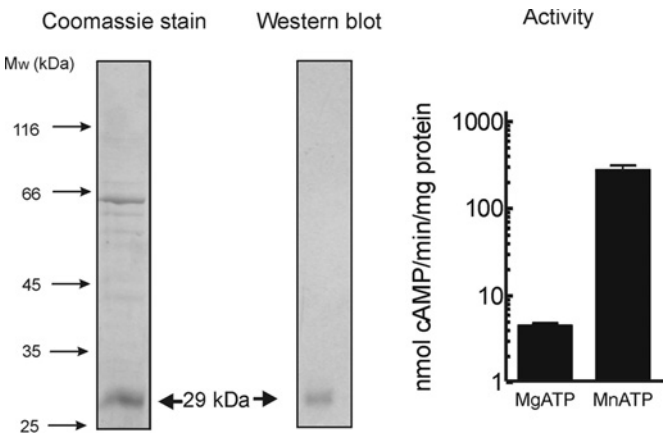

Figure 8 Expression and activity of the ML1399 protein

Coomassie-stained SDS/polyacrylamide gel showing the ML1399 catalytic domain protein of molecular mass approx. 29 kDa purified using Ni-NTA Sepharose. Western-blot analysis of the purified protein was performed with affinity-purified antibody to Rv1647. Adenylate cyclase activity of the protein (~680 nM) was performed using 1 mM ATP and 12 mM Mn at pH 8.5. Results shown represent the means ± S.E.M. for assays performed twice in duplicates ($n = 4$).

tyrphostin inhibition appeared to be the presence of two *cis*-hydroxy groups in the cyanocinnamonitrile scaffold and we have suggested that tyrphostins bind at a site close to the nucleotide-binding site [30]. We tested the inhibitory potential of tyrphostin A25 on the activities of both Rv1647 and Rv1625c enzymes. As shown in Figure 7, both enzymes were inhibited by tyrphostin A25, with similar IC_{50} values (30 ± 1 μM for Rv1647 and 40 ± 8 μM for Rv1625c), indicating that the region of tyrphostin A25 binding is conserved in mammalian and bacterial class III nucleotide cyclases.

Catalytic activity of ML1399

Only four class III cyclases are identifiable in the genome of *M. leprae*, but none have been shown to have catalytic activity to date [2]. In a previous study, we had predicted that ML0201 and ML1399 are most probably active adenylate cyclases [2]. ML1399 contains the conserved metal-binding aspartic residues (Asp^{144} and Asp^{188}; Figure 2A) and the transition state stabilizing residues (Asn^{245} and Arg^{249}). The substrate specifying pair of residues is formed by Lys^{184} and Asp^{238} and therefore the protein is probably an adenylate cyclase.

We cloned and expressed the full-length protein, and in a manner similar to our observations with Rv1647, proteolysis was observed (results not shown). In agreement with the observations made with Rv1647, a fragment containing the catalytic domain (amino acids 100–324) of ML1399 was purifiable and showed significant and high adenylate cyclase activity (Figure 8). In addition, this purified protein was recognized with the antibody to Rv1647, showing that the high sequence similarity between Rv1647 and ML1399 also results in immunological cross-reactivity (Figure 8).

Expression of Rv1647 and Rv1625c in *M. tuberculosis* and ML1399 in *M. leprae*

To date, there is no evidence to show that any of the adenylate cyclases biochemically characterized from mycobacteria are expressed in the organism. We therefore used the antibody raised against Rv1647 to detect the presence of the protein in whole-cell lysates of *M. tuberculosis* H37Rv (Figure 9A). The major cross-reacting band observed in subcellular fractions was similar to the size predicted to represent the full-length protein of molecular mass of approx. 35 kDa. In addition, the band seen at approx. 28 kDa could represent a proteolysed product. A band of approx. 42 kDa was also observed in the whole-cell lysates, cell wall and membrane fractions from *M. tuberculosis*, and could represent a post-translationally modified product. These results therefore indicate that the Rv1647 protein is expressed in *M. tuberculosis*, under the conditions used for culturing the organism and preparing the subcellular fractions.

Since we had shown that the antibody against Rv1647 could cross-react with the ML1399 protein, we performed Western-blot analysis using the Rv1647 antibody and lysates obtained from *M. leprae*. The results obtained showed a reactive band of size similar to that seen in *M. tuberculosis* (Figure 9A). Interestingly, only a single major cross-reacting band was seen, of a size predicted for the full-length protein in *M. leprae*. With the fractions that were available to us, it appeared that most of the protein was present in the cell wall, and only a minor fraction in the cytosol.

The relative purity of the subcellular fractions was confirmed by Western-blot analysis using a mAb against the phosphate transporter (PstS-1; Figure 9B), which is known to be localized only to the membrane and cell wall fraction in *M. tuberculosis* [24]. The localization of a significant fraction of Rv1647 to the membrane and cell wall may indicate its interactions with components of the plasma membrane, perhaps with the N-terminal region of the protein. This might make it more resistant to proteolysis in mycobacteria, in contrast with what we observe for the expressed full-length protein (see above).

We have earlier raised an antibody to the Rv1625c protein [9], and Western-blot analysis indicated that this adenylate cyclase was also expressed in *M. tuberculosis* H37Rv. Protein of the expected mass (47 kDa) was seen only in the membrane fraction (Figure 9B). Rv1625c has six transmembrane helices and is therefore expected to localize to the membrane fractions, and has been shown to do so on heterologous expression in bacteria and in mammalian cells [5]. The 88 kDa band seen with this antibody could represent a stable dimer of Rv1625c [5] or is a cross-reactive band, probably due to the presence of antibodies against a heat-shock protein, which co-purifies with Rv1625c protein

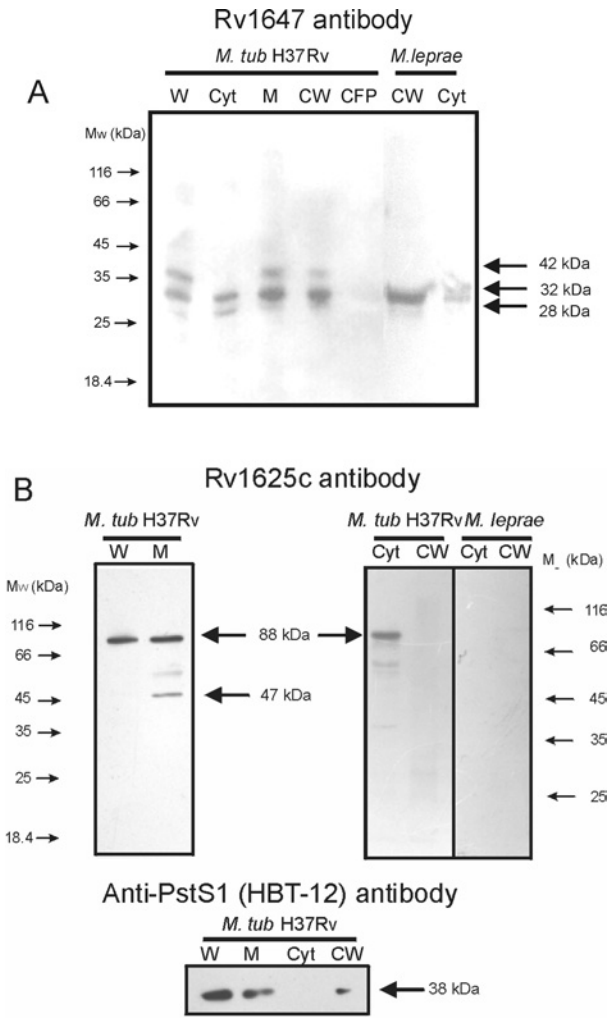

Figure 9 Expression of Rv1647 and Rv1625c in *M. tuberculosis* and ML1399 in *M. leprae*

(**A**) Whole-cell lysates and subcellular fractions (20 μg of protein) of the *M. tuberculosis* H37Rv strain (M.tubH37Rv) and *M. leprae* were obtained from Colorado State University and subjected to Western-blot analysis using an affinity-purified antibody raised against the Rv1647 catalytic domain. (**B**) Lysates were used for Western-blot analysis using the affinity-purified antibody against Rv1625c. The same fractions were also probed using a mAb (HBT-12 at 1:100 dilution) against a phosphate transporter (PstS-1). W, whole-cell lysates; Cyt, cytosol; M, plasma membrane; CW, cell wall; CFP, culture filtrate proteins. Proteins were visualized using enhanced chemiluminescence.

from *E. coli* that was used as the immunogen. The orthologue of Rv1625c in *M. leprae* is a pseudogene [2] and Western blots performed with the Rv1625c antibody on *M. leprae* cytosol and cell wall fractions showed no reactivity. These results also indicate that the antibodies that we have are specific for their respective antigens and do not cross-react with any of the other cyclases present in these organisms. This, therefore, represents the first demonstration of expression of any class III nucleotide cyclase in these organisms, and paves the way for a study of the role of these proteins in mycobacteria.

DISCUSSION

In the present study, we describe a distantly related adenylate cyclase from *M. tuberculosis*, and its orthologue from *M. leprae*. This represents the first demonstration of the biochemical properties of these gene products, hitherto annotated as 'conserved hypothetical proteins' in various databases. Our earlier analysis using bioinformatic approaches had suggested that the proteins could be adenylate cyclases [2]. Interestingly, orthologues of Rv1647 are found in a number of mycobacterial species, and our results indicating that the protein is expressed in *M. tuberculosis* and *M. leprae*, suggest that they could have an important role in the biology of these organisms.

The divergence from the mammalian enzyme is evident from a number of differences at the sequence level, most noticeably the deletion of a critical loop in Rv1647, which in the mammalian adenylate cyclases contributes to the formation of a stable dimer [15]. Moreover, a search of the simple modular architecture research tool [32] database identifies the class III cyclase domain in Rv1647 as a putative GGDEF domain [33]. However, the Gly-Gly-Asp-Glu-Phe sequence motif (after which they are named) is absent from Rv1647, and is instead replaced by Leu^{189}-Gly-Asp-Gly-Ile^{193} in which the Asp^{191} corresponds to the second metal-binding aspartate present in class III cyclases (Figure 2A). Indeed, the GGDEF domain itself has been suggested to be homologous with the class III cyclase domain [34], and a GGDEF protein from *Caulobacter crescentus* has been shown to possess diguanylate cyclase activity [35]. Rv1647 could thus be a divergent adenylate cyclase that bears some sequence similarity with the GGDEF proteins. This is further supported by the distance calculations shown in Table 1.

In view of this dissimilarity in the sequences of Rv1647 and the mammalian enzymes, it is conceivable that the dimer interface of this protein is different from that of the mammalian enzymes. The dimerization may be driven by hydrophobic interactions in Rv1647, as is suggested from the activation of the enzyme by detergents. Furthermore, the activity of the enzyme is not affected by 500 mM NaCl, at which concentration it was expected that the protomers in a dimer would fall apart, if ionic interactions mainly contributed to their association. Rv1625c was found to be inhibited by even 100 mM NaCl (A. R. Shenoy and S. S. Visweswariah, unpublished work), suggesting that its dimer interface is therefore quite different from that of Rv1647. In addition, robust activity of Rv1647 was seen at very low concentrations of protein (Figure 4A), indicating a high affinity between the protomers. This is again in contrast with that seen for Rv1625c, where increasing protein concentrations enhanced activity, whereas at high concentrations, a decrease in specific activity of Rv1625c was observed, as a consequence of aggregation [5].

Mutational studies also revealed differences in the properties of Rv1625c and Rv1647. Although the oligomeric status of Rv1625c was altered by mutations in its substrate specifying residues [9], this was not seen with Rv1647. This may at least partly be attributed to the low sequence identity (22 % in the catalytic domain) between the two proteins, and more importantly to the deletions in the β4–β5 loop in Rv1647. Both Rv1625c and Rv1647 proteins failed to gain guanylate cyclase activity on mutation of substrate-specifying residues, in contrast with the relatively easy conversion observed in the mammalian enzymes [36–38]. This could be due to additional structural changes in the mycobacterial enzymes that disallow the substrate specificity switch by mutations in just two amino acids. The fact that C1- and C2-like proteins, generated by mutagenesis in Rv1647, were capable of complementing each other indicates that the overall reaction mechanism of the protein, i.e. formation of an active site by contributions from both protomers, is similar to other class III cyclases [16]. However, Rv1625c was unable to interact with Rv1647 in the complementation assay (Figure 6C), even though Rv1625c has been shown to form heterodimers with other nucleotide cyclases [39].

Rv1647 was also not inhibited by the P-site inhibitor but showed an IC_{50} value for inhibition by tyrphostin A25 higher than that seen with the mammalian adenylate and guanylate cyclases [30]. Therefore its active site is probably different from that of the mammalian cyclases, which are inhibited by P-site compounds efficiently by forming dead-end complexes of the P-site inhibitor and the enzymes [40].

Transcripts corresponding to Rv1319c and Rv2435c from *M. tuberculosis* H37Rv have been found to be regulated under hypoxic conditions in a microarray analysis [41]. However, to date, there is no report on the presence of proteins corresponding to any of the class III cyclase-like genes in *M. tuberculosis*. Using an affinity-purified antibody to Rv1647, we demonstrate that this protein is expressed in *M. tuberculosis*, and its orthologue is also expressed in *M. leprae*. This suggests that the ML1399 gene in *M. leprae*, which is flanked by pseudogenes, is in fact functional. Western-blot analysis also indicated that a significant fraction of the protein is found in the membrane fraction, although no transmembrane regions were identifiable in the protein sequence (Figure 9). Whether this localization is due to its direct recruitment to the plasma membrane or through association with other protein(s) is not clear at the moment. Studies of the factors that control this localization as well as the activity of the different nucleotide cyclases are relevant, since more than one nucleotide cyclase may be expressed at any given time, and the local concentration of cAMP is what is critical for downstream signalling events.

Rv1625c is also expressed in *M. tuberculosis* but a cross-reacting protein is absent from *M. leprae*, which was expected since the orthologue of Rv1625c is predicted to be a pseudogene in *M. leprae*.

Rv1647 and Rv1625c are phylogenetically distant and biochemically distinct adenylate cyclases. *M. tuberculosis* may express these adenylate cyclases simultaneously, each of which may be regulated uniquely and involved in different, non-overlapping biochemical pathways. Since many of the mycobacterial adenylate cyclases can be expressed to high levels in *E. coli*, one can attempt to obtain structural information, which will provide a greater understanding of the evolution of the nucleotide cyclase domain in a structural and biochemical context through the various phyla, as well as contribute to the structural genomics programme initiated for *M. tuberculosis* [42].

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