Amplification of Six Putative RD1 Genes of *Mycobacterium tuberculosis* for Cloning and Expression in *Escherichia coli* and Purification of Expressed Proteins

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Key Words

Mycobacterium tuberculosis • RD1 genes • Cloning and expression • Protein purification

Abstract

Objectives: To amplify, clone and express in *Escherichia coli* six open reading frames (ORFs) predicted in the RD1 DNA segment of *Mycobacterium tuberculosis* and purify the expressed proteins to homogeneity. **Materials and Methods:** DNA corresponding to the coding regions of six RD1 ORFs, i.e. ORF10 to ORF15, was amplified from genomic DNA of *M. tuberculosis*, cloned in the plasmid vector pPCR-Script and subcloned in expression plasmid vectors pET29a and/or pGEX-4T for expression in *E. coli* as fusion proteins. The recombinant fusion proteins were identified by sodium dodecyl polyacrylamide gel electrophoresis and Western immunoblotting. Attempts were made to obtain purified proteins, free of the fusion partner, using affinity and fast protein liquid chromatography. **Results:** DNA corresponding to all six targeted RD1 ORFs was amplified from the genomic DNA of *M. tuberculosis* and five of the six ORFs, with the exception of ORF13, were cloned in the plasmid vectors and expressed in *E. coli*. Because of extensive degradation of ORF10 and ORF12 fusion proteins or nonbinding to the affin-

ity columns of ORF15 fusion proteins, only ORF11 and ORF14 proteins were purified, free of the fusion partner, to homogeneity. **Conclusion:** All of the six targeted RD1 genes were amplified and five expressed using *E. coli* hosts, but only two of the expressed proteins were purified to homogeneity. Alternative expression systems are required to obtain all RD1 proteins for functional characterization.

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Introduction

Tuberculosis (TB) is a major infectious disease problem around the world with an estimated incidence of 8.9 million cases and 1.7 million deaths annually [1]. The global problem of TB is worsening mainly due to the increase in multidrug-resistant TB and coinfection with HIV [1]. The effective control of TB requires identification and availability of *Mycobacterium tuberculosis*-specific proteins as immunological reagents for diagnosis of TB. Although a large number of proteins of *M. tuberculosis* have been identified in the past, most of them are shared with the vaccine strains of *Mycobacterium bovis* BCG [2–5], and thus differentiation between individuals infected with *M. tuberculosis* and vaccinated with *M. bo-*

vis BCG is not possible. The availability of purified *M. tuberculosis*-specific proteins is also required to delineate the mechanisms of virulence, identify targets for new and pathogen-specific drugs and develop subunit vaccines against TB [6, 7].

Comparisons of mycobacterial genome sequences have led to the identification of several regions of differences (RD) that are conserved in *M. tuberculosis* but absent in *M. bovis* BCG [8–10]. Among these regions, RD1 (a 9.5-kb DNA segment) is of special interest because it is present in all virulent laboratory and clinical strains/isolates of pathogenic *M. tuberculosis* but deleted in all vaccine strains of *M. bovis* BCG [8–10]. The reintroduction of RD1 into *M. bovis* BCG resulted in a protein expression profile almost identical to that of virulent *M. tuberculosis* [8]. The immunological and pathological importance of proteins encoded by RD1 was predicted in studies in which two major *M. tuberculosis*-specific antigenic proteins, i.e. ESAT6 and CFP10, were found encoded by genes present in RD1 [11, 12], and deletion of RD1 region from *M. tuberculosis* decreased its capacity to cause disease in an experimental animal model of TB [13].

A careful bioinformatics analysis of the RD1 sequence has revealed the presence of 14 open reading frames (ORF2 to ORF15) deleted in *M. bovis* BCG [14]. These ORFs were predicted to vary between 0.3 and 2.2 kb in size, and 13 were expressed at the mRNA level [15]. However, to study the functional relevance of these ORFs, it was essential to obtain the encoded proteins in purified form. A number of previous studies have shown that mycobacterial proteins can be expressed in *E. coli* and purified to homogeneity for functional studies [14, 16–19]. Therefore in the present study, we aimed to amplify and clone RD1 ORFs in plasmid vectors, express them in *E. coli* and purify the mycobacterial proteins to homogeneity.

Materials and Methods

Plasmids and *E. coli* Strains

The following plasmids and *E. coli* strains were used: pPCR-Script (Stratagene, La Jolla, Calif., USA) was used for cloning of mycobacterial DNA and the recombinant plasmids were propagated in XL1-Blue strain of *E. coli* (Stratagene). pGEX-4T-1 and pGEX-4T-2 vectors (Amersham-Pharmacia Biotech AB, Uppsala, Sweden) were used to clone mycobacterial genes for expression in BL-21 (Novagen, Madison, Wisc., USA) and XL-1 blue strains of *E. coli*. pET29a vector was used for cloning and expression of mycobacterial genes in BL-21-DE3 strain of *E. coli* (Novagen). DH5 α strain of *E. coli* (Life Technologies, USA) was used to propagate all the parent plasmid vectors.

Isolation and Manipulation of *M. tuberculosis* Genomic DNA

M. tuberculosis H37Rv was obtained from the American Type Culture Collection (Rockville, Md., USA), grown at 37°C in Middlebrook 7H9 medium (Difco, USA) and used for isolation of genomic DNA, as previously described [15]. The isolated genomic DNA served as the source for the amplification of RD1 genes by PCR. All DNA manipulations, restriction endonuclease digestion, sequencing and transformation were performed using standard procedures [20].

Oligonucleotide Primers

The oligonucleotide primers to amplify full-length ORF10 to ORF15 genes were designed based on the nucleotide sequence of these ORFs in the *M. tuberculosis* genome, as described previously [15, 16]. As examples, the DNA sequences of forward (F) and reverse (R) primers for ORF11 and ORF12 are shown:

- ORF11F: 5'-CGC **GGA TCC** ATG GCT GAA CCG TTG GCC GTC-3';
- ORF11R: 5'-CCC **AAG CTT** TCA CAA CGT TGT GGT TGT TGA-3';
- ORF12F: 5'-C **GAG CTC** GTG GGC AGC GTA GGC CCG GAA-3';
- ORF12R: 5'-CCC **AAG CTT** TCA TTG GTC ACG GCG ACG CAT-3'.

The F primers for ORF10, ORF11 and ORF14 had a *Bam*HI and ORF12, ORF13 and ORF15 had a *Sac*I restriction site incorporated at the 5' end (bold-face nucleotides) and an initiation codon (underlined). All of the R primers had a *Hind*III restriction site (bold-face nucleotides) at the 5' end and a termination codon (underlined).

PCR Amplification of Target DNA and Cloning in Plasmid Vectors

PCR procedures for amplification of ORF10 to ORF15 from the genomic DNA of *M. tuberculosis* were performed as described previously [15]. The DNA products were resolved by agarose gel electrophoresis, purified and ligated to pPCR-Script to transform *E. coli* cells using standard procedures [16]. To confirm the identity of the cloned DNA, restriction digestion analysis and DNA sequence analysis were performed according to standard procedures [15].

For expression in *E. coli*, ORF10, ORF11 and ORF14 genes were subcloned from pPCR-Script into pGEX-4T-2 vector to be expressed as fusion proteins with glutathione S-transferase (GST) as the fusion partner at the N-terminus of the protein, using standard procedures [16]. ORF11 and 15 could not be cloned directly from pPCR-Script into pGEX vectors because the *Bam*HI site of ORF11 was not in frame with that of the vector, and the latter has no *Sac*I site suitable for cloning ORF15. The other restriction sites common for the two vectors were also present inside the ORFs. For these reasons, the two ORFs were digested from pPCR-Script with *Bam*HI, *Hind*III (ORF11) and *Sac*I, *Hind*III (ORF15) and ligated into pET29-a. From this vector, ORF11 and 15 were excised with *Bam*HI, *Xho*I and *Eco*RI, *Xho*I, respectively, and finally ligated to pGEX4T-1 vectors suitable for expression [16]. The recombinant plasmids were transformed into appropriate *E. coli* strains (BL-21-DE3, XL-1 blue and BL-21) for protein expression, as described previously [16].

Induction, Identification and Purification of Recombinant Proteins

Recombinant proteins were expressed in *E. coli* cells using standard procedures [19], and processed for detection of expressed proteins using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining of the gels with Coomassie blue [19]. The identity of the expressed proteins was further confirmed. Their stability in different *E. coli* strains was determined by Western immunoblotting using anti-GST antibodies [19]. The recombinant proteins purified from *E. coli* lysates upon loading onto glutathione-Sepharose columns and cleavage of the fusion proteins occurs upon reaction with thrombin protease, as described previously [19]. The GST-free recombinant proteins were further purified to remove other contaminating proteins by fast protein liquid chromatography (FPLC, Pharmacia, UK). Any endotoxins contaminating the recombinant proteins were removed by using prepacked endotoxin removing columns (Detoxi-Gel™, Pierce, Rockford, Ill., USA) according to the manufacturer's instructions.

The amplification of ORF10 and ORF15 was achieved by using the reaction mixture described previously, but the amplification of ORF11 and ORF13 required addition of 4% formamide and 8% DMSO, respectively, and the amplification of ORF12 and ORF14 required addition of both 8% formamide and 8% DMSO [15]. The PCR-amplified ORFs were purified and ligated to the pPCR-Script vector to transform the cells of XL-1 blue strain of *E. coli*. Plasmids isolated from transformed cells were digested with the appropriate restriction enzymes to confirm the presence of the inserts.

Results

Amplification of RD1 ORFs by PCR and Cloning in pPCR-Script

Five of the six ORFs, with the exception of ORF13, were successfully cloned in pPCR-Script. The identity of the cloned ORFs was confirmed by DNA sequencing.

Cloning and Expression of RD1 ORFs in Expression Vectors

ORF10 and ORF14 were directly subcloned from pPCR-Script into pGEX-4T-2 and the fusion proteins were expressed in BL-21 and XL-1 blue strains of *E. coli*, as described previously. However, ORF11 and ORF15 could not be subcloned into the pGEX-4T vectors directly from pPCR-Script because of incompatible restriction sites. These ORFs were first subcloned in pET29a followed by cloning in pGEX-4T-1 and the proteins were expressed in *E. coli* from both vectors, as detected by SDS-PAGE and Western immunoblotting (fig. 1, data shown for ORF11 in pGEX-4T-1). Although full-length ORF12 could be cloned in pGEX-4T-2 as well as in pET29a, the expected recombinant protein was not ex-

Fig. 1. Expression of ORF11 in *E. coli* as a GST fusion protein from the pGEX4T-1-ORF11 recombinant plasmid using XL-1 blue and BL-21 strains of *E. coli*. The expression of recombinant protein molecules was induced in *E. coli* cells that were transformed with the recombinant plasmids and the expressed proteins were resolved by SDS-PAGE, as described in Materials and Methods. Lane 1: Low-molecular-weight protein marker. Lane 2: Proteins expressed in *E. coli* cells without any plasmid (negative control). Lane 3: Proteins expressed in *E. coli* cells transformed with non-recombinant pGEX4T-1. The band corresponding to GST protein (29 kDa) is indicated by arrow 2. Lane 4: Proteins expressed in *E. coli* strain XL-1 blue transformed with recombinant pGEX4T-1-ORF11. The band corresponding to the recombinant fusion protein (60 kDa) is marked by arrow 1. Lane 5: Proteins expressed in *E. coli* strain BL-21 transformed with recombinant pGEX4T-1-ORF11. The band corresponding to the recombinant fusion protein (60 kDa) is marked by arrow 1.

pressed in transformed *E. coli* cells, when detected by SDS-PAGE and Western immunoblotting. Therefore, ORF12 was digested arbitrarily into several overlapping fragments and attempts were made to express them individually. The largest fragment successfully expressed was 1.3 kb covering about 73% of the protein from N-terminus.

Confirmation of Expression and Stability of Recombinant Proteins Expressed in *E. coli*

To confirm the expression of recombinant proteins and study their stability, *E. coli* lysates were probed with anti-GST antibodies in Western immunoblotting using anti-GST antibodies. The results confirmed that all the five recombinant proteins were expressed but appeared to be degraded in both XL-1 blue and BL-21 strains of

Fig. 2. Western immunoblotting with anti-GST antibodies to confirm the identity of fusion proteins expressed in *E. coli*. The proteins from SDS-PAGE gels were transferred to nitrocellulose membranes and probed with anti-GST antibodies, as described in Materials and Methods. **a** Detection of ORF11 fusion protein with anti-GST antibodies. The same controls were used: *E. coli* cells alone (lane 2) and *E. coli* cells transformed with the parent plasmid pGEX4T-1 (lane 3); GST protein appears at 29 kDa, marked by arrow 2. The ORF11 fusion protein was detected in XL-1 blue (lanes 4 and 5) and BL-21 (lane 6) strains of *E. coli*, which were transformed with pGEX4T-1-ORF11. In both cases

the protein appears at 60 kDa, as expected (arrow 1). Lane 1: Prestained low-molecular-weight protein marker. **b** Detection of ORF12 fraction (F1) protein with anti-GST antibodies. The negative (lane 3) and positive controls (lane 2) were the same as in **a**. The F1 fraction of ORF12-GST fusion protein was detected at around 50 kDa (arrow 1) using anti-GST antibodies in both BL-21 (lane 4) and XL-1 blue (lane 5) strains of *E. coli*. The protein was further degraded to other products between 50 and 21 kDa, as shown in the figure. Lane 1: Prestained low-molecular-weight protein marker.

Fig. 3. SDS-PAGE analysis of eluted ORF11 protein after cleavage of the GST-ORF11 fusion proteins with thrombin protease on an anti-GST affinity column. The recombinant GST-ORF11 proteins were cleaved from the GST fusion partner on an anti-GST column after treating with thrombin protease, as described in Materials and Methods. The eluted fractions were resolved on SDS-PAGE gels. Lane 1: Low-molecular-weight protein marker. Lane 2: The eluted ORF11 protein free of the GST fusion partner (35 kDa, arrow 2). However, an *E. coli* protein at 73 kDa (arrow 1) was also eluted in these fractions.

E. coli (fig. 2a, results shown for ORF11 only). The protein degradation was so extensive in case of ORF10 and the expressed fragment of ORF12 that the band corresponding to recombinant protein of expected size was hardly visible in the Western immunoblots, whereas the degradation products of various sizes were quite marked (fig. 2b, results shown for ORF12 only).

Purification of Recombinant Proteins

Attempts were made to purify the recombinant RD1 proteins, free of the *E. coli* components and the fusion partner by affinity chromatography and FPLC of those ORFs that showed a high level of expression and a low level of degradation as fusion proteins, i.e. ORF11, ORF14 and ORF15. The recombinant ORF11 and ORF14 fusion proteins were abundant in the soluble fraction of *E. coli* lysates, which were loaded on glutathione-Sepharose 4B columns for purification. Upon cleavage with thrombin protease, RD1 proteins corresponding to the molecular weights of ORF11 and ORF14 were eluted (fig. 3, data shown for ORF11). However, both of these proteins were contaminated with an *E. coli* cellular protein appearing at around 73 kDa (fig. 3, data shown for ORF11). The contaminating protein was successfully removed by FPLC to obtain highly homogeneous recombinant ORF11 and ORF14 proteins (fig. 4).

Fig. 4. SDS-PAGE analysis of recombinant ORF14 and ORF11 proteins after further purification by FPLC. The recombinant ORF14 and ORF11 proteins eluted from anti-GST columns after treatment with thrombin protease were further purified by FPLC as described in Materials and Methods. The purified proteins were analyzed by SDS-PAGE. Lane 1: Low-molecular-weight protein marker. Lane 2: Purified ORF14 protein (30 kDa, arrow 2). Lane 3: Purified ORF11 protein (35 kDa, arrow 1).

In case of ORF15, most of the fusion protein was present in the form of pellets and very little was found in the soluble fraction of *E. coli* lysates. The pellets were treated with several concentrations of urea (1, 2, 4 and 6 M urea) to dissolve the fusion protein. Although the fusion protein was optimally solubilized in 6 M urea, all efforts (dilution with phosphate-buffered saline and dialysis to remove urea) failed to facilitate its binding to the glutathione columns. Thus, the recombinant ORF15 protein could not be purified.

Discussion

In this work we have studied six ORFs of RD1 (ORF10 to ORF15) for amplification from genomic DNA of *M. tuberculosis* by PCR, cloning in plasmid vectors, expression in different *E. coli* strains and purification of the expressed *M. tuberculosis* proteins. Although all of the ORFs could be amplified by PCR using genomic DNA from *M. tuberculosis* H37Rv, the reaction conditions varied. ORF10 and ORF15 were amplified using standard protocols of PCR with an annealing temperature of 65°C [15]. However, nonspecific DNA fragments were amplified when using standard protocols with primers for ORF11, ORF12, and ORF14, and no products were obtained for ORF13. Nonspecific products or absence of any

distinct product could be due to the high GC contents of these regions (ORF11 66.7%, ORF12 70.4%, ORF13 67.5% and ORF14 75.4%) [14]. The GC contents of ORF10 and ORF15 (60.4 and 61.8%, respectively) are slightly less [14], and this could have made the amplification of these easier than the others. The annealing temperature was varied between 53 and 70°C in attempts to improve specificity, without success. Formamide and DMSO have been reported to improve the specificity and yield of PCR [21, 22], and the use of these chemicals helped the amplification of specific products of ORF11, ORF12, ORF13 and ORF14 [15]. However, the use of these reagents remains empirical, and it is not known which parameters of the reaction are affected. For example, these reagents may affect primer-melting temperature (T_m), the catalytic activity of the enzyme, or template and product structure [21, 22].

All targeted RD1 ORFs were cloned in pPCR-Script, except ORF13, which is a 2.2-kb fragment encoded on the complementary strand of the genomic DNA overlapping ORF12 and ORF14 [15]. The amplified ORF13 was tailed with poly-adenine (A) by adding Taq DNA polymerase at the last cycle of the PCR amplification reaction, with subsequent attempts to clone the product into pGEM-T easy vector which has thymidine at the 3'-terminal of both ends [18]. However, these trials were unsuccessful (data not shown). In addition, we tried to clone this ORF using other plasmid systems, such as the pZero system described to give zero background of nonrecombinant product [23], but again the attempts were unsuccessful, and ORF13 was dropped from further work.

ORF10 and ORF15 were initially subcloned into pQE-30 vector to be expressed as proteins fused to a 6× histidine tag at the N-terminal [24]. However, the expression of these proteins could not be detected either by Coomassie blue staining or by Western immunoblotting using antihistidine antibodies. These observations confirm the previous reports that most of the target proteins of *M. tuberculosis* did not show any expression and only 22% were well expressed in *E. coli* when an N-terminal His-tag was used [25]. However, a fusion protein approach enhanced the percentage of well-expressed proteins to 63% [26]. Therefore, attempts were made to express ORF10 and ORF15 proteins in The pGEX-4T vectors that use GST gene fusion system to express foreign proteins fused to the relatively large and stable GST (29-kDa) protein. The pGEX-4T vectors provide high level expression of the fused protein, which is induced chemically by isopropyl β-D-thiogalactoside [16]. Cleavage of the desired protein from GST is achieved using a site-specific thrombin

protease whose recognition sequence is located immediately upstream from the multiple cloning site on the pGEX plasmids [16]. Proteins can then, theoretically, be purified from bacterial lysates by affinity chromatography [16]. ORF10 and ORF14 were successfully expressed as GST fusion proteins, although Western immunoblot using anti-GST antibodies showed extensive (in case of ORF10) to some (in case of ORF14) degradation [15].

Since ORF10 and ORF12 proteins were extensively degraded in *E. coli*, we proceeded with purification of the expressed proteins from ORF11, ORF14, and ORF15. Success was achieved with the proteins from ORF11 and ORF14. After cleavage from the GST fusion partner, the two proteins were seen at 30 and 35 kDa, respectively, on SDS-PAGE, whereas their predicted molecular weights are 27 and 21 kDa, based on the size of respective genes [14]. Both proteins have several short repetitive motifs containing proline residues at the C-terminal end, a feature shared among several antigenic mycobacterial proteins, including MTC28 and 36 kDa antigens [29, 30]. A characteristic feature of these proteins is that they migrate slowly in SDS-PAGE gels compared to their expected molecular weights [29, 30]. The discrepancies between the calculated and apparent molecular weights of these antigenic proteins can therefore probably be attributed to the rigidity and reduced mobility of the protein backbone due to the abundance of proline residues.

Conclusion

Genes for all six selected RD1 proteins were amplified, five of which were cloned in plasmid vectors for expression in *E. coli*. However, obtaining full-length proteins was successful for only two, i.e. ORF11 and ORF14, due to extensive degradation or nonbinding to affinity columns of other recombinant proteins. Another disadvantage of the *E. coli* expression system is the lack of post-translational modifications, often observed with *M. tuberculosis* proteins expressed naturally. These observations limit the use of *E. coli* as a host to express and purify putative mycobacterial proteins for functional studies and support the argument that efficient mycobacterial expression systems should be developed to obtain large quantities of *M. tuberculosis* proteins for functional characterization.

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