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The *Mycobacterium tuberculosis* desaturase DesA1 (Rv0824c) is a Ca^{2+} binding protein

Veena C. Yeruva**, Mamata Savanagouder, Radhika Khandelwal, Apoorva Kulkarni, Yogendra Sharma, Tirumalai R. Raghunand*

CSIR - Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, India

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ABSTRACT

The hallmark feature of *Mycobacterium tuberculosis* (*M.tb*) the causative agent of human tuberculosis, is its complex lipid rich cell wall comprised primarily of mycolic acids, long chain fatty acids that play a key role in structural stability and permeability of the cell wall. In addition, they are involved in inhibiting phagosome-lysosome fusion and aid in granuloma formation during the pathogenic process. *M.tb* DesA1 is an essential acyl-acyl carrier protein desaturase predicted to catalyze the introduction of position specific double bonds during the biosynthesis of mycolic acids. This protein is one among three annotated desaturases (DesA1-3) in the *M.tb* genome but is unique in containing a βγ-crystallin Greek key signature motif, a well-characterized fold known to mediate Ca^{2+} binding in both prokaryotic and eukaryotic organisms. Using Isothermal Titration Calorimetry and $^{45}CaCl_2$ overlay, we demonstrate that Ca^{2+} binds to DesA1. Spectroscopic measurements suggested that this binding induces changes in protein conformation but does not lead to significant alterations in the secondary structure of the protein, a feature common to several βγ-crystallins. An *M. smegmatis* strain over-expressing *M.tb desA1* showed a Ca^{2+} dependent variation in surface phenotype, revealing a functional role for Ca^{2+}in DesA1 activity. This study represents the first identification of a Ca^{2+} binding βγ-crystallin in *M.tb*, emphasizing the implicit role of Ca^{2+} in the pathogenesis of *M.tb*.

1. Introduction

Mycolic acids are the primary component of the lipid rich cell wall of *Mycobacterium tuberculosis* (*M.tb*), the causative agent of human tuberculosis (TB). These complex molecules, are composed of long-chain alpha-alkyl, beta-hydroxy fatty acids which are extensively modified to form diverse mycolic acids containing different functional groups such as double bonds, keto, ester, epoxy, methoxy, and cyclopropane rings. These fatty acids are esterified to trehalose and arabinogalactan and are either inserted in the mycomembrane as trehalose dimycolates (TDM) and monomycolates (TMM), or linked covalently to the underlying arabinogalactan, itself bound to peptidoglycan, to form the Mycolic acid-Arabinogalactan-Peptidoglycan complex (MAPc) [1].

Several components of the cell wall are implicated in *M.tb* pathogenesis *via* modulation of its own cellular processes like permeability, neutralization of free radicals and alteration of host immune responses [2]. Mycolic acids confer unique properties to mycobacteria such as low cell wall permeability, increased survival rates in the host and resistance to commonly used antibiotics [3]. Inhibition of various steps in mycolic acid biosynthesis by antitubercular drugs such as ethambutol, isoniazid and ethionamide has proven to be a useful strategy in the treatment of TB. As a result, a detailed understanding of mycolic acid biosynthesis has been a major research objective over the last decade. Characterization of key enzymes involved in mycolic acid biosynthesis not only allows understanding their role in mycobacterial physiology but also may lead to identification of new drug targets.

Desaturation is an essential step in the biosynthesis of structurally and chemically diverse mycolic acids. Desaturases catalyze the oxidation of alkyl-saturated fatty acids to yield alkyl-unsaturated fatty acids by introducing two *cis*-double bonds at the proximal or distal end. This process facilitates introduction of various functional groups into the mero chain of α-mycolic acids to form diverse mycolic acids [4]. Three potential aerobic desaturases (encoded by *desA1*, *desA2* and *desA3*) have been identified by whole genome analysis of *M.tb* [5]. Of these, DesA1 (Rv0824c) and DesA2

* Corresponding author.
** Corresponding author.
E-mail addresses: veenachaitanya@ccmb.res.in (V.C. Yeruva), raghu@ccmb.res.in (T.R. Raghunand).

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(Rv1094) are annotated as acyl-ACP desaturases as they introduce position-specific double bonds to convert stearoyl-ACP to oleoyl-ACP. The involvement of DesA1 and DesA2 in mycolic acid synthesis has been predicted based on sequence annotation [5,6] and structural characterization of DesA2 which contains a ferritin-like domain involved in iron binding [7]. However, experimental evidence of their biochemical function is currently unavailable.

Interestingly, in addition to a desaturase domain, DesA1 is annotated to contain a βγ-crystallin Greek key signature motif. βγ-crystallins are a diverse group of structurally similar proteins involved in Ca^{2+} binding in prokaryotes. These proteins are evolutionarily related with lens βγ-crystallins which render high refractive index and transparency to the eye lens [8]. A typical βγ-crystallin domain is characterized by a pair of Greek key motifs, consisting of two signature sequences (Y/FXXXXY/FxG) that forms the β-hairpin loops [9] and two Ca^{2+} binding sites, with (N/D)(N/D)XX(T/S)S sequence stretches which run in opposite directions to coordinate Ca^{2+} binding [8–10]. The Greek key signature motifs and the Ca^{2+} binding sites come together to form a distinct double-clamp to co-ordinate Ca^{2+} binding [11–13]. The βγ-crystallin fold is considered to have evolved from a common ancestor and is found to confer higher intrinsic stability to the protein in many bacterial species [14,15]. The stability of these proteins is increased several-fold upon binding to Ca^{2+}, projecting a Ca^{2+} dependent protective role of these proteins in the respective organisms [14,16].

Of the few calcium binding proteins (CaBP) of *M.tb* identified thus far, is Ca^{2+} dodecin which is found only in *M.tb* and *M. bovis*, and has a unique Ca^{2+} binding motif not seen in other CaBPs. Its identification as an immunodominant antigen, suggests that it is a secreted antigen and has a role in host immune response [17]. Recent work from our laboratory has identified two immunomodulatory CaBPs, Rv1818c and Rv3653. These are involved in Ca^{2+} dependent immune signaling events with possible implications in the establishment and maintenance of *M.tb* infection [18].

In this study, we report *M.tb* DesA1 to be the first mycobacterial member of the βγ-crystallin family. We demonstrate that Ca^{2+} binds DesA1, altering its structural properties and functionally influencing its activity. Our findings will assist in understanding the importance of Ca^{2+} in *M.tb* physiology and consequently help in designing effective therapeutic approaches against this pathogen.

2. Materials and methods

2.1. Bacterial strains, media and growth condition

Mycobacterium smegmatis mc^2155 and *Escherichia coli* (*E. coli*) strains were cultured as described [19]. The following antibiotics were added when necessary - kanamycin (15 μg/ml for *M. smegmatis*), ampicillin (200 μg/ml for *E. coli*).

2.2. Isothermal titration calorimetry (ITC)

The Ca^{2+} binding isotherm of DesA1 was determined on a Microcal VP-ITC (Microcal Inc., USA). DesA1 (60 μM) and 5 mM $CaCl_2$ were prepared in Chelex-purified 50 mM Tris, pH 7.5, 10 mM KCl. 1.4 ml of protein was used for the binding experiment at 30 °C and aliquots of 6 μl of $CaCl_2$ were injected for each titration. Blanks were obtained by titration of the buffer with identical concentrations of Ca^{2+}. Curve fitting was performed using the Origin software (version 7.0) supplied by Microcal after subtraction with the appropriate buffer blank.

2.3. Fluorescence and circular dichroism spectroscopy

Intrinsic fluorescence emission spectra of DesA1 were recorded on an F-4500 fluorescence spectrofluorimeter (Hitachi Inc., Japan) using 0.1 mg/ml protein in 50 mM Tris-HCl, pH 8.5 and 10 mM KCl. The spectra were recorded from 300 to 450 nm at an excitation wavelength of 295 nm using excitation and emission band passes of 5 nm each. Fluorescence spectra were plotted after subtraction with the appropriate buffer blank. Changes in surface hydrophobicity of DesA1 (0.1 mg/ml) in response to varying concentrations of $CaCl_2$ (10 μM–5 mM) were monitored using bis-ANS(4,4′-Dianilino-1,1′-Binaphthyl-5,5′-Disulfonic Acid, Dipotassium Salt) (10 mM) as a probe. The samples were excited at 365 nm, and emission spectra were recorded between 400 nm and 650 nm. The spectra were corrected for equal concentrations of bis-ANS in buffer. Far-UV CD spectra of DesA1 (0.1 mg/ml) were recorded on a Jasco J-815 spectropolarimeter in various concentrations of Ca^{2+} (10 μM–5 mM) in 50 mM Tris buffer, pH 7.5, 10 mM KCl. Samples were scanned from 200 to 250 nm using a 0.1 cm path length quartz cuvette. The α-helical and β-strand content of DesA1 were derived from its far-UV CD spectrum using the K2D3 software (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/) [20].

2.4. Calcium dependent spot phenotype of DesA1

To investigate the effect of Ca^{2+} on DesA1 function, an expression construct of *M.tb desA1* was generated by amplifying its ORF from *M.tb* H37Rv genomic DNA using the gene specific forward (5′-ATCGAGGATCCATGTCAGCCAAGCTGACCG-3′) and reverse (5′-TGCATGAATTCCTAACGACGGCTCATCGC-3′) primers, and cloning this product between the *EcoR*1 and *BamH*1 sites of pMV261, downstream to the constitutive *hsp60* promoter. The recombinant plasmid was transformed into *M. smegmatis* and for gene expression analyses, total RNA was isolated from the recombinant strain at its exponential phase of growth using TRIzol reagent (Invitrogen). Following DNAse I treatment, cDNA synthesis was performed using Superscript II (Invitrogen) and subsequently used as a template for PCR amplification using the *desA1* specific forward (5′- CACCTGAGCATGCACAAGC-3′) and reverse (5′-TGATACGACGGCAGGTTGTC-3′) primers to generate 200 bp amplicons. Samples without reverse transcriptase (-RT) served as negative controls in the analysis. For assessment of the Ca^{2+} dependent function of DesA1, recombinant *M. smegmatis* strains expressing *M.tb desA1* as well as the empty vector (pMV261) were grown in Middlebrook 7H9 broth containing kanamycin in the presence and absence of 1 mM EGTA to the logarithmic phase of growth. 1 μl of these cultures were spotted on Middlebrook 7H10 agar plates with and without 1 mM EGTA and the spot phenotypes examined following incubation of the plates at 37 °C for 4 days.

Materials and methods described in supplementary information include - DNA manipulations, purification of recombinant proteins and multiple sequence alignment.

3. Results

3.1. DesA1 contains an atypical βγ-crystallin signature

M.tb DesA1 (Rv0824c) is annotated as a fatty acyl desaturase type 2 and is listed to contain the βγ-crystallin signature motif (http://tuberculist.epfl.ch/). Based on this annotation, we scanned the sequence for the presence of the βγ-crystallin signature (Fig. 1A). The amino acid sequence of DesA1 has a stretch of WXXFXXXXFXG (from amino acid residues 270 to 280) followed by a serine residue at position 306, which is characteristic of a βγ-crystallin domain. The WRIFEREDFTG stretch of aa likely forms the β-hairpin loop between the first and second β-strand, the WRIF sequence denotes the Trp corner of the first β-strand, and FTG is the second β-strand of the four-stranded Greek key motif (Fig. 1A,

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marked in green). Thus, based on sequence analysis, we identified the above-mentioned sequence to be the βγ-crystallin Greek key signature motif. Multiple sequence alignment of this motif from known βγ-crystallins with the identified signature in DesA1 showed that these sequences share significant similarities (Fig. 1B). The Greek key motifs in a βγ-crystallin domain exist in pairs, and the Trp corner is generally located upstream to the second motif, implying that this motif is represented in DesA1 by the above-identified sequence. The first Greek key motif is not obvious as per its known sequence features, but it is likely that the sequence FQVPEFRR from 235 to 242aa (Fig. 1A, marked in red) represents this motif with the absence of the conserved Gly residue (Fig. 1A). This is not unusual, as variations in sequences of this kind have been observed in other βγ-crystallins such as nitrollin and DdCaD [11,21], leading us to conclude that DesA1 possesses a non-canonical βɣ-crystallin signature.

3.2. Ca^{2+} binds DesA1

It is known that many bacterial βγ-crystallins bind Ca^{2+} at the N/DN/DXXS/TS motif [11,13]. Based on this property, we analyzed the DesA1 sequence for the presence of a putative Ca^{2+} binding motif and the amino acid stretch 301-DKFEVS (Fig. 1A, marked in orange) appears to be the likely Ca^{2+} binding motif. We investigated the Ca^{2+}binding propensity of DesA1 using $^{45}CaCl_2$ overlay and ITC. In the $^{45}CaCl_2$ overlay, binding of Ca^{2+} to DesA1 was comparable to that of secretagogin (Fig. 2A), a known Ca^{2+} binding neuroendocrine marker [22]. We quantitated the affinity of this binding using ITC, as depicted by the thermogram in Fig. 2B. Ca^{2+} was observed to bind DesA1 with a dissociation constant (k_d) of 53 μM - the reaction is exothermic with a negative enthalpy change, suggesting that this binding is specific (Fig. 2B Table). These observations clearly demonstrate the Ca^{2+} binding property of DesA1.

3.3. Ca^{2+} binding induces conformational changes in DesA1

Having observed that Ca^{2+} binds DesA1, we investigated the role of Ca^{2+} in inducing conformational changes in the protein using intrinsic Trp fluorescence and far-UV CD spectroscopy. We observed measurable changes in the fluorescence intensity upon titration of the protein with increasing concentrations of Ca^{2+} (Fig. 3A), indicating minimal changes in the local Trp microenvironment upon ion binding. To probe changes in the surface hydrophobicity of DesA1 upon Ca^{2+} titration we performed fluorescence measurements of the protein in the presence of bis-ANS. This probe binds to DesA1 with a λ_{max} of emission at 480 nm and addition of Ca^{2+} increases the intensity of bis-ANS fluorescence marginally with a λ_{max} of 483 (Fig. 3B). This increase is a reflection of Ca^{2+} induced changes in surface hydrophobicity, strongly suggesting that DesA1 undergoes significant alterations to its conformation on Ca^{2+} binding. To determine the effect of Ca^{2+} on the secondary structure of DesA1, far-UV CD spectra were recorded in the presence and absence of Ca^{2+}. The secondary structure of apo DesA1 is composite in nature with a mixture of α-helices (50%) along with β-sheets (6%) - no considerable changes in the far-UV CD spectra were observed upon addition of 1 mM Ca^{2+} (Fig. 3C) to the apo-protein, suggesting that Ca^{2+} binding does not significantly alter the secondary structure of DesA1.

3.4. Recombinant M. Smegmatis expressing M.tb DesA1 shows a Ca^{2+} dependant surface morphotype

Since Ca^{2+} was seen to bind DesA1 and induce conformational changes, we proceeded to investigate the functional consequence of this binding on the activity of the enzyme. Since DesA1 is involved in mycolic acid biosynthesis, we hypothesized that over-expression of this gene would affect the lipid composition of the mycobacterial cell wall leading to a change in surface texture. To test this hypothesis, *M.tb desA1* was expressed in *M.smegmatis* and its expression confirmed by RT-PCR (Fig. 4A). We then examined the morphologies of transformants containing the recombinant plasmid as well as the vector alone spotted on Middlebrook 7H10 agar plates. As shown in Fig. 4B, the spot corresponding to the *desA1* over-expression strain showed a smooth mucoid texture distinct from the control transformant which was characterized by the presence of grooves and ridges, validating our hypothesis. Interestingly, in cultures grown in the presence of 1 mM EGTA spotted on 7H10 agar plates, the spot morphology phenotype was partially restored (Fig. 4B), suggesting that this change is reversible and that the phenotype is Ca^{2+}dependent. This observation clearly implies a functional role for Ca^{2+} in DesA1 activity.

4. Discussion

Desaturases catalyze the introduction of *cis*-double bonds at specific positions in the growing mero chain of α-mycolic acids in

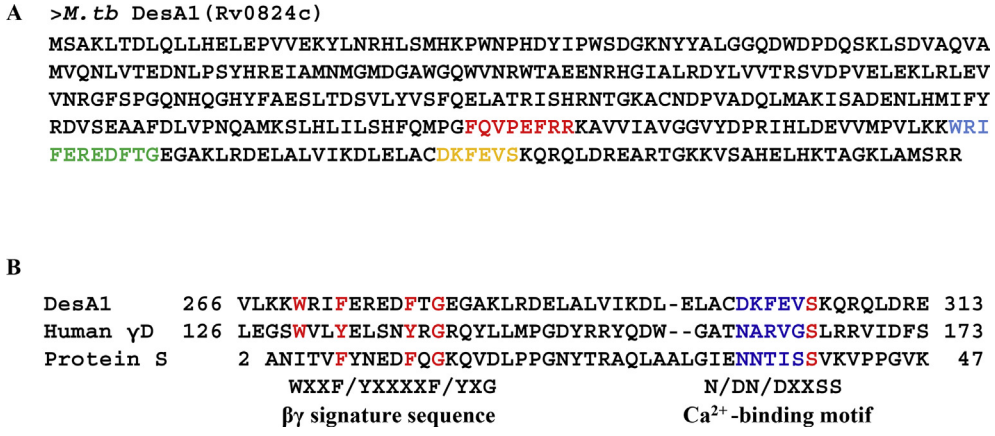

Fig. 1. Sequence features of *M.tb* DesA1. (A) Sequence of *M.tb* DesA1. The βγ-crystallin domain with the predicted Greek key signature motifs are highlighted in green (canonical) and red (non-canonical). The residues in blue represent the Trp corner and the residues in orange represent the predicted Ca^{2+} binding site (B) The folding and packing requirements of a βγ-crystallin motif define a characteristic sequence signature. The crucial residues in DesA1 are highlighted using motif 4 of human γD-crystallin and motif 1 of the Spore coat Protein S of *Myxococcus xanthus* as archetypes. The consensus βγ-crystallin type Ca^{2+} binding motif (N/D-N/D-X-X-S/TS) is marked in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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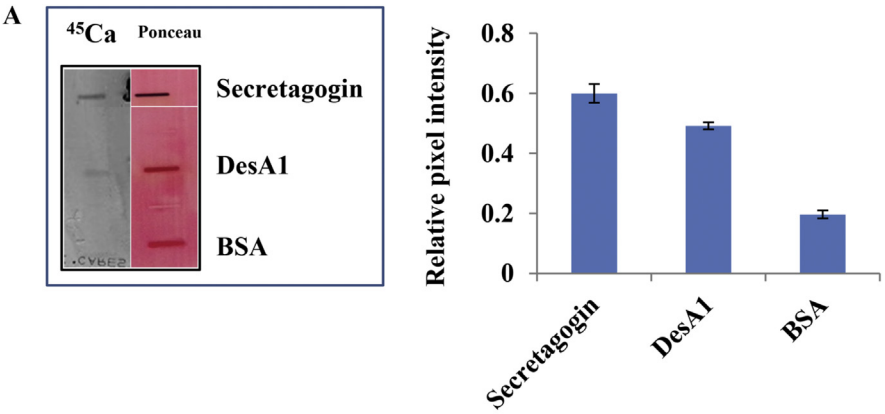

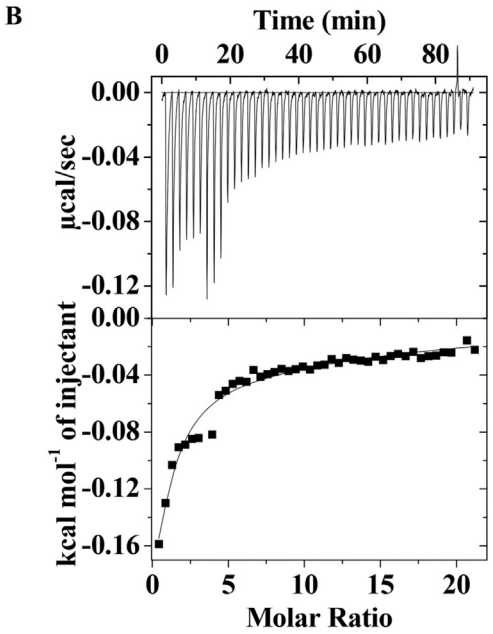

Protein	k_a (M^{-1})	k_d (µM)	ΔH (cal/mol)	ΔS (cal/mol/deg)
DesA1	$1.89 \times 10^4 \pm 1.5 \times 10^2$	53	-343.6 ± 14.7	18.4

Fig. 2. Binding of Ca^{2+} to *M.tb* DesA1. (A) $^{45}Ca^{2+}$ overlay assay for DesA1 probed with radioactive $^{45}CaCl_2$. Bovine Serum Albumin (BSA) and Secretagogin were used as negative and positive controls respectively. The bar graph represents the pixel intensity of Ca^{2+} binding to the above proteins normalized to the loading controls. The data shown are representative of three independent experiments. (B) Isothermal titration calorigrams of Ca^{2+} binding to DesA1. The concentrations of protein and Ca^{2+} used in ITC experiments were 50 µM and 5 mM respectively. The protein in the sample cell was titrated with 45 injections of 6 µl each from a stock of 5 mM $CaCl_2$. The table represents the binding kinetics of Ca^{2+} binding to DesA1. k_a-macroscopic association constant, k_d - dissociation constant, **Δ**H - change in enthalpy, Δ*S* - change in entropy.

mycobacteria. Key modifications like addition of cyclopropyl, methoxy and keto groups radiate from this double bond, which can be easily transformed into various functional groups. These modified derivatives take part in Claisen-Condensation, the penultimate step in mycolic acid biosynthesis to form a mature α-mycolate, which can now be transported to form the MAPc and TDM [23]. The importance of these modifications, made possible due to the introduction of double bonds by desaturases is emphasized by the fact that both DesA1 and DesA2 are essential genes in *M.tb* [24]. In this study, we highlight a unique feature of DesA1, that in addition to its ferritin-like domain it contains a single βγ-crystallin-like domain at its C-terminal end (Supplementary Fig. 1), unlike DesA2 and DesA3, the other two desaturases in the *M.tb* genome (data not shown). Interestingly DesA1, which shares sequence similarity with DesA2 in its N-terminus, has a unique C-terminal region which we have identified to contain the βγ-crystallin domain (Supplementary Fig. 2).

We report for the first time that *M.tb* DesA1 is a βγ-crystallin that binds Ca^{2+} with an affinity of 53 µM, typical of bacterial βγ-crystallins that have Ca^{2+} binding affinities ranging from 4 to 60 µM [13,25]. Ca^{2+} binding to DesA1 is likely to occur through the putative βγ-crystallin domain, which we have identified to contain two Greek key signature motifs and a Ca^{2+} binding site. The majority of βγ-crystallins contain two βγ-crystallin domains but single domain βγ-crystallins have been reported in both eukaryotes (Spherulin 3a) and prokaryotes (Nitrollin) [11,12]. On structural

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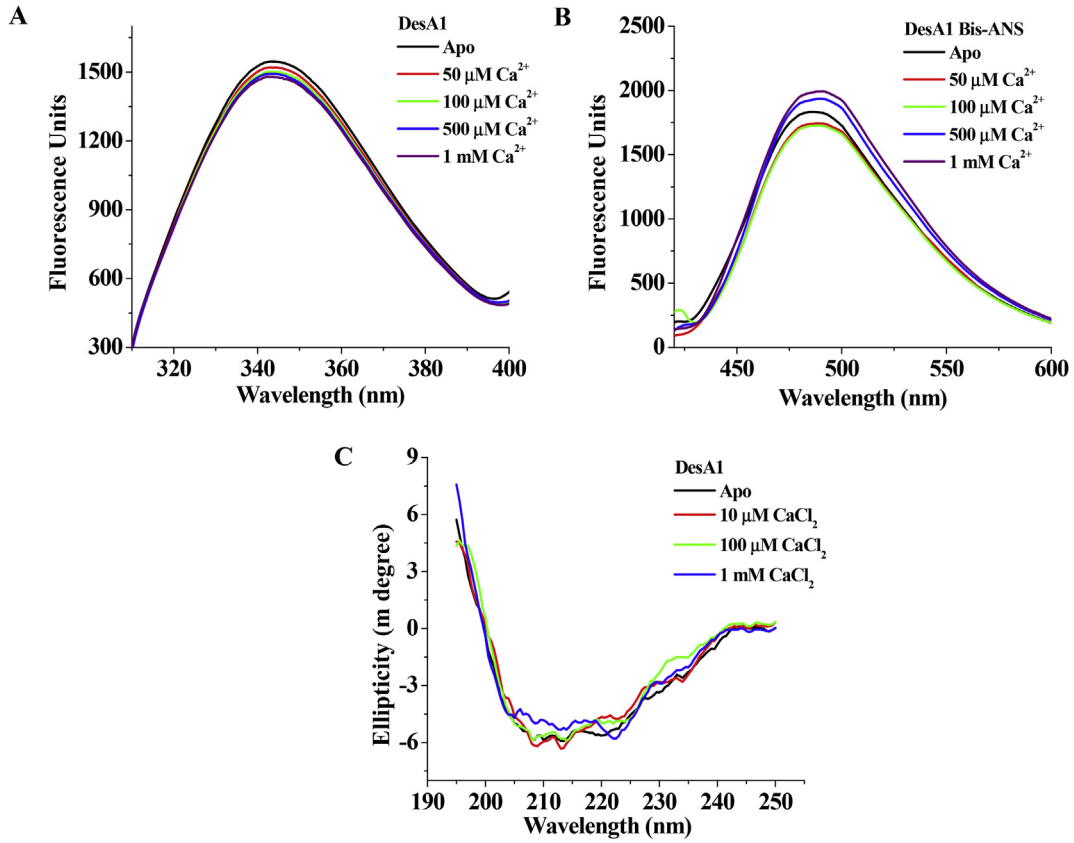

Fig. 3. Ca^{2+} induced conformational changes in DesA1. Effect of Ca^{2+} titration on - Intrinsic tryptophan fluorescence spectra of DesA1 (A), Bis-ANS fluorescence spectra of DesA1 (B) and Far-UV CD spectra of DesA1 (C). Ca^{2+} was used at concentrations of 0–1 mM. The data shown are representative of three independent experiments.

analysis, these proteins were found to retain the characteristic βγ-crystallin fold, which is known to contribute to protein stability. In DesA1, the first signature motif is atypical with few conserved residues and the second signature motif contains residues typical of a βγ-crystallin, namely a tryptophan residue preceding the motif, followed by a serine residue in the Ca^{2+} binding site, typical of a βγ-

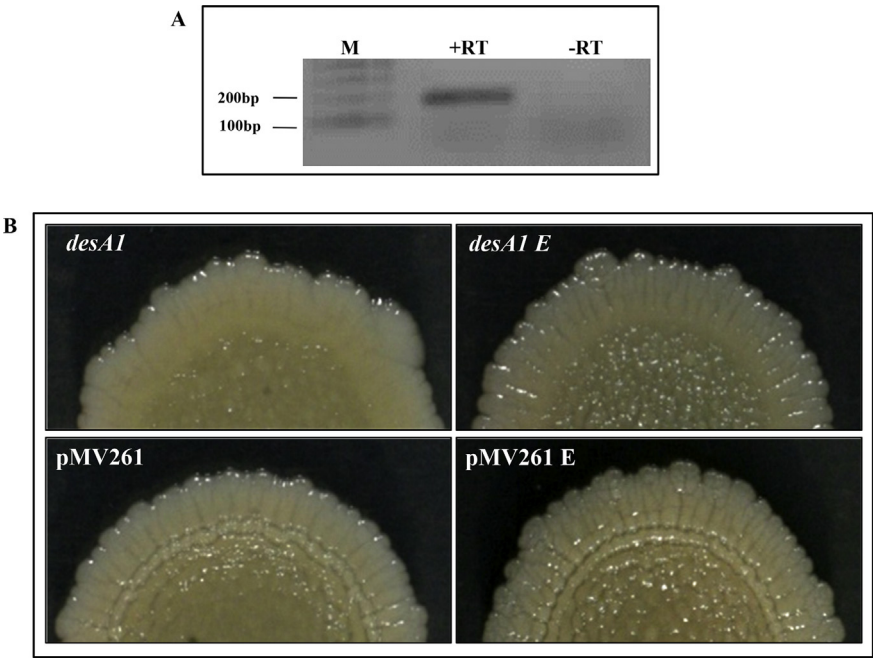

Fig. 4. Calcium dependant spot phenotypes of *desA1* overexpression. (A) RT-PCR based detection of the *desA1* transcript in recombinant *M. smegmatis* expressing *M.tb desA1* M-100bp DNA Ladder. (B) Spot morphologies of *M. smegmatis* expressing *desA1* and its empty vector (pMV261) control. The recombinant strains were grown in the presence or absence of 1 mM EGTA (E) - approximately 10^5 cells were spotted on Middlebrook 7H10 agar plates at the logarithmic phase of growth.

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crystallin Greek key motif. βγ-crystallins such as DdCAD and nitrollin have variations in their sequences but structural analyses show that they can form the characteristic βγ-crystallin fold [11,21,25]. βγ-crystallin domains, which have very few conserved residues in their sequence are the result of convergent evolution, and thus are not ancestrally related to the typical βγ-crystallins [9].

In our study, Ca^{2+} binding to DesA1 does not induce marked changes in its conformation. These results are consistent with known βγ-crystallins which unlike Ca^{2+} sensors, do not generally undergo large conformational changes upon binding to Ca^{2+} [25]. Although βγ-crystallins do not undergo drastic structural changes on binding Ca^{2+}, they are said to thermodynamically reach a state of higher structural stability suggesting that Ca^{2+} may play the role of an external stabilizer [14]. It is believed that the crystallin domain evolved in proteins of extraordinary stability and the βγ-crystallin fold confers this stability [13]. Consistent with the absence of a βγ-crystallin domain in DesA2, the structure of this protein clearly indicates that it does not possess the capability to bind to Ca^{2+} [7].

The saprophytic mycobacterium *M. smegmatis* has been widely used as a surrogate host to study the physiology of the highly pathogenic *M.tb*, based on the assumption that essential processes such as the building of the mycomembrane are conserved in both species [26]. In our study, a mucoid surface morphology with loss of striations was observed upon over expression of *M.tb desA1* in *M. smegmatis* compared to that of the empty vector control. Several studies have reported that overexpression or deletion of genes involved in mycolic acid biosynthesis such as *hadC* and *pca2* leads to changes in lipid composition and cellular morphotype of the cell wall [27,28]. The highly mucoid phenotype we observe could be a consequence of an alteration in the composition of mycolic acids in the cell wall. We also observed that the phenotype of *M. smegmatis* overexpressing *M.tb* DesA1 is Ca^{2+} dependent, clearly implying that Ca^{2+} could be necessary for proper functioning of the protein. Ca^{2+} may either be required as a co-factor for the proper functioning of the protein or its binding to DesA1 may be required to stabilize the protein. In either scenario, restricted Ca^{2+} availability results in the loss of DesA1 activity as reflected in the phenotype of the over expressing strain resembling the empty vector control in the absence of Ca^{2+}.

DesA1 is conserved across the mycobacterial species (Supplementary Fig. 3) an indication of its functional importance in mycobacterial physiology. Several steps in the mycolic acid biosynthesis pathway are targeted by first and second line anti-TB drugs. However, there are currently no molecules that target the essential desaturation step in this pathway. Our observations suggest that as a consequence of its binding to Ca^{2+}, *M.tb* DesA1 plays a Ca^{2+} dependent role in mycolic acid synthesis, which is intimately linked to the integrity of the mycomembrane. A detailed structural analysis will help in defining the molecular nature of Ca^{2+} coordination to DesA1 and the resulting conformational changes to the protein. This could lead to the development of small molecule inhibitors targeting the Ca^{2+} dependent desaturase function of DesA1, a new therapeutic approach against *M.tb*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.10.014.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.10.014.

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