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Characterisation of potential antimicrobial targets for tuberculosis.

II. Branched-chain amino acid aminotransferase and methionine regeneration in Mycobacterium tuberculosis.

E.S. Venos, M.H. Knodel, C.L. Radford and B.J. Berger
Defence R&D Canada – Suffield

Technical Report
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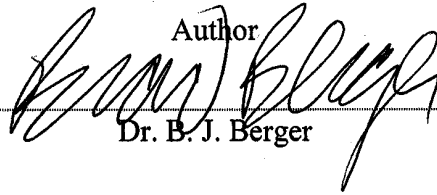
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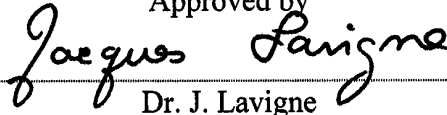
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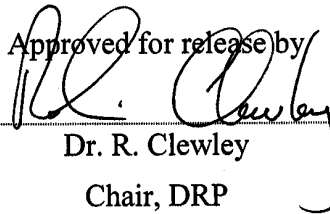
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Abstract

Tuberculosis remains an important problem for the Canadian Forces in many of its overseas deployments. With the spread of drug-resistant strains of *Mycobacterium tuberculosis*, there is an increased need to characterise novel drug targets in the organism. The final step of methionine recycling from methylthioadenosine has been examined in *M. tuberculosis*, and has been found to be catalysed by a branched-chain amino acid aminotransferase. The enzyme was found to be a member of the aminotransferase IIIa subfamily, and closely related to the corresponding aminotransferase in *Bacillus subtilis*, but not to that found in *B. anthracis* or *B. cereus* (Berger et al., *Journal of Bacteriology*, 185, p. 2418-2431, 2003). The amino donor preference for the formation of methionine from ketomethiobutyrate was isoleucine, leucine, valine, glutamate, and phenylalanine. The enzyme catalysed branched-chain amino acid and ketomethiobutyrate transamination with a K_m of 1.77 - 7.44 mM and a V_{max} of 2.17 - 5.70 $\mu\text{mol}/\text{min}/\text{mg}$ protein, and transamination of ketoglutarate with a K_m of 5.79 - 6.95 mM and a V_{max} of 11.82 - 14.35 $\mu\text{mol}/\text{min}/\text{mg}$ protein. Aminoxy compounds were examined as potential enzyme inhibitors, with O-benzylhydroxylamine, O-t-butylhydroxylamine, carboxymethoxylamine, and O-allylhydroxylamine yielding mixed-type inhibition with K_i values of 8.20 - 21.61 μM . These same compounds were examined as antimycobacterial agents in a *M. marinum* model and were found to completely prevent cell growth. O-allylhydroxylamine was the most effective growth inhibitor with an MIC of 78 μM and an IC₅₀ of 8.49 μM .

Résumé

La Tuberculose est un problème encore important dans de nombreux déploiements outremer des Forces canadiennes. Avec la dissémination des souches pharmacorésistantes de *Mycobacterium tuberculosis*, il existe un besoin accru de caractériser de nouvelles cibles pour les médicaments. L'étape finale de recyclage de la méthionine à partir de méthylthioadénosine a été examinée pour la *M. tuberculosis* et on a trouvé qu'elle était catalysée par une transaminase aminoacide de chaîne ramifiée. On a trouvé que l'enzyme était un membre de la sous-famille transaminase IIIa et étroitement liée à la transaminase correspondante du *Bacillus subtilis* mais pas à celle que l'on trouve dans le *B. anthracis* ou *B. cereus* (Berger et al., *Journal of Bacteriology*, 185, p. 2418-2431, 2003). La préférence des donneurs amines pour la formation de méthionine à partir de kétométhiobutyrate étaient l'isoleucine, la leucine, la valine, le glutamate et la phénylalanine. L'enzyme a catalysé l'acide aminé de chaîne ramifiée et la transamination du kétométhiobutyrate avec une K_m de 1,77 - 7,44 mmole et une V_{max} de 2,17 - 5,70 $\mu\text{mol}/\text{min}/\text{mg}$ protéine et une transamination de kétoglutarate avec une K_m de 5,79 - 6,95 mM et une V_{max} de 11,82 - 14,35 $\mu\text{mol}/\text{min}/\text{mg}$ protéine. Les composés amino-oxy ont été examinés comme inhibiteurs potentiels d'enzymes, avec O-benzylhydroxylamine, O-t-butylhydroxylamine, carboxyméthoxylamine et O-allylhydroxylamine produisant des inhibitions de type mixte ayant des valeurs K_i de 8,20 - 21,61 μM . Ces mêmes composés ont été examinés comme agents antimycobactériens dans un modèle *M. marinum* et il a en été conclu qu'ils empêchent complètement la croissance de la cellule. O-allylhydroxylamine était l'inhibiteur de croissance le plus efficace avec un CMI de 78 μM et une CI 50 de 8,49 μM .

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Executive summary

Tuberculosis remains one of the world's greatest disease threats, with approximately 8 million new infections and 1-2 million deaths per year. In addition, tuberculosis is one of the three endemic disease threats (along with malaria and dengue fever) that are of particular concern to the Canadian Forces during overseas deployment. Due to various features in its biology, such as its dense waxy coat, its ability to live inside human white blood cells, and its ability to remain dormant for decades, tuberculosis is difficult to treat and requires multiple drugs over a long period of time. In addition, the spread of multidrug-resistant tuberculosis has placed enormous pressure on the few existing chemotherapeutic compounds.

Given the current state of antitubercular chemotherapy, there is a strong need for the identification and characterisation of novel drug targets in the organism. This laboratory has been investigating enzymes involved in polyamine biosynthesis and its associated methionine salvage pathways as potential drug targets in a number of organisms. Polyamines are small molecular weight nitrogenous compounds that are essential for cellular replication. The biosynthesis of polyamines consumes the amino acid methionine in a one-to-one ratio, yielding methylthioadenosine as a byproduct. As methionine is an essential compound in its own right, is present in limiting amounts in the cell, and is energetically expensive to synthesize de novo, cells have a unique pathway for regenerating methionine from methylthioadenosine. It is known that inhibition of enzymes in this pathway leads to cell death in a number of organisms, including malaria. The final step in this recycling pathway is the conversion of ketomethiobutyrate to methionine by an aminotransferase. In *M. tuberculosis*, we have discovered that the particular aminotransferase catalysing this reaction is a branched-chain amino acid aminotransferase. This enzyme appears to be closely related to a similar aminotransferase found in *Bacillus subtilis*, but not to that found in *B. cereus* or *B. anthracis*. The tuberculosis enzyme has been successfully cloned and recombinant protein expressed, purified, and characterised. The kinetic constants for the enzyme were consistent with those previously found for branched-chain amino acid aminotransferase in *B. subtilis*.

A number of potential inhibitors have been tested against the tuberculosis enzyme, with several yielding potent inhibition of methionine production. These inhibitors were also screened as antimycobacterial agents in a *Mycobacterium marinum* growth model. All of the compounds were able to completely prevent cell growth, with the best inhibitor being O-allylhydroxylamine. This class of inhibitor, which contains an aminoxy substituent, shows promise as the basis of future antimycobacterial development.

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Sommaire

La tuberculose demeure une maladie dont la menace est une des plus importantes au monde, avec approximativement 8 millions de nouvelles infections et 1 à 2 millions de décès par an. De plus, la tuberculose est une des trois menaces de maladies endémiques (avec la malaria, et la dengue) qui préoccupent particulièrement les Forces canadiennes durant les déploiements outremer. La tuberculose est difficile à traiter du fait que plusieurs de ses caractéristiques biologiques, telles que son revêtement cireux et dense, sa capacité à vivre à l'intérieur des globules blancs d'un être humain et à demeurer dormante pendant des décennies ; elle requiert la prise de multiples médicaments durant une période prolongée. De plus, la dissémination de la tuberculose ayant une résistance multiple aux médicaments fait pression sur les quelques composés chimiothérapeutiques existants.

Étant donné l'état actuel de la chimiothérapie antituberculeuse, il faut absolument identifier et caractériser de nouvelles cibles de médicaments dans l'organisme. Ce laboratoire a examiné, comme cibles de médicaments, les enzymes participant à la biosynthèse de polyamine et des voies de récupération de méthionine qui leur sont associées dans un certain nombre d'organismes. Les polyamines sont des espèces azotées de poids moléculaire léger qui sont essentielles à la réplication cellulaire. La biosynthèse de polyamines consomme l'acide aminé méthionine dans un rapport égal produisant de la méthylthioadénosine comme sous-produit. Du fait que la méthionine est un composé essentiel en lui-même, qu'elle existe en petite quantité dans la cellule et qu'elle est coûteuse en énergie à synthétiser de novo, les cellules ont une voie unique pour régénérer la méthionine à partir de la méthylthioadénosine. On sait déjà que l'inhibition d'enzymes dans cette voie amène à la mort de la cellule dans un certain nombre d'organismes, y compris la malaria. L'étape finale dans cette voie de recyclage est la conversion de kétométhiobutyrate en méthionine par une transaminase. Dans la *M. tuberculosis*, on a découvert que la transaminase particulière qui catalyse la réaction est une transaminase aminoacide à branche ramifiée. Cette enzyme apparaît être étroitement liée à une transaminase similaire que l'on trouve dans le *Bacillus subtilis* mais que l'on ne trouve pas dans le *B. cereus* or *B. anthracis*. On a réussi à cloner l'enzyme de la tuberculose, à exprimer la protéine recombinante, à la purifier et à la caractériser. Les constantes cinétiques de l'enzyme correspondaient à celles qui avaient été trouvées auparavant pour les transaminases aminoacides à branche ramifiée du *B. subtilis*.

Un certain nombre d'inhibiteurs potentiels a été testé contre les enzymes de la tuberculose dont plusieurs exerçant une inhibition potente de la production de méthionine. Ces inhibiteurs ont aussi été criblés comme agents antimycobactériens dans un modèle de croissance *Mycobacterium marinum*. Tous les composés ont pu complètement empêcher la croissance de la cellule, le meilleur inhibiteur étant O-allylhydroxylamine. Cette classe d'inhibiteurs qui contient un substituant amino-oxy montre des promesses comme fondement aux futurs développements antimycobactériens.

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Introduction

Tuberculosis remains one of the leading causes of worldwide mortality and morbidity, infecting an estimated 8 million people annually with approximately 2 million deaths [1]. The situation regarding the control of tuberculosis has significantly worsened over the last decades, with the spread of multidrug resistant strains. In the absence of an effective vaccine for tuberculosis, there is an urgent need for the development of novel antimycobacterial agents. The study of mycobacterial biochemistry assists this development through the identification and characterisation of cellular enzymes amenable to therapeutic inhibition.

Polyamine synthesis and its associated methionine (Met) regeneration pathway (Figure 1) are known to be potential drug targets in a variety of microorganisms [2-4]. The synthesis of polyamines is essential during periods of DNA replication, although the exact physiological role of these compounds remains unclear [2]. The production of spermidine from putrescine, or spermine from spermidine, consumes the amino acid Met in a 1:1 stoichiometry yielding methylthioadenosine (MTA) as a byproduct. As Met biosynthesis is energetically expensive, and many organisms lack the ability to synthesize the amino acid, a unique pathway exists which recycles Met from MTA. To date, the entire pathway has only been fully characterised in the Gram-negative bacterium *Klebsiella pneumoniae* [5-11], and analogous enzymes have been identified in silico for the Gram-positive bacterium *Bacillus subtilis* [12,13]. Selected individual enzymes active in the pathway have been studied in a wide variety of eukaryotic and prokaryotic organisms [5,14-18]. For *Mycobacterium spp.*, only methionine adenosyltransferase has been cloned, expressed, and fully characterised [19].

The final step in Met regeneration is the transamination of ketomethiobutyrate (KMTB) by an aminotransferase. The specific aminotransferase responsible for the reaction has been identified and characterised in a number of microorganisms, including malaria, African trypanosomes, *K. pneumoniae*, *B. subtilis*, and *B. anthracis* [5,16,17]. In the lower eukaryotes *Plasmodium falciparum*, *Trypanosoma brucei brucei*, *Giardia intestinalis*, and *Crithidia fasciculata*, this reaction is catalysed by the subfamily Ia enzyme aspartate aminotransferase [16]. In *K. pneumoniae*, however, the reaction was performed by the close homologue tyrosine aminotransferase, which is also a member of subfamily Ia [5]. Gram-positive bacteria and archaeobacteria appear to lack any subfamily Ia homologues in their genomes, and *B. subtilis*, *B. cereus*, and *B. anthracis* were recently found to catalyse Met regeneration via a branched-chain amino acid aminotransferase (BCAT) [17]. This enzyme is a member of family III, along with D-amino acid aminotransferase (DAAT), and is unrelated structurally to family I enzymes [20]. Intriguingly, *B. subtilis* and *B. cereus/B. anthracis* utilised BCAT enzymes from separate subfamilies (IIIa vs. IIIb respectively). As *Mycobacterium spp.* are Gram-positive bacteria, it would be expected that *M. tuberculosis* also catalyses the conversion of KMTB to Met via a BCAT. In this paper, we report the identification, cloning, and functional expression of a single BCAT from *M. tuberculosis*. In addition, this enzyme has been demonstrated to actively catalyse Met regeneration and is subject to inhibition by a variety of aminoxy compounds.

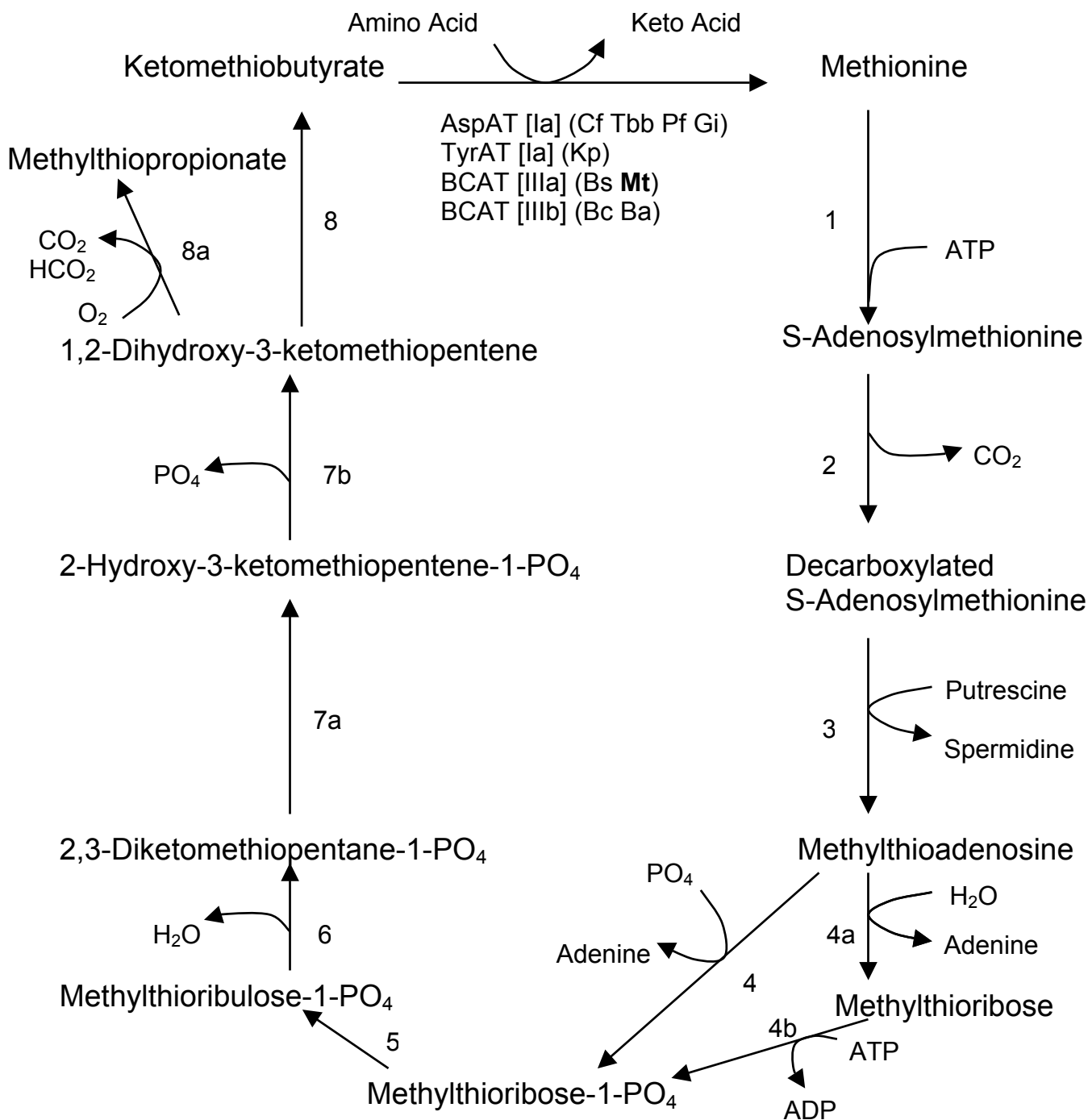


Figure 1. The methionine regeneration pathway. The labelled enzymes are as follows: 1, methionine adenosyltransferase; 2, S-adenosylmethionine decarboxylase; 3, spermidine/spermine aminopropyltransferase; 4a, methylthioadenosine nucleosidase; 4b, methylthioribose kinase; 4, methylthioadenosine phosphorylase; 5, unidentified isomerase; 6, unidentified dehydratase; 7a and 7b, bifunctional enolase-phosphatase; 8, non-enzymatic or dioxygenase; 8a, dioxygenase. The specific aminotransferases experimentally found to catalyze KMTB to Met conversion are shown, with the subfamily membership in square brackets. The organism abbreviations are as follows: Cf, *Crithidia fasciculata*; Tbb, *Trypanosoma brucei brucei*; Pf, *Plasmodium falciparum*; Gi, *Giardia intestinalis*; Kp, *Klebsiella pneumoniae*; Bs, *Bacillus subtilis*; Mt, *Mycobacterium tuberculosis*; Bc, *Bacillus cereus*; Ba, *Bacillus anthracis*.

Materials and Methods

Cells and Reagents

M. tuberculosis H37Rv was acquired from Dr. J. Talbot, University of Alberta, and *M. marinum* Aronson (ATCC927) was obtained from the National Collection of Type Cultures (London, UK). Cells were cultured in liquid Middlebrook 7H9 medium or on Middlebrook 7H10 plates at 37°C for *M. tuberculosis* or 30°C for *M. marinum*. All substrates and inhibitors were obtained from Sigma Chemical Co. or Aldrich Chemical Co. (Oakville, ON, Canada).

Cloning and Functional Expression

Genomic DNA was isolated from *M. tuberculosis* by vortexing packed cells in a minimal volume of 50 mM Tris-HCl (pH 8.0)/10 mM EDTA/100 mM NaCl containing 500 µm acid washed glass beads (Sigma). After allowing the glass beads to settle, the supernatant was added to an equal volume of 10 mM Tris-HCl pH 8.0/100 mM NaCl/25 mM EDTA/0.5% w/v sodium dodecyl sulfate/0.1 mg/ml proteinase K and incubated for 1 hr at 37°C with occasional gentle mixing. The mixture was then subjected to extraction with phenol and chloroform:isoamyl alcohol (24:1), and the DNA ethanol precipitated.

The sequence of the putative *M. tuberculosis* BCAT gene was discovered by a BLAST search of the complete *M. tuberculosis* H37Rv genome using the *B. subtilis* YbgE, YwaA, or YheM gene products as the query proteins [17,21,22]. The single resulting putative BCAT gene was used to construct oligonucleotide primers for PCR amplification. The 5' primer was TCGAGGCGGCCGCAAATGACCAGCGGCTCCCTTCA and incorporated a NotI restriction site and an in-frame start codon. The 3' primer was ATCGAGCTCGAGTTACCCAGCCGCGCCATCCAG and incorporated a XhoI restriction site and an in-frame stop codon. The BCAT gene was then amplified using a 5:1 mixture of Taq:Pfu polymerases (Promega; Madison, WI, USA) and the following program: 1 cycle of 95°C for 1.5 min; 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min; and 1 cycle of 72°C for 10 min. The resulting PCR product was excised from a 1% agarose gel and recovered using the Qiaex II kit (Qiagen; Mississauga, ON, Canada). The purified product was digested with NotI and XhoI and ligated into a similarly digested pET 19m (a modified pET19b (Novagen; Madison, WI, USA) which contains extra restriction sites in the multiple cloning site; see Figure 2) using a Rapid Ligation kit (Fermentas; Burlington, ON, Canada). The recombinant plasmid was then transformed into *Escherichia coli* XL10 cells (Stratagene; La Jolla, CA, USA) and was subsequently recovered using the Qiaspin miniprep kit (Qiagen). Positive clones were determined by digesting the plasmid with NotI and XhoI, and confirming the presence of the insert on a 1% agarose gel.

The plasmid from positive clones was transformed into *E. coli* BL21(DE3) CodonPlus RIL cells (Stratagene) for functional expression. Cells were grown in liquid LB medium containing 50 µg/ml ampicillin and 50 µg/ml chloramphenicol at 37°C and 250 rpm until the culture reached an A_{600nm} of 0.6-0.8. The culture was then cooled to 20°C for 30 min at 250

EDTA/trace pyridoxal-5-phosphate (PLP) overnight at 4°C. The concentrated enzymes were stored at 4°C for several days, or with 20% v/v glycerol at -20°C for several weeks without appreciable loss of activity. Recombinant protein samples were examined by electrophoresis on 10% SDS polyacrylamide gels followed by Coomassie brilliant blue R250 staining. Protein concentration was measured using the Bio-Rad reagent (Bio-Rad; Mississauga, ON, Canada).

Enzyme Assays and Inhibition Studies

Aminotransferase activities were assayed by an HPLC method [16]. Five or 10 µl of recombinant enzyme was added to 100 µl of substrate mix (100 mM PO₄ (pH 7.4)/50 µM PLP/various concentrations of amino acid/various concentration of keto acid) and incubated for 30 min at 37°C. The samples were then stored at -20°C until analysis by HPLC. All samples were analysed by pre-column derivatisation and reverse-phase HPLC. 10 µl of sample was mixed with 50 µl of 400 mM borate (pH 10.5) and then with 10 µl of 10 mg/ml o-phthalaldehyde/12 µl/ml mercaptopropionate/400 mM borate (pH 10.5) prior to the injection of 7.0 µl onto a 2.1 x 200 mm ODS-AA column (Agilent; Mississauga, ON, Canada). The column was eluted using 2.72 mg/ml sodium acetate (pH 7.2)/0.018% v/v triethylamine/0.3% v/v tetrahydrofuran as Buffer A and 2.72 mg/ml sodium acetate (pH 7.2)/40% v/v methanol/40% v/v acetonitrile as Buffer B with a linear gradient of 0 – 17% B over 16 min followed by a linear gradient of 17-100% B over 1 min and 6.0 min at 100% B. The flow rate was 0.45 ml/min from 0 – 16 min and 0.80 ml/min from 17-24 min. The elution of derivatised amino acids was monitored at 338 nm and fluorometrically with an excitation of 338 nm and an emission of 450 nm. All separations were performed on an Agilent 1100 HPLC equipped with an autosampler, variable wavelength ultraviolet/visible spectrophotometric detector, fluorescence detector, and Chemstation operating system.

The amino donor range for Met regeneration was determined by incubating 2 mM of each individual amino acid and 1 mM KMTB, followed by HPLC for Met quantification. Amino acids that were effective amino donors were further studied at 0.1 - 10 mM amino acid and 10 mM KMTB to determine the kinetic constants. Similar assays were performed with 0.1 - 10 mM KMTB and 10 mM Leu. Replacement of KMTB with ketoglutarate (KG) in these experiments and subsequent HPLC analysis of Glu formation allowed for the determination of BCAT activity. The apparent K_m and V_{max} values for each substrate was assessed by non-linear curve fitting using the Scientist software programmed with the Michaelis-Menton equation (Micromath; Salt Lake City, UT, USA).

Initial inhibition studies screened 13 aminooxy compounds against *M. tuberculosis* BCAT using 2.0 mM Leu/1.0 mM KMTB/0.1 or 1.0 mM inhibitor in the enzyme incubation. Inhibitors which demonstrated better than 50% reduction of activity at the 0.1 mM concentration were further studied for the determination of K_i values. These reactions involved 0.5, 1.0, 2.0, or 3.0 mM Leu and 1.0 mM KMTB in the reaction mixture together with 0, 25, 50, 75, 100, 150, or 200 µM of inhibitor. The K_i values were determined by non-linear curve fitting with the Scientist software programmed with competitive, uncompetitive, and mixed inhibition equations [23].

In vitro growth inhibition studies were performed on *M. marinum* using the most effective enzyme inhibitors. A culture of *M. marinum* at mid-log growth in Middlebrook 7H9 medium was diluted to 2×10^5 cfu/ml and 100 μ l added to a 96 well microtitre plates containing 100 μ l of doubling dilutions of each inhibitor. The final inhibitor concentration ranged from 10 mM - 298 pM. Positive and negative controls consisted of 100 μ l Middlebrook 7H9 medium replacing the inhibitor or cells respectively. The plates were incubated at 30°C and no agitation for 8 days before measurement of growth at A_{650nm} using a Molecular Devices 96-well spectrophotometer (Sunnyvale, CA, USA). The MIC was determined as the lowest dilution that completely prevented microbial growth and the IC_{50} was determined by non-linear curve fitting with the Scientist software programmed with the Chou equation [24].

Phylogenetic Analysis

Additional BCAT and DAAT sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein>) [25]. *Mycobacterium spp.* BCAT sequences from preliminary genome projects were made available from The Institute for Genomic Research (<http://www.tigr.org>) for *M. smegmatis* and *M. avium*, from The Sanger Centre (<http://www.sanger.ac.uk>) for *M. marinum*, and from The Institut Pasteur (<http://www.pasteur.fr>) for *M. ulcerans*. These sequences were aligned using the Clustal algorithm and the BLOSUM sequence substitution table in the ClustalX program [26]. Aligned sequences were viewed using the Bioedit program [27] and then were then used with the ProtDist component of the PHYLIP [28] to construct a distance matrix that was the basis for tree construction using the neighbour-joining method [29]. All trees were visualized using Treeview [30].

Results

Branched-chain Amino Acid Aminotransferase in *M. tuberculosis*

The complete, published genome of *M. tuberculosis* H37Rv was found to contain a single gene with a very high sequence homology to either *B. subtilis* ybgE or ywaA, which are both known to be subfamily IIIa BCATs [17,22]. In contrast, the tuberculosis genome did not contain a homologue to *B. subtilis* yheM, *B. cereus* BCAT, or *B. anthracis* BCAT, which are all subfamily IIIb aminotransferases [17]. The putative *M. tuberculosis* BCAT gene, Rv2210c, has not been previously cloned, expressed, or characterised. It is interesting to note that the *M. tuberculosis* genome contains a single BCAT homologue and no obvious DAAT homologue.

Examination of complete and incomplete genome projects for *Mycobacterium* spp. uncovered a single gene in *M. leprae*, *M. bovis*, *M. marinum*, *M. ulcerans*, *M. avium*, and *M. smegmatis* with an extremely high identity to Rv2110c. Together, with other family III aminotransferases, the putative mycobacterial sequences were aligned and a cladogram constructed using the neighbor-joining method [29] (Figure 3). The *M. tuberculosis* and *M. bovis* sequences were identical, as were the *M. marinum* and *M. ulcerans* sequences. Aside from *M. bovis*, all the mycobacterial BCAT sequences were found to be 85 - 88% identical to the *M. tuberculosis* sequence. However, the tuberculosis sequence was only 57% to the putative BCAT from *Streptomyces coelicolor* and 45% identical to *B. subtilis* ybgE. There was little sequence conservation with enzymes found in subfamily IIIb, with only 27% identity to the *E. coli* BCAT, 18% to the *B. anthracis* BCAT, and 15% to *B. subtilis* yheM.

This low level of sequence conservation outside of the genus can be seen in the alignment of selected BCAT sequences shown in Figure 4. Only 19 residues are completely conserved across even this small sequence sampling. Interestingly, of the residues found by X-ray crystallography to be important in substrate binding to the *E. coli* BCAT [31], only K228(K159) and T339(T257) were conserved across the 13 sequences in Figure 4. The residues in parentheses represent the corresponding position in the *E. coli* BCAT. Of these two residues, K228(K159) is the PLP binding site and would be expected to be invariant. If one excludes the only DAAT in Figure 4, then Y91(Y31), F96(36), Y233(164), and A340(A258) can be added to this conserved list of residues important for substrate binding in the *E. coli* BCAT. Clearly, sequence conservation is very low across family III.

Cloning and Functional Expression of Branched-chain Aminotransferase

The putative *M. tuberculosis* BCAT was cloned as a deca-histidine fusion protein for expression in *E. coli*. To prevent complete inclusion of the recombinant protein, it was necessary to induce expression with a relatively low concentration of IPTG (0.1 mM) at 20°C for 20 hr. Even under these conditions, the majority of the protein was localised to inclusion

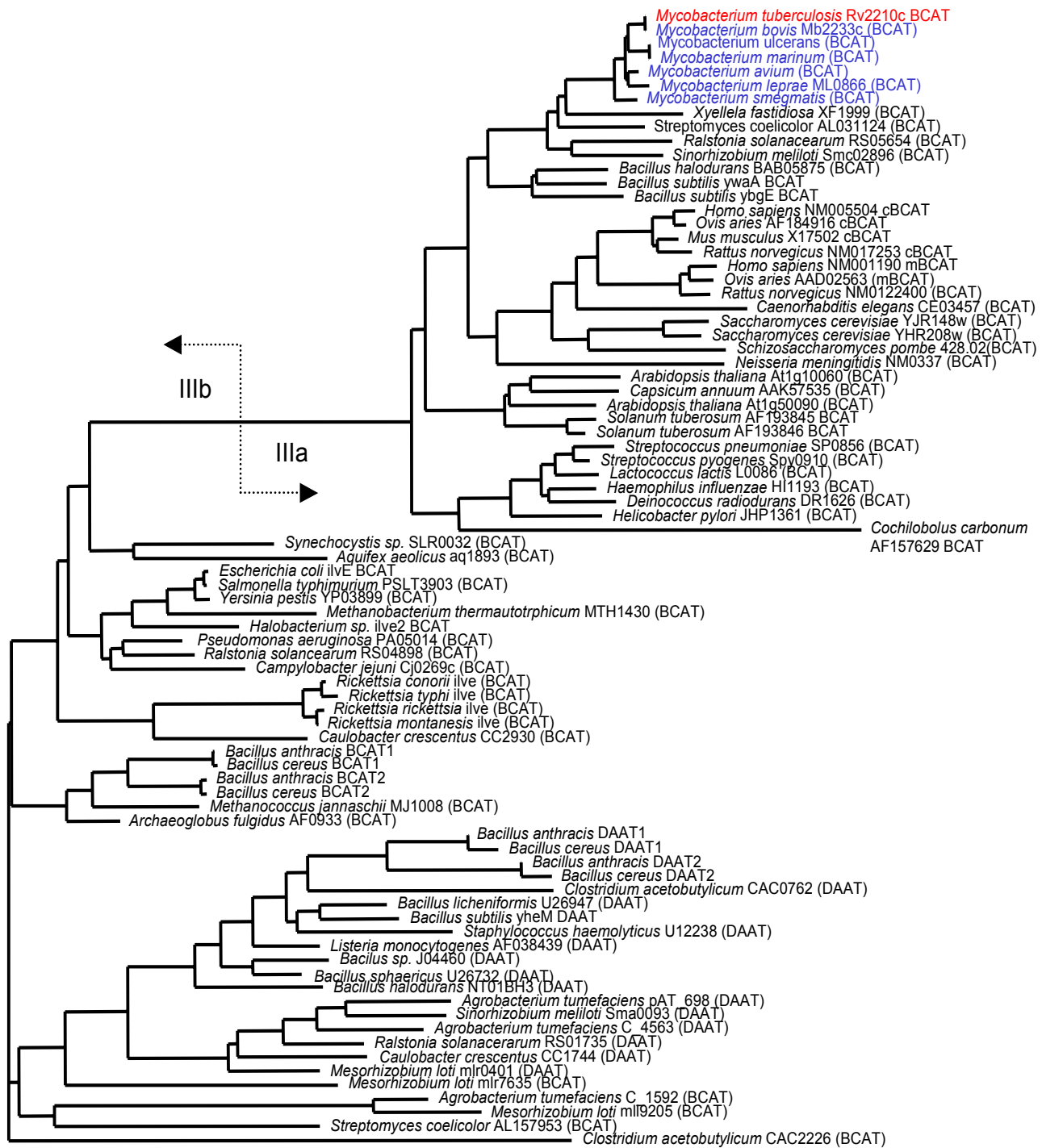


Figure 2. Family III aminotransferases. The BCAT and DAAT sequences were aligned with the Clustal algorithm and used for tree construction with the neighbor joining method. The division between subfamilies IIIa and IIIb is shown by arrows. The *Mycobacterium tuberculosis* BCAT is highlighted in red. Other *Mycobacterium* spp. BCATs are highlighted in blue.

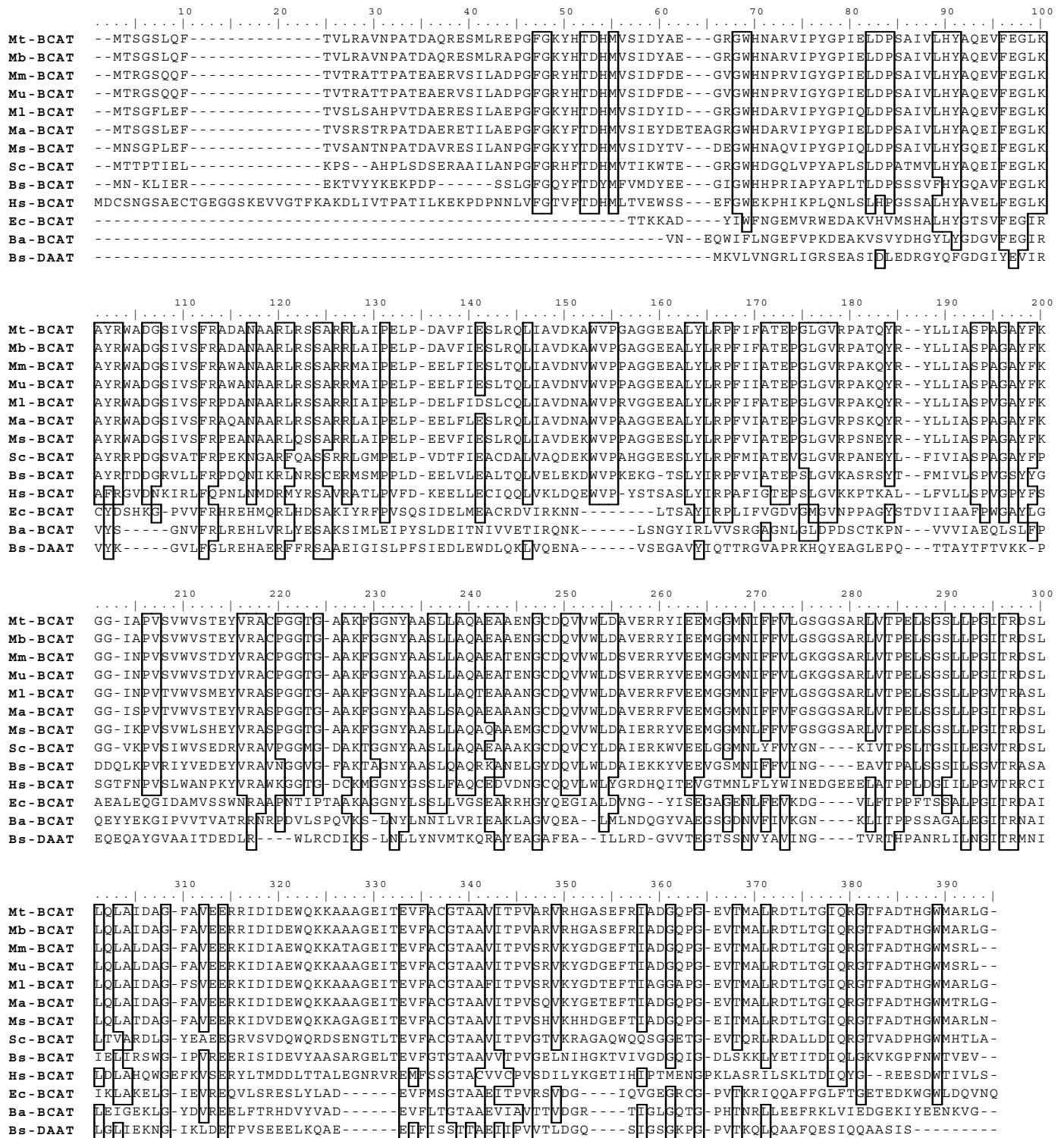


Figure 3. Alignment of selected family III aminotransferases. The following sequences were aligned with the Clustal algorithm: Mt-BCAT, *M. tuberculosis* BCAT [22]; Mb-BCAT, *M. bovis* BCAT [42]; Mm-BCAT, *M. marinum* BCAT; Mu-BCAT, *M. ulcerans* BCAT; Ml-BCAT, *M. leprae* BCAT [43]; Ma-BCAT, *M. avium* BCAT; Ms-BCAT, *M. smegmatis* BCAT; Sc-BCAT, *Streptomyces coelicolor* BCAT [44]; Bs-BCAT, *Bacillus subtilis* BCAT [21]; Hs-BCAT1, human BCAT1 [45]; Ec-BCAT, *Escherichia coli* BCAT [46]; Ba-BCAT, *B. anthracis* BCAT [17]; Bs-DAAT, *B. subtilis* DAAT [21]. Residues conserved by 75% of the sequences are boxed.

bodies, although sufficient soluble material was produced and purified over Ni²⁺ affinity columns (Figure 5). Assay of the eluted material with 2 mM each of the amino acids ADEFGHIKLNQRSTVWY and 1 mM KMTB resulted in appreciable Met production (data not shown), demonstrating that the enzyme was active and catalysed Met regeneration.

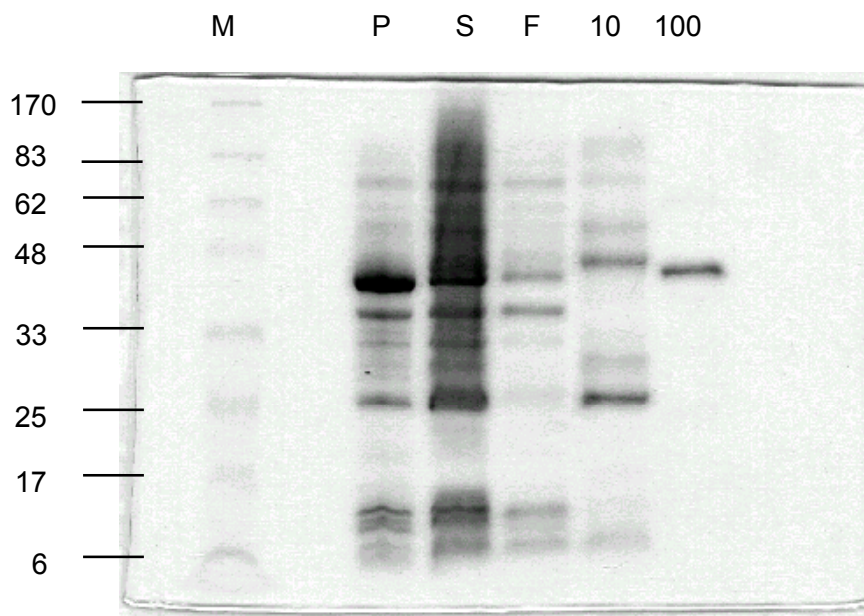


Figure 5. Purification of recombinant *M. tuberculosis* BCAT. *E. coli* BL21(DE3) CodonPlus-RIL cells were induced with IPTG and prepared as described in the Materials and Methods section. The cell lysate was separated by centrifugation into pellet (P) and supernatant (S) fractions. The supernatant was loaded onto an Ni²⁺-charged metal ion affinity column and flowthrough (F), 10% (v/v) elution buffer (10), and 100% (v/v) elution buffer (100) fractions were collected. Aliquots of each fraction were analysed on a 10% polyacrylamide gel under reducing conditions. Lane (M) contains molecular weight markers (units in kDa).

Enzyme Characterisation and Inhibition

The purified enzyme was screened against 2 mM of each individual amino acid and 1 mM KMTB to determine the amino donor range for Met regeneration. Isoleucine, leucine, and valine were found to be the most effective substrates (Figure 6), while glutamate and phenylalanine were also active as amino donors. Tyrosine and tryptophan were found to have a much lesser ability to transaminate KMTB and all other amino acids were inactive. The five most active amino donors were more closely examined in order to determine their kinetic parameters (Table 1). The Km for Leu, Ile, and Val ranged from 1.77 - 2.85 mM, while that for Glu was 9.53 mM and Phe 7.44 mM. The Vmax for all five amino acids was similar at 2.17 - 5.70 $\mu\text{mol}/\text{min}/\text{mg}$ protein. KMTB was found to have a Km of 4.20 mM. The enzyme was also examined for branched-chain amino acid and KG aminotransfer in order to characterise the "classic" reactions associated with a BCAT (Table 1). The Km of the

substrates was found to be similar, while the V_{max} ranged from 11.82 - 14.35 $\mu\text{mol}/\text{min}/\text{mg}$ protein. Therefore, the tuberculosis BCAT catalyzes aminotransfer of KG about 3 times more readily than KMTB. This result is broadly similar to that seen with the *B. subtilis* BCAT, which also transaminates KG at a higher rate than KMTB [17].

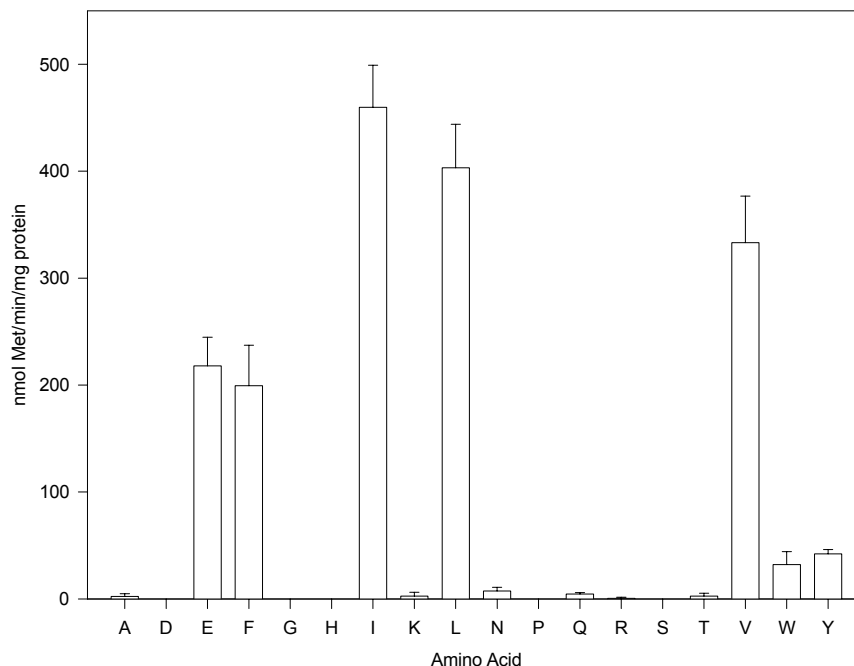


Figure 6. The amino donor range for Met regeneration. The enzyme was mixed with 1.0 mM KMTB, 2.0 mM of an individual amino acid, and PLP for 30 min at 37 °C before HPLC analysis of Met production.

Thirteen aminoxy compounds were assayed for inhibitory effects on the tuberculosis BCAT. The enzyme was incubated with 2.0 mM leucine, 1.0 mM KMTB and 0.1 or 1.0 mM inhibitor to assay for the effect on Met regeneration (Figure 7). With the exception of O-trimethylsilylhydroxylamine, all of the compounds inhibited Met formation to some extent. The four most active compounds at 0.1 mM were O-allylhydroxylamine, carboxymethoxylamine, O-benzylhydroxylamine, and O-t-butylhydroxylamine, and these inhibitors were further examined in order to determine K_i values (Table 2). For all four compounds, the inhibition data was not consistent with a simple competitive or uncompetitive model, but fit very well with a model of mixed mode inhibition [23]. The competitive component of inhibition yielded a K_{ic} of 8.20 - 21.61 μM , while the uncompetitive component gave a K_{iu} of 84.08 - 386 μM . Therefore, the inhibition of the tuberculosis BCAT by these four aminoxy compounds is primarily competitive.

These four inhibitors and canaline, an aminoxy analogue of ornithine that has been

Table 1. Kinetic characterization of *M. tuberculosis* branched-chain aminotransferase. The enzyme was incubated with varying concentrations of substrate and 10 mM cosubstrate, as described in the Materials and Methods section.

SUBSTRATE	COSUBSTRATE	APPARENT K_m (mM)	APPARENT V_{MAX} ($\mu\text{mol}/\text{min}/\text{mg}$ protein)
Leu	KMTB	2.50 \pm 0.90	3.65 \pm 0.43
Val	KMTB	1.77 \pm 0.86	2.58 \pm 0.41
Ile	KMTB	2.85 \pm 0.56	4.28 \pm 0.32
Glu	KMTB	9.53 \pm 3.43	5.70 \pm 1.20
Phe	KMTB	7.44 \pm 1.40	2.17 \pm 0.22
KMTB	Leu	4.20 \pm 1.79	4.22 \pm 0.72
Leu	KG	6.02 \pm 0.94	13.44 \pm 0.84
Val	KG	5.79 \pm 0.99	11.82 \pm 0.80
Ile	KG	6.16 \pm 1.14	14.35 \pm 1.08
KG	Leu	6.95 \pm 1.44	12.80 \pm 1.12

Table 2. K_i determination for selected aminoxy inhibitors. The enzyme was incubated with variable amounts of leucine and inhibitor and fixed amounts of KMTB, as described in the Materials and Methods section. K_{ic} and K_{iu} refer to the competitive and uncompetitive components of mixed-type inhibition [23].

INHIBITOR	K_{ic} (μM)	K_{iu} (μM)
O-(tert-Butyl)hydroxylamine	11.02 \pm 2.76	85.60 \pm 44.79
Carboxymethoxylamine	20.97 \pm 7.27	142.42 \pm 69.76
O-allylhydroxylamine	21.61 \pm 11.08	386 \pm 345
O-benzylhydroxylamine	8.20 \pm 2.56	84.08 \pm 31.91

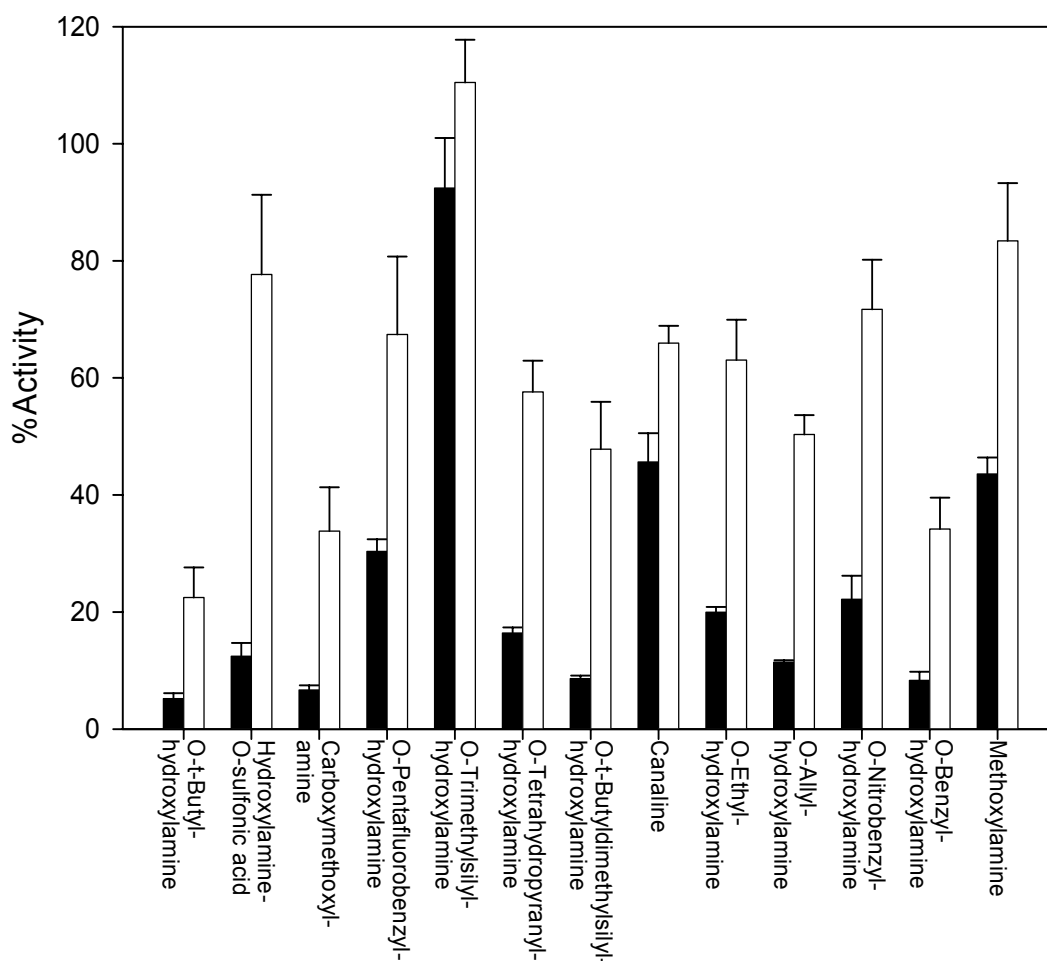


Figure 7. Inhibition of branched-chain aminotransferase by aminoxy compounds. Leucine, KMTB, PLP and 1 mM (black bars) or 0.1 mM (white bars) of inhibitor were incubated with Mt-BCAT as described in the Materials and Methods section. Percent residual activity was calculated by measuring amount of Met produced when the inhibitor was incubated with enzyme versus a positive control with no inhibitor.

Table 3. In vitro growth inhibition of *M. marinum* by aminoxy compounds. One hundred μL of a mid-logarithmic culture of *M. marinum* at a concentration of 2×10^5 cfu/ml was added to 100 μL of serial doubling dilutions of inhibitor in a 96-well microtitre plate. The drug plates were grown for eight days at 30 °C with no agitation before checking for cell growth at $A_{650\text{nm}}$. The minimum inhibitory concentration (MIC) and inhibitory concentration 50% (IC_{50}) were calculated as described in the Materials and Methods section.

INHIBITOR	MIC (mM)	IC_{50} (μM)
O-allylhydroxylamine	0.078	8.49 ± 1.96
Carboxymethoxylamine	0.313	89.32 ± 7.65
O-benzylhydroxylamine	1.25	84.08 ± 31.91
Canaline	1.25	335.29 ± 13.64
O-(tert-Butyl)hydroxylamine	10	43.18 ± 10.51

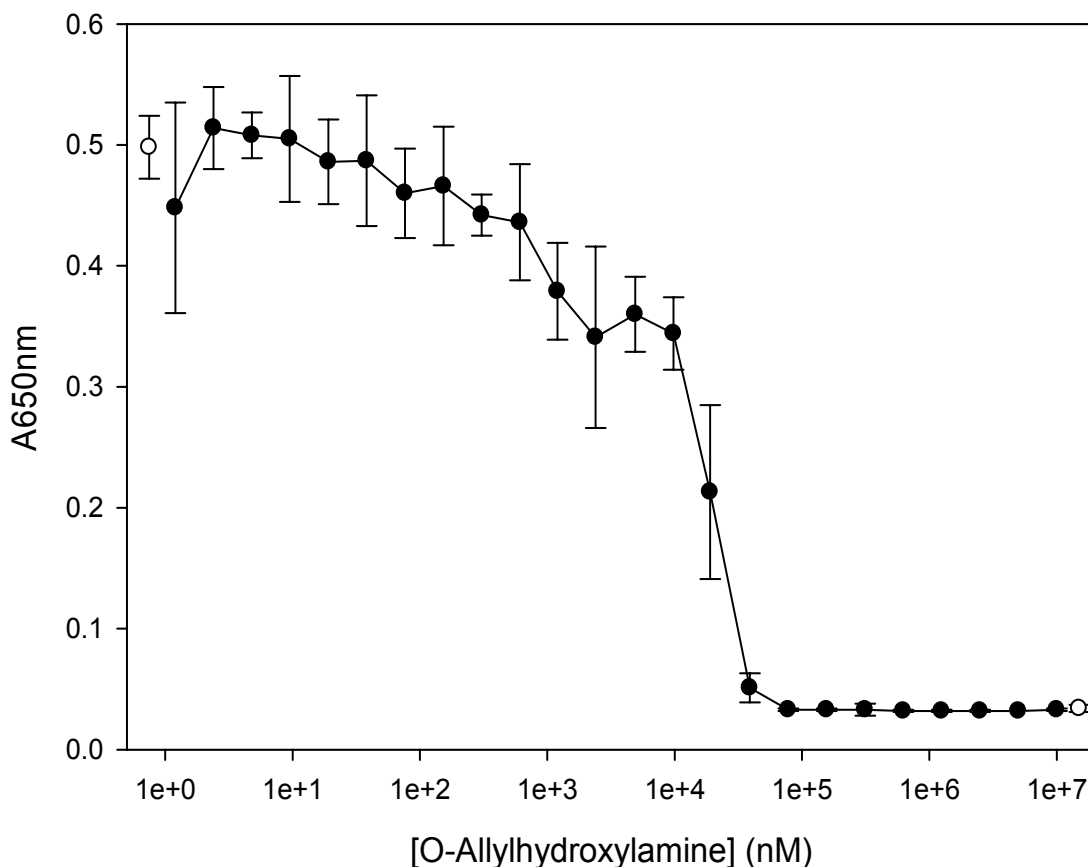


Figure 8. The inhibition of *Mycobacterium marinum* growth by O-allylhydroxylamine. One hundred μL of *M. marinum* culture growing in Middlebrook 7H9 media was inoculated into a 96 well microtitre plate containing 100 μL of sequential serial doubling dilutions of inhibitor, as described in the Materials and Methods section. After 8 days of growth at 30 °C, the plates were examined for growth at $A_{650\text{nm}}$ by a 96 well spectrophotometer.

demonstrated to be an effective aminotransferase inhibitor in other systems [16,17,32-34], were screened against *M. marinum* in vitro to determine potential antimicrobial activity. *M. marinum* is a close relative of *M. tuberculosis* that causes a similar disease in fish, grows faster than *M. tuberculosis* in culture, and does not cause serious infections in humans [35]. As such, it is an excellent surrogate for the initial screening of antimycobacterial agents. All the inhibitors were found to have some degree of antimycobacterial activity (Table 3), with MIC values ranging from 78 μM - 10 mM and IC_{50} values of 8.49 μM - 335 μM . The best inhibitor was found to be O-allylhydroxylamine (Figure 8). While O-t-butylhydroxylamine and O-benzylhydroxylamine appeared to be the best enzyme inhibitors, they were significantly less effective than O-allylhydroxylamine as growth inhibitors. Unlike other organisms examined to date [32,36], canaline was not a particularly good inhibitor of both enzyme activity and cell growth.

Discussion

The specific aminotransferase involved in the formation of Met from KMTB has been examined in a number of eukaryotic and prokaryotic organisms [5,16,17]. However, within the Gram-positive bacteria, only *B. subtilis*, *B. cereus*, and *B. anthracis* have been studied [17]. In all of these *Bacillus spp.*, a BCAT has been found to be responsible for catalysing the reaction, with *B. subtilis* and *B. cereus/B. anthracis* utilising enzymes from different aminotransferase subfamilies. Like *B. subtilis*, *M. tuberculosis* has been found to catalyse Met regeneration using a subfamily IIIa aminotransferase. In fact, the kinetic parameters for the two aminotransferases were almost identical. The *M. tuberculosis* BCAT had Km values of 1.77 - 2.85 mM and Vmax values of 2.58 - 4.28 $\mu\text{mol}/\text{min}/\text{mg}$ protein for branched-chain amino acids and KMTB, while the *B. subtilis* ybgE had the corresponding values of 2.36 - 3.20 mM and 1.84 - 2.03 $\mu\text{mol}/\text{min}/\text{mg}$ protein. For branched-chain amino acids and KG, the values were 5.79 - 6.16 mM and 11.82 - 14.35 $\mu\text{mol}/\text{min}/\text{mg}$ protein for the *M. tuberculosis* BCAT, and 2.82 - 3.99 mM and 13.93 - 16.61 $\mu\text{mol}/\text{min}/\text{mg}$ protein for *B. subtilis* ybgE. Therefore, a 45% sequence identity between the two enzymes is sufficient to conserve both the substrate range and kinetic properties of the BCATs. Structural information is only available for the *E. coli* BCAT (ilvE) and the human mitochondrial BCAT [31,37], but the key residues involved in substrate specificity appear to be conserved in the *M. tuberculosis* BCAT. However, while the human mitochondrial BCAT is also a family IIIa aminotransferase, there are some clear differences when compared to the *M. tuberculosis* enzyme. The human enzyme will not accept aromatic amino acids, whereas the tuberculosis BCAT would use phenylalanine as an amino donor. In addition, the human enzyme contains the redox-active motif CXXC at positions 311-314 (positions 341-344 in Figure 3) which is essential for maintaining activity, while the tuberculosis BCAT lacks these residues. Structural analysis of the *M. tuberculosis* and/or *B. subtilis* enzymes would clarify these issues.

The *M. tuberculosis* BCAT was also screened with a variety of aminooxy compounds as potential inhibitors. These compounds are known aminotransferase inhibitors and act by forming a stable Schiff-base with the PLP cofactor [38]. Unlike previous studies [5,16,17,39], canaline was not found to be one of the better inhibitors of aminotransferase activity. Instead, O-benzylhydroxylamine, O-t-butylhydroxylamine, carboxymethoxylamine, and O-allylhydroxylamine were the most efficient inhibitors of Met formation from KMTB. In addition, these compounds demonstrated mixed type inhibition with a lower Ki for the competitive component. This result contrasts with that previously found for canaline with the *Bacillus spp.* enzymes, where inhibition was uncompetitive [17]. It may be possible that this difference is due to the structure of the inhibitors, as canaline is a γ -substituted amino acid analogue, while the present inhibitors are α -substituted or non-amino acid analogues. In other words, the inhibitors examined in this study do not present an α -amino group suitable for participation in the transamination reaction whereas canaline does.

Further screening of the inhibitors against *M. marinum* in vitro demonstrated that the compounds can act as effective antimycobacterial agents. Interestingly, there was no direct correlation between the Ki of the compounds against recombinant *M. tuberculosis* BCAT and the MIC/IC₅₀ against *M. marinum* growth. It is possible that there may be differences in the uptake rate of the various compounds into viable cells. Alternatively, the most effective

growth inhibitors act by inhibiting other PLP-dependent enzymes in addition to BCAT. In any case, O-allylhydroxylamine was the most effective antimycobacterial agent with an MIC of 78 μ M and an IC₅₀ of 8.49 μ M. Unfortunately, the compound is corrosive, and is thus unsuitable for further in vivo study. O-benzylhydroxylamine, O-t-butylhydroxylamine, canaline, and carboxymethoxylamine are less toxic, but the concentration of these compounds required to completely kill *M. marinum* in vitro (313 μ M - 10 mM) are unrealistic from an in vivo perspective. We are interested in the synthesis and characterisation of additional aminoxy compounds. In particular, the α -aminoxy analogues of branched-chain amino acids could have the potential to be more effective and selective inhibitors of BCAT activity.

Several interesting findings arose during the course of this investigation. First, *M. tuberculosis* contains no putative gene product with significant homology to a DAAT. In fact, the organism appeared to contain no subfamily IIIb aminotransferases. The physiological significance of a lack of a DAAT is unclear, but many organisms do not contain a homologue of this enzyme. With DAAT, there might be a diminished capacity to catabolise D-amino acids for energy, although the same reactions could be performed by a D-amino acid oxidase. *M. tuberculosis* is known to be reliant on carbohydrate catabolism during the active growth phase and lipid metabolism during the chronic, dormant phase [40]. Therefore, the lack of a DAAT might be reflective of a lifestyle where protein and peptide catabolism is relatively unimportant.

Similarly, *M. tuberculosis* was found to lack clearly identifiable homologues of several enzymes in the Met regeneration pathway. The most glaring omission is the lack of an S-adenosylmethionine decarboxylase (SAMdc) homologue (see Figure 1). *M. tuberculosis* contains the preceding enzyme, methionine adenosyltransferase [19], and has an easily identifiable homologue for the succeeding enzyme, spermidine synthase [22]. Therefore, *M. tuberculosis* must catalyse SAMdc activity via another enzyme in order to be able to synthesize polyamines. A previous study has demonstrated SAMdc activity in *M. bovis* homogenates, but has not identified the enzyme responsible [41]. Resolution of this issue is critical for a more complete understanding of polyamine biosynthesis in tuberculosis, and may yield a novel enzyme as an additional drug target. The *M. tuberculosis* genome also appears to be missing homologues of the enzymes converting methylthioribose to KMTB (see Figure 1). However, outside of *K. pneumoniae*, these enzymes have not been well studied. The enzymes implicated in these steps in *B. subtilis* in silico have no obvious relationship to the enzymes discovered in *K. pneumoniae* [12,13]. Therefore, there is much left to examine before concluding that *M. tuberculosis* contains neither homologues nor analogues to these Met recycling enzymes.

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List of symbols/abbreviations/acronyms/initialisms

DND	Department of National Defence
Met	methionine
BCAT	branched-chain amino acid aminotransferase
DAAT	D-amino acid aminotransferase
KMTB	ketomethiobutyrate
KG	ketoglutarate
PLP	pyridoxal-5-phosphate
MIC	minimum inhibitory concentration
IC50	inhibitory concentration 50%
SAMdc	S-adenosylmethionine decarboxylase

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Tuberculosis remains an important problem for the Canadian Forces in many of its overseas deployments. With the spread of drug-resistant strains of *Mycobacterium tuberculosis*, there is an increased need to characterise novel drug targets in the organism. The final step of methionine recycling from methylthioadenosine has been examined in *M. tuberculosis*, and has been found to be catalysed by a branched-chain amino acid aminotransferase. The enzyme was found to be a member of the aminotransferase IIIa subfamily, and closely related to the corresponding aminotransferase in *Bacillus subtilis*, but not to that found in *B. anthracis* or *B. cereus* (Berger et al., *Journal of Bacteriology*, 185, p. 2418-2431, 2003). The amino donor preference for the formation of methionine from ketomethiobutyrate was isoleucine, leucine, valine, glutamate, and phenylalanine. The enzyme catalysed branched-chain amino acid and ketomethiobutyrate transamination with a Km of 1.77 - 7.44 mM and a Vmax of 2.17 - 5.70 $\mu\text{mol}/\text{min}/\text{mg}$ protein, and transamination of ketoglutarate with a Km of 5.79 - 6.95 mM and a Vmax of 11.82 - 14.35 $\mu\text{mol}/\text{min}/\text{mg}$ protein. Aminooxy compounds were examined as potential enzyme inhibitors, with O-benzylhydroxylamine, O-t-butylhydroxylamine, carboxymethoxylamine, and O-allylhydroxylamine yielding mixed-type inhibition with Ki values of 8.20 - 21.61 μM . These same compounds were examined as antimycobacterial agents in a *M. marinum* model and were found to completely prevent cell growth. O-allylhydroxylamine was the most effective growth inhibitor with an MIC of 78 μM and an IC50 of 8.49 μM .

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Mycobacterium tuberculosis, aminotransferase, branched-chain amino acid, ketomethiobuytrate, methionine, inhibition