

## Cloning and expression of *aceA* gene encoding isocitrate lyase from *Mycobacterium tuberculosis*

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**The enzyme isocitrate lyase (ICL) reversibly catalyses the conversion of isocitrate to glyoxylate and succinate in the glyoxylate bypass of the tricarboxylic acid (TCA) cycle. We cloned the *Mycobacterium tuberculosis aceA* gene encoding ICL using polymerase chain reaction (PCR). Restriction mapping and sequencing confirmed the cloned gene to be *M. tuberculosis aceA* gene. The cloned gene consists of 2300 nucleotides encoding a protein of 765 amino acids. The cloned fragment has a high degree of similarity of *aceA* gene of *M. leprae*. Expression of the cloned gene was confirmed by assay of the enzyme.**

TUBERCULOSIS (TB) still remains the major killer disease the world over. More people are dying of TB today than ever before and one-third of the world's population is believed to be infected with *Mycobacterium tuberculosis*<sup>1</sup>. The increased incidence of TB in both developing and industrialized countries, the widespread emergence of drug-resistant strains and a deadly synergy with the human immunodeficiency virus, prompted WHO to declare TB as a global health emergency in April 1993 (ref. 2).

There is an urgent need to develop new strategies to combat *M. tuberculosis*. During the last decade the basic biology of mycobacterial pathogens has benefited greatly from a molecular biological approach. Investigations can now be focused on issues which have been either not attempted, due to lack of information or because of the cumbersome nature of targeting the right gene for expression.

One such area of research, which is ill-understood, is dormancy in mycobacteria. The metabolic processes which lead to dormancy have not been completely explored, though earlier workers have suggested glyoxylate pathway to be one of the possible switch-over mechanisms<sup>3</sup>, just prior to or during dormancy.

Glyoxylate pathway is one of the important metabolic pathways which bypasses the tricarboxylic acid (TCA) cycle. Isocitrate lyase (ICL) (threo-Ds isocitrate glyoxylate-lyase) (EC 4.1.3.1) is the first of the two specific enzymes of the glyoxylate cycle. The enzyme reversibly catalyses the reaction wherein isocitrate is converted to glyoxylate and succinate. ICL has been isolated from

various organisms and has been characterized very well. ICL has also been demonstrated from *M. leprae*, *M. tuberculosis* H37Rv strain and various other mycobacteria<sup>4-6</sup>.

In mycobacteria, ICL activity has been reported to increase continuously with age of the culture in *M. tuberculosis* H37Rv<sup>7</sup>, but not in *M. tuberculosis* H37Ra or *M. smegmatis*<sup>8</sup>. Another study reports enhanced glyoxylate cycle enzyme activity under low oxygen tension<sup>9</sup>.

Persistence of live organisms despite chemotherapy for long periods is a significant problem in TB. The mechanisms of this dormant state are ill-understood, and as an explanation a switch-over to alternate modes of metabolism such as glyoxylate bypass and other shunts has been suggested<sup>3</sup>. In order to understand whether ICL, the first enzyme of the glyoxylate cycle, has a role to play in dormancy, we have cloned and expressed the *aceA* gene of *M. tuberculosis* encoding ICL.

*Escherichia coli* (DH5 $\alpha$  recA lacZ $\delta$  M15) was obtained from Stratagene, USA and was routinely grown in Luria Bertani medium at 37°C. *M. tuberculosis* H37Rv, the virulent strain, was obtained from Bacteriology Laboratory, Tuberculosis Research Centre, Chennai, and was grown in Middlebrook 7H9 medium (Difco) supplemented with glycerol (0.2%) and ADC (glucose, 0.2%; BSA fraction V, 0.5%; and NaCl, 0.085%) at 37°C. Kanamycin (25  $\mu$ g/ml), ampicillin (100  $\mu$ g/ml) and carbenicillin (100  $\mu$ g/ml), were all obtained from Sigma, USA.

The commercial primers (M13 forward and reverse, SP6 and T7) used in this study, were from GIBCO-BRL, USA. The custom primers, listed in Table 1, were from Integrated DNA Technologies, Iowa, USA.

For small-scale preparations of plasmid, modified method of Birnboim and Dolly<sup>10</sup> was used. For large-scale preparations, Qiagen columns (Qiagen GmbH) were used. All commercial enzymes (Amersham Pharmacia Biotech., USA or Boehringer Mannheim, Germany) were used according to standard protocols. For manual sequencing with T7 and SP6 primers, either Sequenase or Thermosequenase version 2.0 kit (Amersham Pharmacia Biotech., USA) was used according to the manufacturer's protocols. Automated sequencing of clone pSAP1 with M13 forward and reverse primers was done using an ABI PRISM 377 system.

Homology searches were performed against GenBank, EMBL and Sanger Centre databases using BLAST or FASTA algorithms.

TA cloning kit (Invitrogen, USA) was used according to the manufacturer's protocol using DyNAzyme (Finnzyme) for PCR cloning of the *aceA* gene and other fragments.

For expression of the *aceA* gene, either PinPoint Xa1, part of Xa protein purification system (Promega Corp, USA) or pCR2.1, part of TA cloning kit (Invitrogen, USA) was used according to the manufacturer's protocol.

Spectrophotometric assay for enzyme activity of ICL was done<sup>11</sup>. Briefly, a mixture 1.5 ml of Tris-Mg<sup>++</sup> buffer,

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**Table 1.** Sequence of the primers and their locations in the cosmid

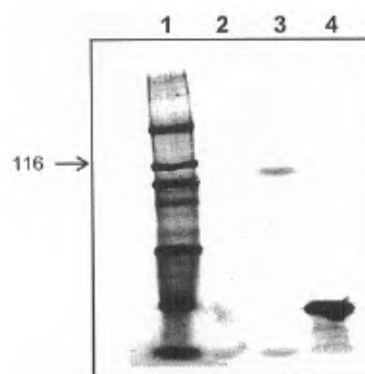
Primer	Sequence of the primer	Location in cosmid MTCY 180
SAP3	5'-GGGGTACCCCGCAGAGGAGCGCAAGGCCAT-3'	30-mer primer corresponding to base pair position 4830 to 4849 of the cosmid; Complementary strand
SAP4	5'-GGGGTACCCCTGGCTACGCCTCCTTCGTGA-3'	30-mer primer corresponding to base pair position 2529 to 2548 of the cosmid
SAP15	5'-CCAAGCTTGGGCAGAGGAGCGCAAGGCCAT-3'	30-mer primer corresponding to base pair position 4830 to 4849 of the cosmid; Complementary strand

0.2 ml of glutathione-SH, 0.1 ml of crude extract, and 0.2 ml of threo-Ds-isocitrate solution (40 mM) was incubated for 10 min at 30°C. The reaction was stopped by the addition of 1.0 ml of 10% trichloroacetic acid. 6.0 ml of oxalic acid–phenylhydrazine hydrochloride mixture was added to 1.0 ml of the reaction mixture and heated until just boiling. The solution was cooled at room temperature for 5 min, and chilled in an ice bath for 2 min. To this, 4.0 ml of concentrated HCl and then 1.0 ml of 5% potassium ferricyanide solution were added in a known, timed sequence. Seven minutes after the addition of ferricyanide, the optical density (OD) was read at 520 nm against a water blank in suitable colorimeter. If the path length is 1.0 cm, the yield of glyoxylate in micromoles per reaction vessel, (i.e. 2.0 ml of original incubation mixture) is given by:  $(OD_{520}-0.05)/1.15$ . One unit of the enzyme is that amount which catalyses the disappearance of 1 micromole of threo-Ds-isocitrate per minute at 30°C under conditions of the assay. The amount of isocitrate that disappears is equivalent to that of glyoxylate produced.

Five recombinant clones from a previous partial genomic library<sup>12</sup> of *M. tuberculosis* H37Rv in pGEM4Z were chosen randomly for sequencing. The homology searches performed with the sequence data thus obtained revealed that one of the fragments, TRC9, had high similarity to the cosmid MTCY180 in the predicted *aceA* gene of *M. tuberculosis* in the Sanger Centre database.

Using the custom designed primer pairs SAP3 and SAP4 and SAP15 and SAP4, and MTCY180 and *M. tuberculosis* DNA as templates, we amplified the 2.3 kb *aceA* gene by PCR. The amplified fragments from *M. tuberculosis* DNA were cloned into pCR2.1 vector. The recombinant plasmid pSAP1 had the cloned fragment (amplified using SAP3 and SAP4) in the sense orientation. pSAP3 and pSAP4 recombinant plasmids contained the cloned fragment (amplified using SAP15 and SAP4) in the sense and antisense orientation, respectively. The cloned fragment was restriction mapped (data not shown). Further confirmation of the cloned fragment by automated sequencing was done.

pSAP1 was restriction digested with Kpn I, run on an agarose gel and the 2.3 kb *aceA* gene was eluted from the gel using Gene Clean kit (BIO 101). PinPoint XaI vector was restriction digested with Kpn I and was dephosphorylated



**Figure 1.** Western blot picture showing expression of *aceA* gene in PinPoint XaI vector. *E. coli* DH5 $\alpha$  (lane 2), harbouring pSAP10 (lane 3) and PinPoint control vector (lane 4) were induced with IPTG, heat lysed proteins were run in a 7.5% SDS-PAGE, along with biotinylated broad-range protein molecular weight marker (lane 1), transferred onto a nitrocellulose membrane and detected using the streptavidin–alkaline phosphatase system.

using shrimp alkaline phosphatase (Amersham Pharmacia Biotech., UK).

This linearized vector was eluted from the gel and was ligated to the *aceA* gene at 16°C for 16 to 18 h. The ligation mixture was electroporated into *E. coli* and recombinants were selected on LB plates containing ampicillin and X-gal (Sigma, USA). The recombinant plasmids containing the *aceA* gene in the sense and antisense orientations were named pSAP10 and pSAP11, respectively.

Expression of the *aceA* gene was carried out according to the kit protocol. The *aceA* gene was expressed in the XaI system as biotinylated peptide (13 kDa) – *aceA* gene product fusion protein using the tac promoter. The Pin-Point control vector gave a 40 kDa band (biotinylated peptide – chloramphenicol acetyl transferase fusion protein), as expected. In the case of pSAP10, when ampicillin was used, no band could be detected. Then the protocol was altered slightly in that, carbenicillin was used instead of ampicillin at the same concentration. After this modification, a band was detected at around 100 kDa (Figure 1), which corresponded to the size of the induced fusion product.

The *aceA* gene was expressed in the pCR2.1 system as *lacZ*–*aceA* gene product fusion product, using the lac

promoter. pSAP3 and pSAP4 were used for expression in the pCR2.1 vector. The induced fusion protein could be located at around 100 kDa region in the lanes corresponding to the induced *E. coli* DH5 $\alpha$  harbouring pSAP3. This band was not seen in the induced *E. coli* DH5 $\alpha$  and the induced *E. coli* DH5 $\alpha$  harbouring pSAP4 (Figure 2).

Assay for the enzyme ICL was performed as described earlier<sup>12</sup>. The enzyme assay using crude sonicate revealed that the proteins expressed by both the tac promoter in the PinPoint system and the lac promoter in the pCR2.1 system were functionally active. The yield of glyoxylate obtained after induction of the recombinant clones with IPTG has been shown in Figure 3.

Evolutionary conservation of homologous genes from different organisms is of both theoretical and practical importance. In several cases, a newly isolated gene from an organism may be hitherto unknown and in such cases, its function can be determined on the basis of sequence similarity with a well-studied gene of another organism. Similar homology search approach and PCR cloning using the information of genomics of *M. tuberculosis* provided by Cole *et al.*<sup>13</sup> has resulted in the cloning and expression of *aceA* gene of *M. tuberculosis*.

The PinPoint Xa protein purification system is designed for the production and purification of fusion proteins that are biotinylated *in vivo*. Use of PinPoint system eliminates the necessity to have a specific antibody against the protein which is being over expressed and also circumvents the use of metal chelation steps involved in the purification of the over-expressed proteins in other expression systems.

Computer analysis was done to check the in-frame fusion of the *aceA* gene to the biotin purification tag in all three vectors of the PinPoint Xa system using the software, CLONE ver 4.0. This analysis revealed that the

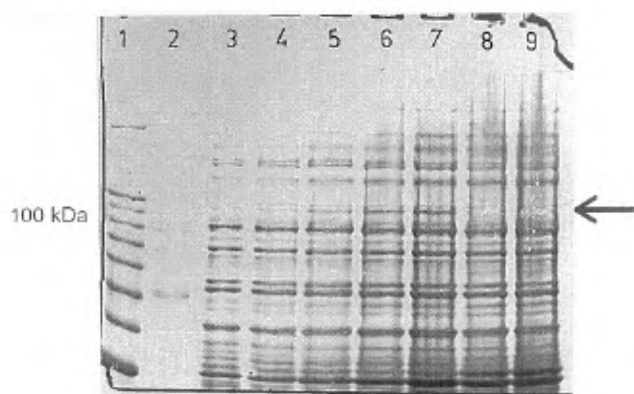
fragment fused in frame in the Xa1 vector. Hence, the 2321 bp fragment obtained by the digestion of pSAP1, was cloned in PinPoint Xa1 expression vector. Initially, when the kit protocol was used without modification, fusion protein could not be detected in Western blots upon induction of the recombinant with IPTG.

One possible explanation could be that, ampicillin selection tends to be lost in cultures, as the drug gets degraded by the secreted  $\beta$ -lactamase enzyme and by the drop in pH, which usually accompanies bacterial fermentation. Hence, a related drug, carbenicillin, which is less sensitive to low pH, was used successfully. After this modification, a band was detected at around 100 kDa, corresponding to the sum of the size of the *aceA* gene product (approximately 90 kDa) and the biotinylated fusion tag (13 kDa).

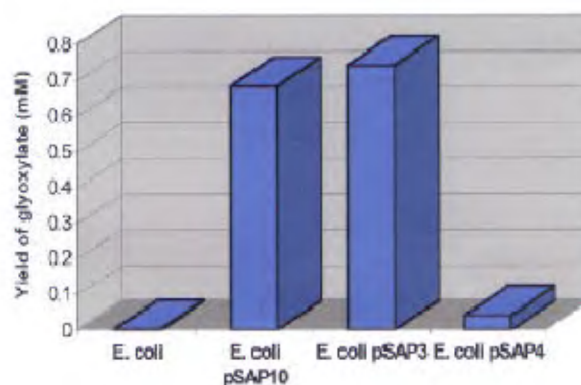
Although it has been predicted in the genome sequence that *aceA* gene could be operative as two modules, there was a suggestion that the organism may be using frame shifting during translation<sup>13</sup>. Our observation of a single large protein of approximately 90 kDa, strengthens this viewpoint. Expression of the *aceA* gene using lac promoter in the pCR2.1 vector also gave a similar picture. Assay for ICL confirmed that the expressed protein was functionally active.

The presence of this enzyme in *M. tuberculosis*, however, is rather surprising because, *M. tuberculosis* is highly adapted to reside within the human macrophages. In the absence of the normal function (utilization of C2 compounds) for which the enzyme is used in other bacteria, the retention of this enzyme by *M. tuberculosis* should have some special reason.

Oxygen limitation causes a four-fold increase in the activity of ICL<sup>14</sup>. Following depletion of nutrition, mycobacteria lose acid-fastness which is attributed to the utilization of the cell wall fatty acids<sup>15</sup>. Mycobacteria produce very long chain fatty acids which serve as the backbone of the mycobacterial cell wall. The use of glyoxylate bypass in the normally growing cells may ensure that additional carbons are available for this anabolic function, though at



**Figure 2.** SDS-PAGE showing expression of *aceA* gene of *M. tuberculosis* in pCR2.1 vector. Proteins from *E. coli* DH5 $\alpha$  harbouring pSAP3 before induction (lane 3) and after 30, 60, 90 and 120 min (lanes 4 to 7) of induction with IPTG, *E. coli* harbouring pSAP4 (lane 8) 120 min after induction, and control *E. coli* (lane 9) 120 min after induction were run along with 10 kDa protein ladder (lane 1) and low molecular weight marker (lane 2) in 10% SDS-PAGE and stained with Coomassie Brilliant Blue. The arrow mark points to the induced fusion product.



**Figure 3.** Graph showing the yield of glyoxylate obtained after induction with IPTG.

the cost of reduced energy production. So, it appears that, ICL is essential for *M. tuberculosis* under normal conditions of growth, the importance of which may be increased in conditions of stress related to dormancy, such as nutrient depletion and anerobic metabolism.

As mentioned earlier, ICL from various bacteria and other organisms has been isolated and biochemically characterized. From these observations, the molecular weight of ICL ranges from a very high value of 123 kDa in *Turbatrix aceti*, a free-living nematode<sup>16</sup>, to a low value of 44.7 kDa in *E. coli*<sup>17</sup>. Our observation is that *M. tuberculosis* ICL has a molecular weight in the range of 90 kDa.

The *aceA* gene encoding ICL, is part of the *aceBAK* operon in *E. coli*. *aceB* encodes malate synthase and *aceK* encodes isocitrate dehydrogenase kinase/phosphatase. In *E. coli* it has been observed that the operon employs a single promoter upstream of *aceB*. It is interesting to note that the expression of *aceK* is downstream of *aceA* gene. It is possible that the competitor enzyme isocitrate dehydrogenase could be regulated by ICL. But in the case of *M. tuberculosis* H37Rv, the organization of this operon seems to be different. From a careful analysis of the genome sequence, it is apparent that *aceB* is situated very far from *aceA*.

Recently Kerstin *et al.*<sup>18</sup>, have characterized the activity and expression of ICL in *M. avium* and *M. tuberculosis* by using two-dimensional gel electrophoresis, of the several proteins expressed by *M. avium* in the intracellular environment. They have reported that only *M. tuberculosis* strain CSU93 expressed detectable levels of *aceA* and not H37Rv. But we have cloned and expressed *aceA* gene of *M. tuberculosis* H37Rv.

Several proteins like crystalline homologue<sup>19</sup>, 27 kDa protein<sup>20</sup> and enzymes like alanine dehydrogenase<sup>21</sup> have been implicated in dormancy.

We therefore concur with the earlier workers that dormancy in *M. tuberculosis* may be controlled by more than one mechanism and further research in this field is necessary to draw a clear-cut conclusion as to how and what exactly triggers dormancy and to see whether ICL has a role to play in dormancy.

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## Digital analysis of induced erythrocyte shape changes in hypercholesterolemia under *in vitro* conditions

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**Under *in vitro* conditions, the incubation of normal erythrocytes in cholesterol-enriched plasma (CEP) for half, one, and two hours leads to increase in the membrane cholesterol level, whereas there is no change in phospholipids composition. This excessive accumulation of cholesterol leads to shape alteration in erythrocytes as observed by phase-contrast microscope. For quantification, these images after digitization are processed to obtain perimeter, area and form factor. These parameters are used to quantify the changes in the shape of erythrocytes induced by hypercholesterolemic process.**

THERE are several *in vivo* mechanisms, which induce changes in erythrocyte membrane lipids and proteins and their interior, leading to alteration in erythrocyte shape, which in turn affects the deformability<sup>1–5</sup>. Such changes under *in vitro* conditions are also observed in erythrocytes

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