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Abstract

The final step of methionine recycling from methylthioadenosine has been examined in the gram-positive bacteria *Bacillus cereus* and *B. anthracis*. Subcellular homogenates were able to convert ketomethiobutyrate to methionine using leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, and alanine as amino donors. Four putative family III aminotransferases, two with homology to branched-chain amino acid aminotransferases and two with homology to D-amino acid aminotransferases, were cloned from *B. cereus*. The two branched-chain aminotransferases were found to have a low sequence identity with the corresponding enzymes from *B. subtilis*, indicative of membership of a different subfamily. After expression of the *B. cereus* enzymes in *Escherichia coli* and subsequent purification, one branched chain aminotransferase, designated Bc-BCAT2, was found to catalyse methionine regeneration using leucine, isoleucine, valine, phenylalanine, tryosine, and tryptophan as amino donors. The homologue of Bc-BCAT2 was cloned from *B. anthracis* and designated Ba-BCAT2. Expression of the recombinant enzyme in *E. coli* and subsequent purification yielded a protein which catalysed methionine regeneration using branched-chain and aromatic amino acids as the amino donors. Kinetic analysis showed that the Km and Vmax values for the enzymes were similar for leucine, valine, and isoleucine as amino donors and ketomethiobutyrate and ketoglutarate as amino acceptors with the $K_m = 0.41 - 4.34$ mM and the $V_{max} = 0.13 - 1.44$ nmol/min/mg protein. Therefore, in both *B. cereus* and *B. anthracis*, BCAT2 would appear to be the primary catalyst of methionine production from ketomethiobutyrate. The aminotransferase inhibitor canaline was found to inhibit the growth of *B. cereus* with an IC50 of 35 μM in minimal medium and 760 μM in nutrient broth. The activity in minimal medium was only marginally antagonised by the addition of exogenous methionine or protein.

Résumé

La phase finale du recyclage de la méthionine à partir de la méthylthioadénosine a été examinée dans la bactérie gram positive des bactéries *Bacillus cereus* et *B. anthracis*. Les homogénats infracellulaires ont été capables de transformer le kétométhiobutyrate en méthionine en utilisant la leucine, l'isoleucine, la valine, la phénylalanine, la tyrosine, le tryptophane et l'alanine comme donneurs amines. Quatre transaminases de la famille III putative, dont deux ayant une homologie avec les transaminases aminoacides de chaîne ramifiée et deux autres ayant une homologie avec les transaminases aminoacides D, ont été clonées à partir du *B. cereus*. On a trouvé que les deux transaminases de chaîne ramifiée avaient peu d'identité de séquence avec les enzymes correspondants du *B. subtilis,* indiquant ainsi leur appartenance à une sous-famille différente. Après avoir exprimé les enzymes du *B. cereus* dans le *Escherichia coli* et les avoir purifiés ultérieurement, on a trouvé que la transaminase de chaîne ramifiée, désignée Bc-BCAT2, catalysait la régénération de méthionine en utilisant la leucine, l'isoleucine, la valine, la phénylalanine, la tyrosine, et la tryptophane comme donneurs amines. L'homologue de la Bc-BCAT2 a été cloné à partir du *B. anthracis* et a été désigné Ba-BCAT2. L'expression de l'enzyme recombiné dans le *E coli* et la purification ultérieure ont produit une protéine qui a catalysé la régénération de la méthionine en utilisant des aminoacides de branches ramifiées et aromatiques comme les donneurs amines. L'analyse cinétique montre que les valeurs de la Km et de la Vmax pour les enzymes sont similaires pour la leucine, la valine, et l'isoleucine comme donneurs amines et pour le kétométhiobutyrate et le kétoglutarate comme receveurs d'amines avec la K_m = 0.41 – 4.34 mM et la V_{max} = 0.13 – 1.44 nmol/min/mg de protéine. Par conséquent, il apparaîtrait que, dans chacun des *B. cereus* and *B. anthracis,* le BCAT2 serait le catalyseur primaire de la production de méthionine à partir du kétométhiobutyrate. On a trouvé que la canaline, un inhibiteur de transaminase, inhibait la croissance de *B. cereus* avec une CI50 de 35 µM dans un milieu minimum et 760 µM dans un bouillon nutritif. L'addition de méthionine exogène ou de protéine antagonise seulement marginalement l'activité dans un milieu minimum.

Executive summary

Anthrax remains one of the most serious biological warfare threats faced by the Canadian Forces and the public at large. The recent events in the United States involving anthrax letters have reinforced the ease with which an anthrax attack can be performed and the large impact such an attack may have. Over the last few years, there has been an increase in the natural resistance of anthrax to penicillin and other beta-lactam antibiotics. Depending on the study, between 10 and 35 percent of soil and veterinary anthrax isolates are now penicillin resistant. Moreover, a single isolate has also been found to be ciprofloxacin resistant.

Given this current state of natural resistance and also the relative ease of genetically modifying *Bacillus anthracis* to create drug resistant strains, there is a need for the development of antibacterial agents which act against novel biochemical targets. This laboratory has been investigating enzymes involved in polyamine biosynthesis and its associated methionine salvage pathway as potential drug targets in anthrax. 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.

In a previous report, we investigated the biochemical identity of the enzyme catalysing the final step of methionine recycling, the transamination of ketomethiobutyrate, in the model system *Bacillus subtilis*. In this organism, the branched-chain amino acid aminotransferase encoded by the ybgE gene was found to catalyse methionine formation. However, *B. subtilis* is not a particularly accurate model for *B. anthracis*, and was chosen due to the fact that it was the only member of the genus *Bacillus* to have a complete genome sequence in the public databases.

During the course of the *B. subtilis* study, a nearly complete genome sequence for *B. cereus* was made public, and a similar sequence for *B. anthracis* was made available for our work by the United States Army Medical Research Institute for Infectious Diseases via the TTCP program. As *B. cereus* and *B. anthracis* are the same species with differences only in plasmid content, the presence of significant genome data makes the former organism a much more suitable non-pathogenic model for *B. anthracis* biochemistry. Analysis of methionine formation in *B. cereus* showed that a branched-chain aminotransferase also catalysed the reaction in this organism. However, the subject enzyme was a member of a different aminotransferase subfamily than that found in *B. subtilis*. Cloning and analysis of the *B. anthracis* homologue of the *B. cereus* enzyme demonstrated that this enzyme was identically active in *B. anthracis*. This result clearly reinforces the use of *B. cereus* as an accurate, non-pathogenic model for *B. anthracis* metabolism. In vitro growth inhibition of *B. cereus* with the aminotransferase inhibitor canaline showed that the compound effectively killed the bacteria when grown in a minimal medium, but less so when in a rich medium. This difference in activity was found to be independent of antagonism by exogenous methionine or

protein found in the rich medium. The study has demonstrated that aminooxy inhibitors of aminotransferases show great potential as antibacterial agents and are worth further study in vitro and in vivo.

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Sommaire

Le charbon bactéridien demeure la menace de guerre biologique la plus sérieuse à laquelle doivent faire face les Forces canadiennes et le public en général. Les évènements récents comprenant notamment les lettres au charbon bactéridien, aux États-Unis, mettent en valeur la facilité avec laquelle une attaque au charbon peut être effectuée et le fait qu'une telle attaque peut avoir un impact important. Depuis quelques années, il existe une croissance de la résistance naturelle du charbon à la pénicilline et aux autres antibiotiques bêta-lactamines. Selon les études, 10 à 30 pourcent des sols et des isolats de charbon vétérinaire sont maintenant résistants à la pénicilline. De surcroît, on a aussi trouvé un isolat résistant à la ciprofloxacine.

Étant donné, l'état actuel de résistance naturelle ainsi que de la facilité relative à modifier le *Bacillus anthracis* pour créer des drogues résistantes aux souches, il existe un besoin de mettre au point des agents antibactériaux agissant contre les cibles biochimiques nouvelles. Ce laboratoire a étudié les enzymes existant dans la biosynthèse de polyamines et leur chemin de récupération de méthionine qui leur sont associés, comme cibles de drogues potentielles dans le charbon. Les polyamines sont des petits composés azotés de poids moléculaire qui sont essentiels à la reproduction cellulaire. La biosynthèse de polyamines consume l'acide aminé méthionine à proportion égale et produit de la méthylthioadénosine comme sous-produit. La méthionine étant un composé essentiel en lui-même qui est présent en quantité limitée dans la cellule et qui nécessite beaucoup d'énergie pour synthétiser de novo, les cellules possèdent un chemin unique de régénération de la méthionine à partir de la méthylthioadénosine. Nous savons déjà que l'inhibition des enzymes, dans ce chemin, aboutit à la mort des cellules dans plusieurs organismes dont la malaria.

Dans un rapport antérieur, nous avons étudié l'identité biochimique de l'enzyme qui catalysait l'étape finale du recyclage de la méthionine, la transamination du kétométhiobutyrate, dans le système modèle du *Bacillus subtilis.* Dans cet organisme, on a trouvé que le transaminase amino-acide de chaîne ramifiée, encodé par le gène ybgE, catalysait la formation de la méthionine. Cependant, le *B. subtilis* n'est pas un modèle particulièrement exact pour le *B. anthracis* et il avait été choisi parce qu'il était le seul membre du genre *Bacillus* dont les bases de données publiques possédaient une séquence complète de génomes.

Durant le cours des recherches effectuées sur le *B. subtilis,* une séquence pratiquement complète de génomes pour le *B. cereus* a été rendue publique et une séquence similaire pour le *B. anthracis* a été rendue disponible pour notre travail par l'institut de l'armée américaine de recherches médicales pour les maladies infectieuses, United States Army Medical Research Institute for Infectious Diseases, par l'entremise du programme TTCP. Puisque le *B. cereus* et le *B. anthracis* sont de la même espèce et qu'ils ne contiennent des différences que dans le contenu plasmide, l'existence de données signifiantes sur les génomes font que le *B. cereus* est un organisme non pathogénique beaucoup plus approprié à la bicochimie du *B. anthracis.* Les analyses de formation de méthionine dans le *B. cereus* ont montré qu'un transaminase de chaîne ramifiée catalysait aussi la réaction dans cet organisme. Cependant, l'enzyme en question était un membre différent de la sous-famille de transaminases qui avait été trouvée dans le *B. subtilis.* Le clonage et l'analyse de l'homologue du *B. anthracis* dans l'enzyme du

B. cereus a montré que cet enzyme était actif de manière identique dans le *B. anthracis*. Ce résultat renforce clairement l'utilisation du *B. cereus* comme modèle non pathogénique exact pour le métabolisme du *B. anthracis*. L'inhibition de la croissance du *B. cereus* in vitro avec la transaminase inhibitrice de la canaline a montré que le composé a efficacement tué la bactérie développée dans un milieu minimum, mais moins efficacement quand elle venait d'un milieu riche. On a trouvé que cette différence d'activité ne dépendait pas d'un antagonisme par la méthionine exogène ou la protéine présente dans le milieu riche. Cette étude a démontré que les inhibiteurs aminooxy des transaminases possédaient un potentiel important en tant qu'agent antibactériaux et méritaient d'être plus amplement étudiés in vitro et in vivo.

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Acknowledgements

Shane English was supported by the DRDC Summer Research Associate Program 2001.

Prelimary genome data for *Bacillus anthracis* Ames was made available via USAMRIID. Sequencing was performed by The Institute for Genomic Research and was funded by DARPA.

Introduction

Polyamines are essential for normal growth and proliferation of all cell types [1-3]. While the exact mode of action of polyamines remains unknown, the compounds play a role in nucleic acid and membrane stabilisation [2,3]. Inhibition of polyamine biosynthesis leads to cell stasis or death in most systems examined to date [1]. The biosynthesis of polyamines requires the transfer of an aminopropyl group from decarboxylated S-adenosylmethionine to putrescine or spermidine, yielding spermidine or spermine respectively. This aminopropylation step effectively consumes the amino acid methionine in a one-to-one stoichiometry, with the formation of methylthioadenosine as a by-product. As methionine is present in limiting amounts and is essential for a broad range of biochemical processes, there exists a unique pathway to recycle the amino acid from methylthioadenosine (Figure 1).

The final step in this methionine regeneration pathway is the conversion of ketomethiobutyrate (KMTB) to methionine via an aminotransferase [4]. The specific aminotransferase which catalyzes this reaction has been studied in the lower eukaryotes *Trypanosoma brucei brucei*, *Crithidia fasciculata*, *Giardia intestinalis*, and *Plasmodium falciparum*, the gram-negative bacterium *Klebsiella pneumoniae*, and partially characterized in pig kidney [5,6,7]. In the case of the protozoa and *K. pneumoniae*, the enzyme responsible was found to be a member of the Ia subfamily of aminotransferases. The enzyme has also proven to be a potential chemotherapeutic target, particularly in *P. falciparum* [8].

In a previous study [9], we had chosen *Bacillus subtilis* to act as an initial model system for biochemical studies of *B. anthracis* due to the fact that the entire genome sequence of *B. subtilis* 168 had been determined. Using this model, it was found that *B. subtilis* had no genes encoding for subfamily Ia aminotransferases. Instead, a member of family III, the branched-chain amino acid aminotransferase (BCAT) encoded by the ybgE gene, was found to be primarily responsible for the conversion of KMTB to methionine in *B. subtilis*. Towards the end of these studies, Integrated Genomics (Chicago, IL, USA) unexpectedly made a public release of a gapped genome sequence (1528 contigs) for *B. cereus* 14579. As recent studies have demonstrated that *B. anthracis*, *B. cereus*, and *B. thuringiensis* are essentially the same species of bacterium that differ in their plasmid content [10], the presence of genome data for *B. cereus* makes this organism a much more accurate, non-BL3 model for *B. anthracis* biochemistry (Figure 2). In the present study, we have extended the work performed on *B. subtilis* to examine the BCATs present in the *B. cereus* genome. Four candidate enzymes were cloned, functionally expressed, and characterized for their ability to catalyse methionine regeneration. As in *B. subtilis*, a single BCAT was found to be responsible for this reaction in *B. cereus*. The *B. anthracis* homologue of this enzyme was also cloned, expressed, and characterized, and was also found to be capable of methionine regeneration.

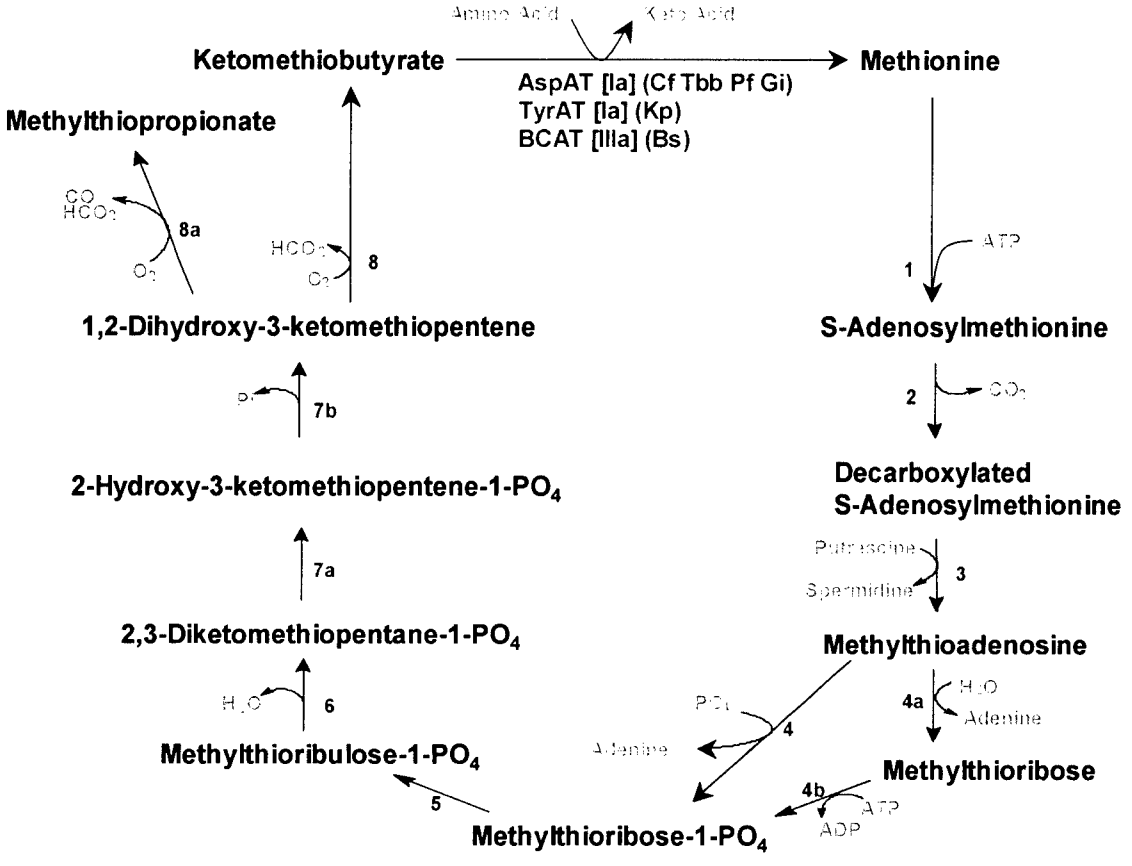

Figure 1. *The Met regeneration pathway. The labelled enzymes are: 1, S-adenosylmethionine synthetase; 2, S-adenosylmethionine decarboxylase; 3, spermidine/spermine synthetase; 4, methylthioadenosine phosphorylase; 4a, methylthioadenosine nucleosidase; 4b, methylthioribose kinase; 5, unidentified isomerase; 6, unidentified dehydratase; 7, enolase-phosphatase; 8, non-enzymatic, or dioxygenase; 8a, dioxygenase. The specific aminotransferases that catalyse the final step are shown in red, with the subfamily membership in square brackets. The organism abbreviations are: Cf, Crithidia fasciculata; Tbb, Trypanosoma brucei brucei; Pf, Plasmodium falciparum; Gi, Giardia intestinalis; Kp, Klebsiella pneumoniae; Bs, Bacillus subtilis.*

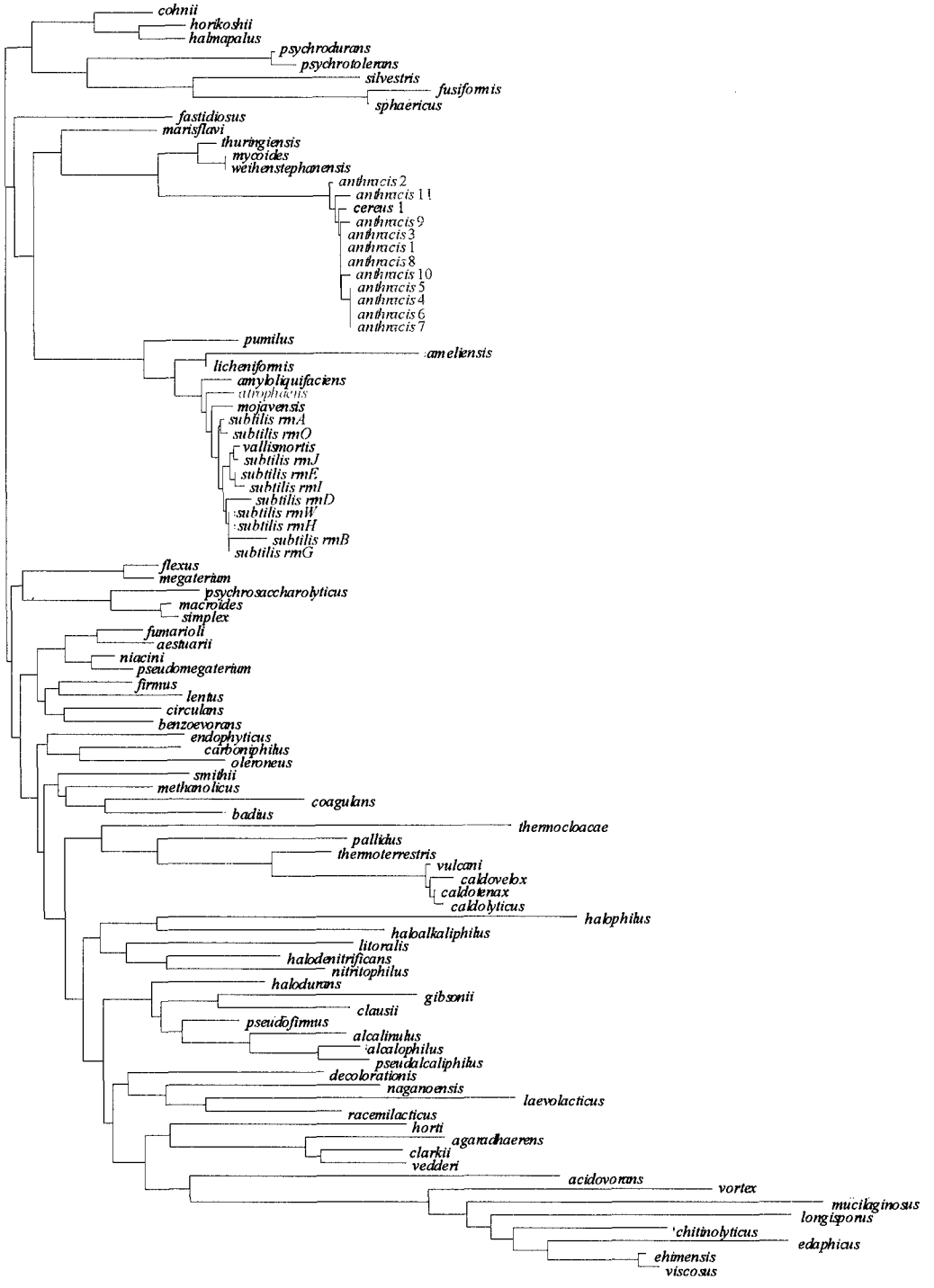

Figure 2. *Phylogenetic relationships within the genus Bacillus. The 16S rDNA sequences for each organism were aligned using ClustalX, and all positions containing gaps or ambiguous bases eliminated. A distance matrix was then constructed using the DNADist program in Phylip, followed by tree construction by the neighbor-joining method. For Bacillus subtilis (in green) all 16S rDNA sequences were obtained from the Subtilist genome database. For Bacillus anthracis (in red), 11 unique 16S rDNA sequences were identified from the nearly-completed genome project data (see Methods). For Bacillus cereus (in blue), only one full length 16S rDNA sequence could be obtained from the gapped genome data available (see Methods), due to the positions of the gaps. Bacillus atrophaeus is highlighted in orange, as this species is the currently correct designation for Bacillus globigii (Bacillus subtilis var. niger), a common biodefence research model.*

Materials and Methods

Cells and Reagents

B. cereus 14579 was obtained from the American Type Culture Collection (Manassas,VA, USA) and was routinely cultured in Nutrient Broth at 30°C and agitation at 250 rpm. For selected experiments, the cells were grown in a minimal medium consisting of 42 mM MES pH 6.1/15 mM $(NH_4)_2SO_4$/0.8 mM $MgSO_4$-$7H_2O$/1.5 µM $MnSO_4$-H_2O/7 mM K_2HPO_4/13.6 mM glutamic acid/30 µM thiamine/30 mM glucose/144 µM $FeSO_4$-$7H_2O$/1.0 mM threonine/1.0 mM serine/1.0 mM leucine/1.0 mM valine/1.0 mM alanine [11]. *B. anthracis* Ames was obtained from DRES reference stocks, and was originally acquired from USAMRIID (Frederick, MD, USA). Growth of *B. anthracis* was under the same conditions as for *B. cereus*, except growth temperature was 37°C.

Subcellular Homogenates

B. cereus cultures were centrifuged at 3500 x g for 20 min at 4°C, and the cell pellet resuspended in 25 mM PO_4 buffer pH 7.4/120 mM KCl/2.5 mM α-ketoglutarate (KG)/0.2 mM pyridoxal phosphate/1 mM dithiothreitol/complete protease inhibitors (Roche Biochemicals; Laval, QB, Canada). Lysozyme (Fisher Scientific; Nepean, Canada) was added to 300 µg/ml and the mixture incubated on ice for 1 hr prior to sonication on ice. The homogenate was then centrifuged at 3500 x g for 20 min at 4°C, and the supernatant dialysed against 100 mM PO4 buffer pH 7.4/1 mM dithiothreitol/1 mM EDTA at 4°C. After dialysis, the samples were stored at 4°C for enzyme assays. For long term storage, glycerol was added to 20% v/v and the samples kept at –20°C.

Biochemical Assays

Aminotransferase activities were assayed by an HPLC method [5]. 10 µl of subcellular homogenate or a variable volume of recombinant enzyme was added to 100 µl of substrate mix (100 mM PO_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 as described below. BCAT activity was assayed using 2.0 mM valine, isoleucine, or leucine/1.0 mM KG mixtures, while D-alanine aminotransferase (DAAT) activity was measured using 2.0 mM D-alanine/1.0 mM KG. Met regeneration was screened using 2.0 mM each of ADEFGHIKLNQRSTWY/1.0 mM KMTB in the substrate mix. The range of effective amino donors for Met formation was determined by using 2.0 mM individual amino acid/1.0 mM KMTB in the substrate mix. For the determination of Michaelis-Menton constants, the substrate mixes contained 0.1-10 mM of substrate and 5 mM or 10 mM of the cosubstrate. Similar kinteic constants were determined using valine, isoleucine, or leucine/KG mixtures at the same concentrations as for Met formation from KMTB.

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 acetontrile 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 17-30 min. The elution of derivatised amino acids was monitored at 331 nm. All separations were performed on an Agilent 1100 HPLC equipped with an autosampler, variable wavelength ultraviolet/visible spectrophotometric detector, and Chemstation operating system.

Protein concentration was determined using the Bio-Rad dye (Bio-Rad; Mississauga, ON, Canada). Recombinant protein samples were examined by electrophoresis on 10% SDS polyacrylamide gels followed by Coomassie Brilliant Blue R250 staining.

Cloning and Functional Expression

Genomic DNA was isolated from *B. cereus* 14579 or *B. anthracis* Ames by digestion with 300 µg/ml lysozyme for 1 hr on ice, followed by incubation with an equal volume of 100mM NaCl/10 mM Tris-HCl pH 8.0/25 mM EDTA/0.5% w/v sodium dodecyl sulfate/0.1 mg/ml proteinase K at 37°C for 1 hr with occasional mixing. The mixture was then subjected to extraction with phenol and chloroform:isoamyl alcohol (24:1), and the DNA ethanol precipitated. The *B. anthracis* DNA was resuspended in ultrapure water and filtered through a 0.2 µm filter. An aliquot of this filtered DNA was used to ensure sample sterility by inoculation into 10 ml brain-heart infusion broth and incubation at 37°C with agitation for 7 days. A sample of the incubated brain-heart broth was then plated on blood-agar plates and incubated at 37°C for 7 days. Upon confirmation of sample sterility, the *B. anthracis* DNA was reprecipitated in ethanol before use.

A gapped genome of *B. cereus* 14579 was obtained from Integrated Genomics (www.integratedgenomics.com/Public/IG_Release.html) and data from the nearly completed genome project for *B. anthracis* Ames was obtained from The Institute for Genomic Research (Rockville, USA) and USAMRIID. The nucleotide sequences of the *B. cereus* and *B. anthracis* aminotransferases were obtained by examination of the appropriate genome data using the BLAST program [12] running within BioEdit [13], and used to design oligonucleotide primers for each enzyme (Table 1). The 5' primers contain a 12 nucleotide LIC (ligation independent cloning, [14]) sequence and an in-frame start codon, while the 3'primers contained a 13 nucleotide LIC sequence and an in-frame stop codon. The target sequences were amplified from the genomic DNA using Taq polymerase (Promega; Madison, WI, USA), 1.5 mM $MgCl_2$, 200 µM dNTP, 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 amplified target sequence was excised from a 1% agarose gel and the DNA extracted using the QiaexII kit (Qiagen;Mississauga, ON, Canada). The genes were then cloned into pCALnFLAG using the LIC procedure outlined by Stratagene (La Jolla, CA, USA), and then transformed into *E. coli* XL10 competent cells (Stratagene). The recombinant plasmid was purified from these cells using the QiaSpin miniprep kit (Qiagen), and the presence of the insert confirmed by digestion with NdeI and SacI and electrophoresis on a 1% agarose gel. The insert was sequenced using the ABI Big-Dye cycle sequencing kit (ABI; Foster City, CA, USA) and an ABI Prism 310 Genetic Analyzer.

Table 1. *Oligonucleotide primers used for amplification of the genes in this study. Primers in both directions contain 5' sequence complementary to the ligation-independent cloning site of pCALnFLAG (Stratagene).*

GENE		SEQUENCE
B. cereus BCAT1	5'	GACGACGACAAGATGGGAAACCAGTACATTTACA
	3'	GGAACAAGACCCGTTTATGCTAAGCTTTCCGTCAG
B. cereus BCAT2	5'	GACGACGACAAGATGAATGAGCAATGGATTTTCTTAA
	3'	GGAACAAGACCCGTTTATCCAACTTTATTTTCTTCGTAAA
B. cereus DAAT1	5'	GACGACGACAAGATGAAAGTTTCACATTACTTACGTACTTA
	3'	GGAACAAGACCCGTTTAAGAAGATGACATATTGGATTGTAA
B. cereus DAAT2	5'	GACGACGACAAGATGAAAGCTACTCATAAAGATTGG
	3'	GGAACAAGACCCGTTTAATTTGTCACTTTAAACAAA
B. anthracis BCAT2	5'	GACGACGACAAGATGAATGAGCAATGGATTTTCTTAA
	3'	GGAACAAGACCCGTTTATCCAACTTTATTTTCTTCGTAAA

The plasmids from positive clones were transformed into *E. coli* BL21 DE3 CodonPlus RIL cells (Stratagene) for functional expression. The BL21 cells containing the recombinant plasmid were grown in LB liquid medium containing 50 µg/ml ampicillin and 50 µg/ml chloramphenicol at 37°C and 250 rpm until the cell density reached an A_{600nm} of 0.6 – 0.8. The culture was then cooled to 28°C and IPTG added to 1.0 mM before 2-5 hr of continued culture at 28°C and 250 rpm. The cells were then pelleted by centrifugation at 3500 x g for 20 min at 4°C, and resuspended in a minimal volume of 10 mM mM HEPES pH 7.8/150 mM NaCl/1.0 mM DTT/1.0 mM imidazole/2.0 mM $CaCl_2$ before storage at –20°C. The sample was thawed, sonicated on ice, and centrifuged at 3500 x g for 20 min at 4°C. The resulting supernatant was loaded onto a 1.6 x 8.0 cm calmodulin-agarose column (Stratagene) equilibrated with the resuspension buffer. The column was eluted with 10 mM HEPES pH 7.8/1.2 M NaCl/1.0 DTT/3.0 EGTA. The eluted enzyme was concentrated to less than 5.0 ml using a 10 Kda molecular weight cut-off centrifugal filter (Pall Filtron; Mississauga, ON, Canada). The concentrated enzyme was kept at 4°C for short term storage and at –20°C with 20% v/v glycerol for long term storage.

Phylogenetic Analysis

Additional aminotransferase sequences were obtained from GenBank and were aligned using the Clustal algorithm and the BLOSUM sequence substitution table in the ClustalX program [15]. Aligned sequences were visualised with the Bioedit program [13].The aligned sequences were then used with the ProtDist component of Phylip [16] to construct a distance matrix which was the basis for tree construction using neighbor-joining [17]. All trees were visualised using Treeview [18].

Results

Methionine Regeneration in *B. cereus* Homogenates

Subcellular homogenates of *B. cereus* were prepared and examined for the range of effective amino donors for the transamination of KMTB. Homogenates prepared from cells grown in Nutrient Broth were found to utilise leucine, isoleucine, valine, tyrosine, phenylalanine, tryptophan, and histidine as preferred amino donors for the reaction (Figure 3A). Alanine, glutamate and glutamine could also act as amino donors to a lesser degree. Cells grown in a minimal medium with sulfate as the only exogenous sulfur source presented a nearly identical amino donor spectrum, with the exception of a greater utilisation of alanine and glutamate, and a lower use of tyrosine (Figure 3B). Therefore, as was seen previously with *B. subtilis* [9], *B. cereus* preferentially uses branched-chain and aromatic amino acids as the amino donor for KMTB regardless of presence of exogenous methionine.

Identification of Family III Aminotransferases in *B. cereus* and *B. anthracis*

As the primary catalyst of KMTB transamination in *B. subtilis* proved to be the ybgE gene product, and was a member of the family III of aminotransferases, *B. cereus* and *B. anthracis* genome data was examined for potential homologues. Both *B. cereus* and *B. anthracis* were found to contain four sequences with high identity to known members of family III aminotransferases (Figure 4). Two of these sequences were similar to BCATs and two were similar to DAATs, and were given the names Bc-BCAT1 (Genbank AF527041), Bc-BCAT2 (Genbank AF527043), Bc-DAAT1 (Genbank AF527045), Bc-DAAT2 (Genbank AF527042), Ba-BCAT1, Ba-BCAT2 (Genbank AF527044), Ba-DAAT1, and Ba-DAAT2.

In a previous study, we had tentatively subdivided family III into two subfamilies: IIIa, which contained eukaryotic and bacterial BCATs, and IIIb, which contained archaeal and bacterial BCATs and DAATs [9]. The *B. subtilis* ybgE gene product was found to be a member of subfamily IIIa. Interestingly, despite their relatively close relationship to *B. subtilis* (Figure 2), neither *B. cereus* nor *B. anthracis* had any aminotransferases with homology to subfamily IIIa. All four *B. cereus* and *B. anthracis* family III aminotransferases are members of subfamily IIIb and are not closely related to the *B. subtilis* ybgE gene product (Figure 4).

Given the extreme similarity of the *B. cereus* and *B. anthracis* genomes, it is not surprising that each of the family III enzymes from *B. cereus* is most identical to its *B. anthracis* counterpart, with identities in excess of 96%. The BCAT1's were also found to be 61% identical to the BCAT2's. All four BCATs were 56% identical to the *Archaeoglobus fulgidus* AF0933 gene product and 50% identical to the *Methanococcus jannaschii* MJ1008 gene product. The best studied member of subfamily IIIb, the *Escherichia coli* ilvE gene product, was 38% identical to the four *Bacillus* BCATs. The *B. subtilis* ybgE gene product, which is responsible for Met regeneration in that organism and is a member of subfamily IIIa, was only 17% identical to the *B. cereus* and *B. anthracis* BCATs.

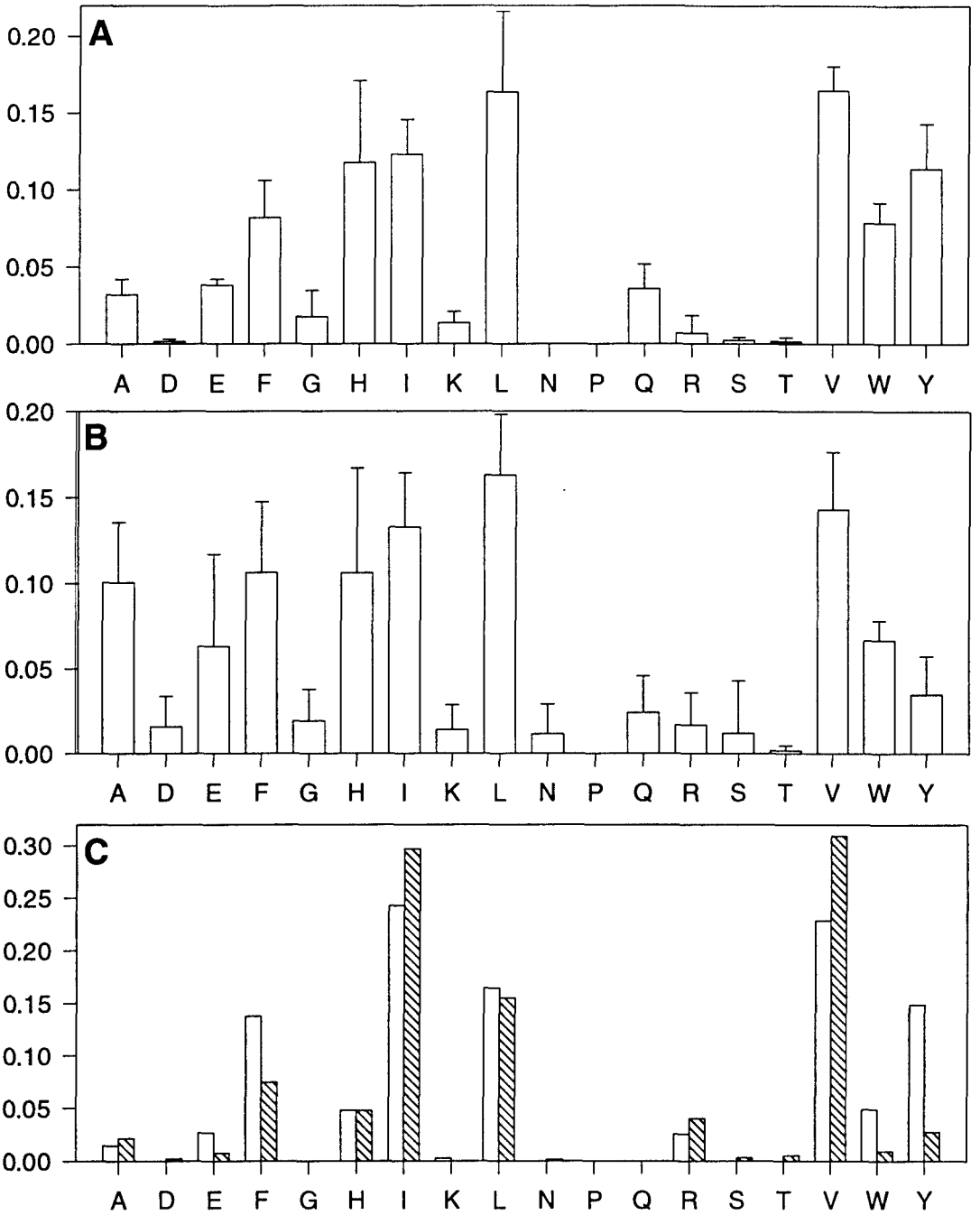

Figure 3. *The amino donor range for Met regeneration in B. cereus. An enzyme source was mixed with 1.0 mM KMTB, 2.0 mM of a single amino acid, and pyridoxal phosphate for 30 min at 37°C before analysis of Met production by HPLC. The enzyme sources are: (A) B. cereus homogenate from cells grown in Nutrient broth, (B) B. cereus homogenate from cells grown in Minimal medium, (C) recombinant B. cereus BCAT2 (white bars) or recombinant B. anthracis BCAT2 (hatched bars).*

Figure 4. *Family III aminotransferases. The 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 with the arrows. The B. cereus sequences are in blue and the B. anthracis sequences in red.*

As with the BCATs, the putative DAAT sequences from *B. cereus* were most identical to their *B. anthracis* counterparts with an identity in excess of 86%. The DAAT1's were also found to be 47% identical to the DAAT2's. All four DAAT sequences were 42% identical to the *B. subtilis* yheM gene product, which is the sole DAAT in that organism, and were also 40% identical to the *Listeria monocytogenes* DAAT (AF038439).

Alignment of the eight *B. cereus* and *B. anthracis* sequences with previously characterised family III aminotransferases highlighted the very small number of conserved residues across the family (figure 5). In this sampling, E104(E37)*, R127(R59), K230(K159), G249(G178), E261(E193), N271(N198), T287(T209), L295(L217), G297(G219), R300(R222), E317(E238), E336(E251), and G367(G278) were completely conserved. When this data set is merged with that found in our previous study on *B. subtilis*, then only E104(E37), R127(R59), K230(K159), G249(G178), E261(E193), T287(T209), L295(L217), E336(E251), and G367(G278) are conserved across 18 members of family III. The sequences used in both these smaller datasets have been cloned and/or characterised in biochemical studies. The alignment used to construct figure 4 had E145(E37), R170(R59), K279(K159), E320(E193), L351(L217), and E376(E251) as the sole conserved residues across the entirety of family III. However, this large data set would be expected to contain a number of sequencing errors as the vast majority of sequences have not been further characterised. In any case, the number of conserved residues in family III is small.

Characterisation of the *B. cereus* Family III Aminotransferases

The four putative family III aminotransferase sequences in *B. cereus* were cloned and functionally expressed as calmodulin-binding peptide fusion proteins in *E. coli*. In all four cases, a large amount of inactive, included material was formed (from 50-100%). However, with the exception of Bc-DAAT2, sufficient soluble, active material was produced and purified. As an example, the purification of Bc-BCAT2 is shown in figure 6. Bc-BCAT1, Bc-BCAT2, and BC-DAAT1 were screened with branched-chain amino acids and KG or KMTB, as well as D-alanine and KG, in order to determine their capacity for transamination of these substrates (figure 7). As would be expected, only Bc-DAAT1 catalysed D-alanine:KG aminotransfer, confirming its identity as a DAAT. Both Bc-BCAT1 and Bc-BCAT2 were active with branched-chain amino acids when KG was used as an amino acceptor, confirming their identities as BCATs. However, Bc-BCAT2 catalysed these reactions 3-4 fold better than Bc-BCAT1. Only Bc-BCAT2 had any appreciable activity with branched-chain amino acids and KMTB as an amino donor. Therefore, while both BCATs were capable of transaminating KG and branched-chain amino acids, only Bc-BCAT has the ability to produce Met from KMTB. Therefore, Bc-BCAT2 is likely the analogue of ybgE in *B. subtilis*, and acts as the primary catalyst of Met regeneration in *B. cereus*.

* As in our previous study [9], the values in parenthases represent the corresponding residue in the *E. coli* ilvE amino acid sequence.

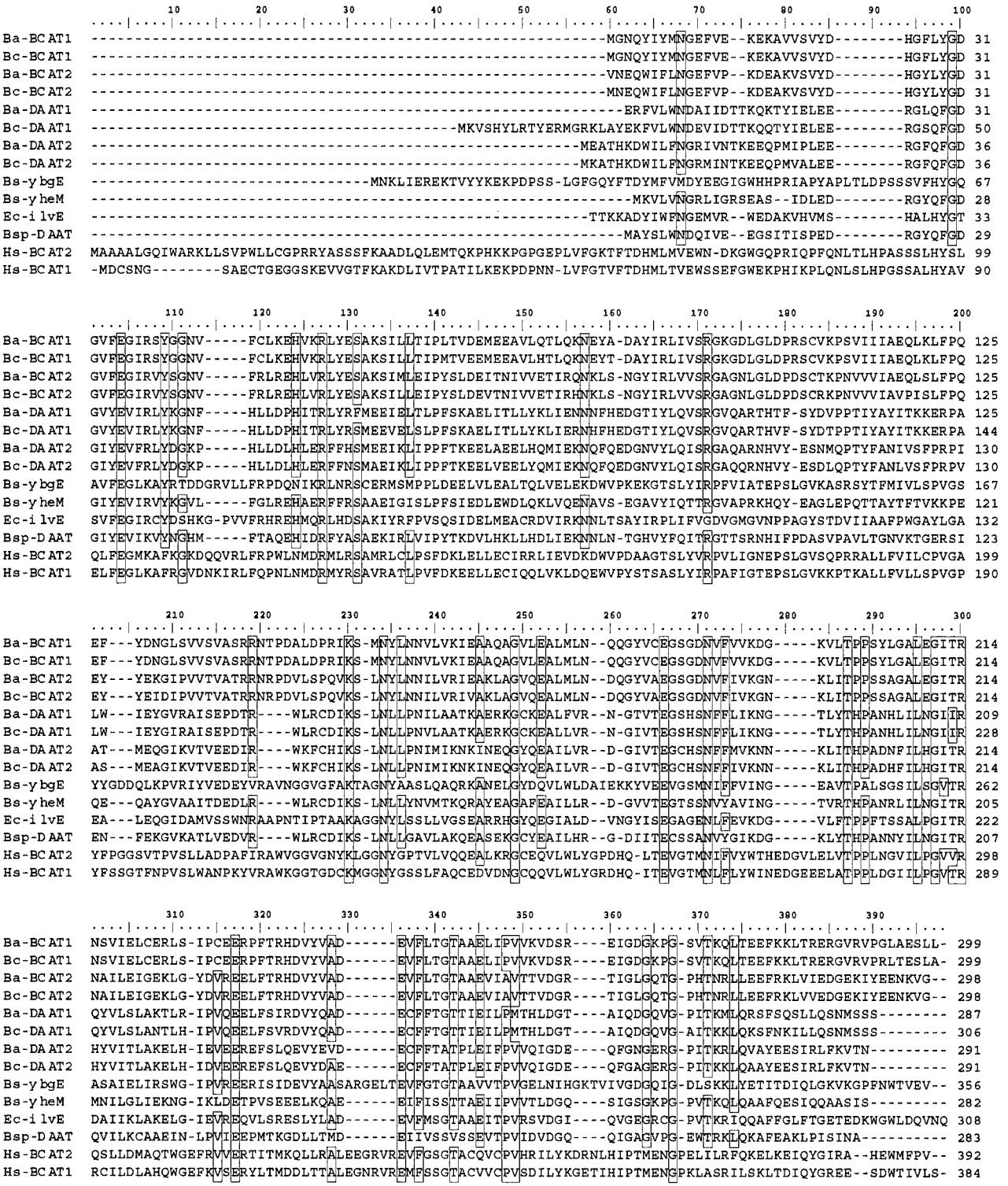

Figure 5. *Alignment of the B. cereus and B. anthracis family III enzymes. The following sequences were aligned with the clustal algorithm: Bs-ybgE [9,19], B. subtilis BCAT [9] [19]; Bs-yheM, B. subtilis DAAT [19]; Ec-ilvE, E. coli BCAT [20]; Bsp-DAAT, B. sphaericus DAAT [21]; Hs-BCAT1, Homo spaiens BCAT1 [22]; Hs-BCAT2, H. sapiens BCAT2 [23]. Residues conserved by 75% of the sequences are highlighted.*

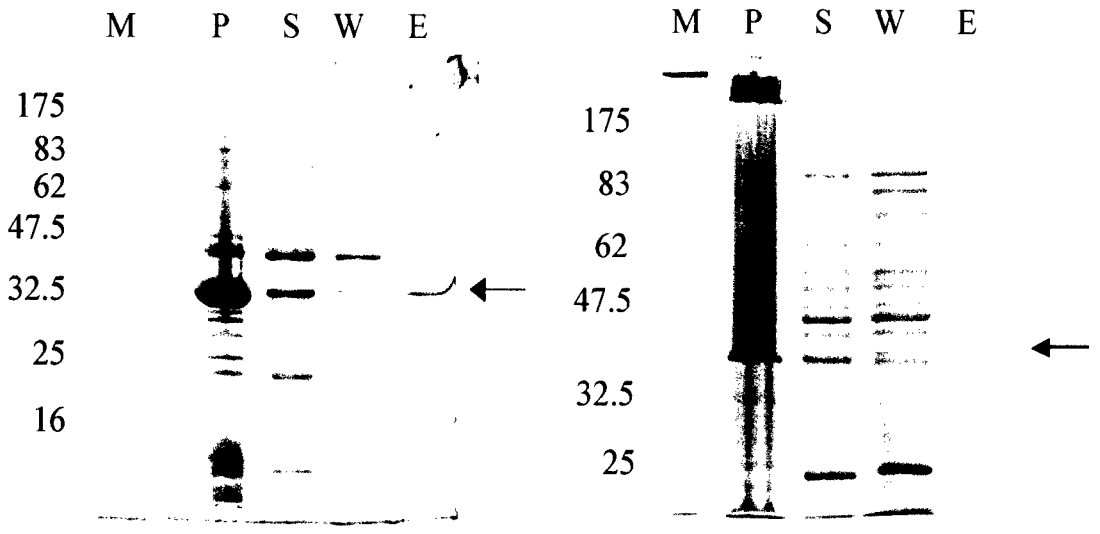

Figure 6. *Purification of recombinant B. cereus or B. anthracis BCAT2. E. coli BL21 codon-plus cells (Stratagene) carrying the transgene were induced with IPTG and prepared as described in the Materials and Methods. The homogenate supernatant (S) was loaded onto a calmodulin-agarose column, and the wash (W) and eluates (E) collected. Aliquots of each of these fractions were analysed by SDS-polyacrylamide electrophoresis along with a sample of the insoluble homogenate pellet (P). The mass of the molecular markers (M) is shown and the target protein is highlighted with the arrows. B. cereus is on the left and B. anthracis on the right.*

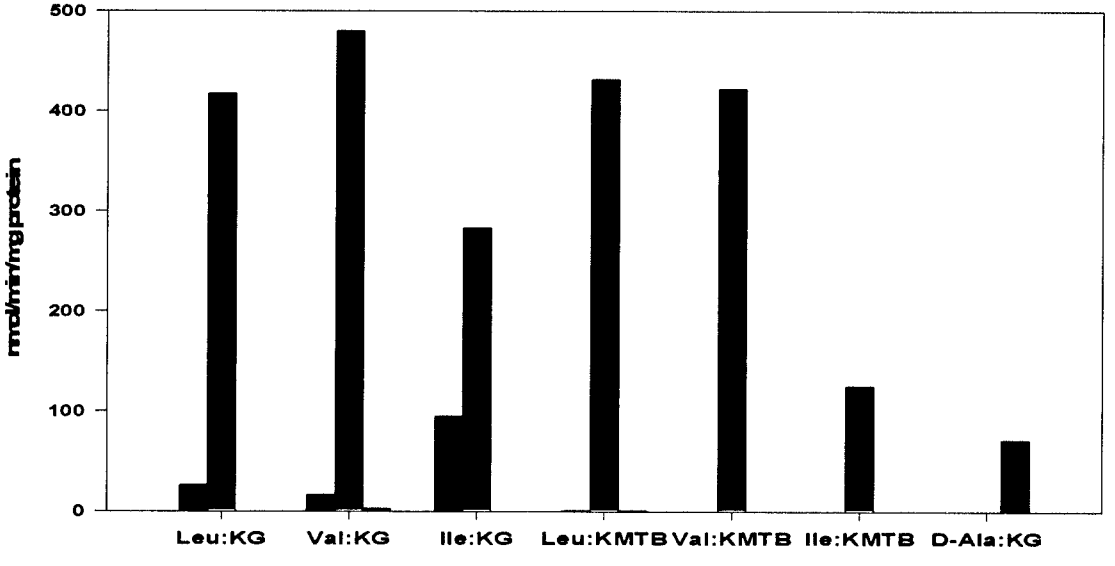

Figure 7. *Substrate preference for the recombinant B. cereus family III aminotransferases. B. cereus BCAT1 (black), BCAT2 (red), or DAAT1 (green) were incubated with 2.0 mM amino acid, 1.0 mM keto acid, and pyridoxal phosphate before HPLC analysis for the production of Met from KMTB or glutamate from KG.*

The recombinant Bc-BCAT2 was screened with single amino acids and KMTB in order to define the amino donor preference of the enzyme (Figure 3C). The enzyme used isoleucine, leucine, valine, tyrosine, phenylalanine, and, to a lesser degree, histidine and arginine.

Therefore, with the exception of alanine and glutamine as amino donors, Bc-BCAT2 would appear responsible for almost all the Met regeneration activity seen in the *B. cereus* homogenates (Figure 3A and 3B). This result is identical to that seen previously in *B. subtilis* [9]. Selected substrate pairings were examined in more detail in order to determine the kinetic parameters of the enzyme (Table 2). The Km and Vmax values were found to be similar regardless of whether KG or KMTB was used as the amino acceptor, with Km values ranging from 0.48 – 4.34 mM and Vmax values from 0.54 – 1.44 µmol/min/mg protein. Therefore, the enzyme appears to be equally active in using branched-chain amino acids for producing glutamate or Met. This result is in variance with the ybgE gene product in *B. subtilis*, where KMTB transamination had a Vmax 10-fold lower than KG transamination. The *B. cereus* enzyme is thus better adapted for Met production than that in *B. subtilis*, and may be a reflection of membership in different subfamilies within family III.

Characterisation of the *B. anthracis* BCAT Homologue

As Bc-BCAT2 was identified as the sole family III enzyme responsible for Met regeneration in *B. cereus*, the *B. anthracis* homologue was cloned and expressed as a calmodulin-binding peptide fusion protein in *E. coli*. Unlike the *B. cereus* counterpart, Ba-BCAT2 was less prone to inclusion-body formation, and active, soluble enzyme was easily purified (Figure 6). Ba-BCAT2 was screened using single amino acids and KMTB as an amino donor, with results nearly identical to that seen with Bc-BCAT2 (Figure 3C). The only difference seen between the two enzymes was a lower preference for aromatic amino acids as amino donor with Ba-BCAT2. Again, selected substrate pairs were further examined in order to define the kinetic properties of the enzyme (Table 2). As with Bc-BCAT2, Ba-BCAT2 had Km and Vmax values that were similar regardless of the amino acceptor. The Km values were 0.41 – 0.83 mM for KG and 0.95 – 3.23 mM for KMTB, while the Vmax values were 0.13 – 0.34 µmol/min/mg protein for KG and 0.42 – 0.44 µmol/min/mg protein for KMTB. Therefore, Ba-BCAT2 catalyses the formation of Met or glutamate equally well, and likely acts as the primary source of Met regeneration in *B. anthracis*.

In vitro Inhibition of *B. cereus* Growth with Canaline

In our previous study, the aminooxy compound canaline was found to be an effective inhibitor of the *B. subtilis* ybgE gene product, and could also inhibit *B. subtilis* growth in minimal medium [9]. These latter experiments were repeated with B. cereus in Nutreint broth and in a defined minimal medium (Figure 8). As was seen with *B. subtilis*, canaline is much more effective as an antibacterial when utilised in minimal medium. However, the degree of difference between these two media was not as marked as with *B. subtilis*. Canaline killed *B. cereus* with an IC50 of 38 µM in minimal medium and 759 µM in Nutrient broth (Table 3), and an MIC of 100 µM and 5000 µM respectively. Total *B. cereus* growth in minimal medium (which has no exogenous protein and uses sulfate as a sulfur source) was approximately half of that seen in Nutrient broth (which contains 30 mg/ml of protein and uses methionine and cysteine as sulfur sources). Addition of 1 mM or 10 mM methionine to the minimal medium reversed most of this inherent growth inhibition, but had little effect on

the IC50 of canaline. Therefore, *B. cereus* grows better with methionine supplementation, but the presence of exogenous methionine does not antagonise the action of canaline. Supplementation of minimal medium with 30 mg/ml bovine serum albumin had no effect on inherent growth rate, and also had little effect on the IC50 of canaline. Therefore, the protein found in Nutrient broth is not essential for optimal growth of *B. cereus*, and the lower IC50 of canaline in Nutrient broth cannot be explained by binding of the drug to exogenous protein.

Table 2. *Kinetic characterisation of Bacillus cereus and B. anthracis the BCATs catalysing methionine regeneration. The enzymes were incubated with varying amounts of substrate and 10 mM cosubstrate before analysis by HPLC as described in the Materials and Methods section.*

GENE PRODUCT	SUBSTRATE	COSUBSTRATE	APPARENT Km (mM)	APPARENT Vmax (µmol/min/mg protein)
B. cereus BCAT2	Leu	KG	0.48 ± 0.29	0.54 ± 0.07
	Val	KG	0.91 ± 0.45	0.74 ± 0.10
	Ile	KG	0.59 ± 0.25	0.63 ± 0.06
	KG	Leu	4.34 ± 1.21	0.80 ± 0.10
	Leu	KMTB	2.09 ± 0.72	0.52 ± 0.06
	Val	KMTB	3.09 ± 1.26	0.90 ± 0.13
	Ile	KMTB	1.37 ± 0.57	1.16 ± 0.15
	KMTB	Leu	2.84 ± 1.01	1.44 ± 0.20
B.anthracis BCAT2	Leu	KG	0.41 ± 0.06	0.25 ± 0.01
	Val	KG	0.71 ± 0.33	0.34 ± 0.04
	Ile	KG	0.67 ± 0.28	0.33 ± 0.04
	KG	Leu	0.83 ± 0.22	0.13 ± 0.01
	Leu	KMTB	1.75 ± 0.58	0.44 ± 0.06
	Val	KMTB	3.23 ± 1.18	0.43 ± 0.06
	Ile	KMTB	1.66 ± 0.52	0.45 ± 0.05
	KMTB	Leu	0.95 ± 0.20	0.42 ± 0.02

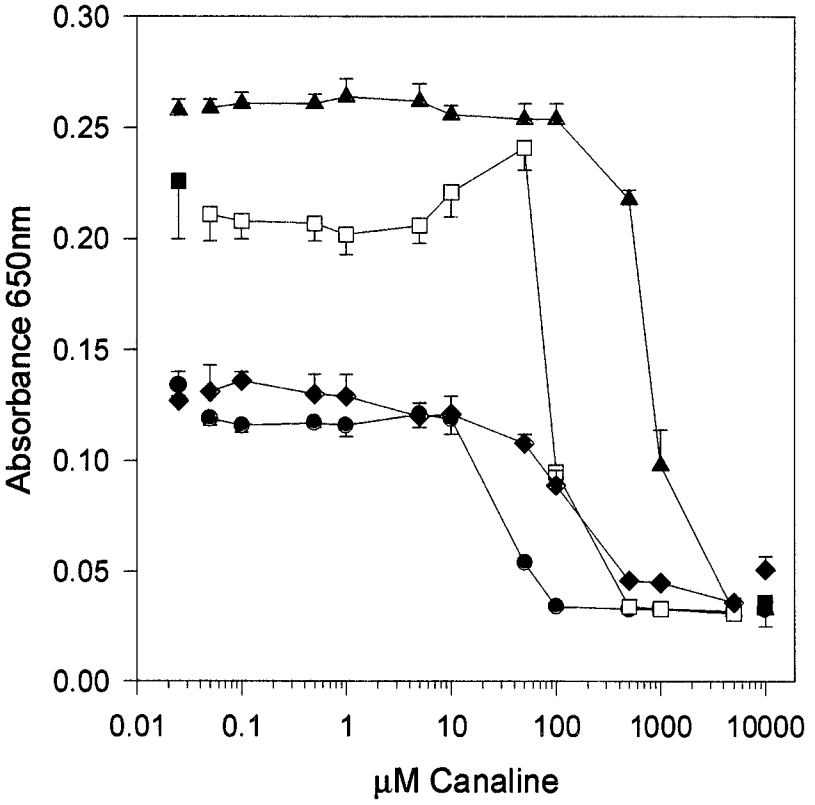

Figure 8. *In vitro inhibition of B. cereus growth by canaline. 2 x 10^4 cfu of B. cereus early log cells were inoculated into Nutrient broth (blue triangles) or minimal medium (red circles) in the presence of varying amounts of canaline. Growth after incubation overnight at 30°C was measured by turbidity at 650nm. The yellow squares and green diamonds represent minimal medium supplemented with 10 mM Met or 30 mg/ml bovine serum albumin respectively. The dark symbols are the appropriate values for growth with no inhibitor and for medium without cells.*

Table 3. *B. cereus growth inhibition by canaline. The IC50 for canaline was determined by non-linear curve fitting of growth inhibition data using the medium dose equation as descibed by Chou [24]. The MIC is the lowest concentration yielding complete prevention of cell growth.*

GROWTH CONDITION	CANALINE IC50 (μM)	CANALINE MIC (μM)
Nutrient Broth	758.9 ± 22.49	5000
Minimal Medium	35.48 ± 5.22	100
Minimal Medium + 1 mM Methionine	38.43 ± 11.41	500
Minimal Medium + 10 mM Methionine	90.00 ± 23.07	500
Minimal Medium + 30 mg/ml BSA	69.67 ± 14.04	500

Discussion

Polyamine biosynthesis, and the associated regeneration of Met from MTA (Figure 1), have been the subject of investigations on experimental chemotherapeutics in a number of organisms [1]. However, unlike in *B. subtilis*, where a number of the enzymes found in this pathway have been characterised [9,25-29], there has been no similar studies in *B. cereus* or *B. anthracis*. The final step of Met recycling from MTA involves the transamination of KMTB via an aminotransferase. Previous studies in *Klebsiella pneumoniae*, *Plasmodium falciparum*, *Crithidia fasciculata*, *Trypanosoma brucei brucei*, and *Giardia intestinalis* had implicated aminotransferases belonging to the Ia subfamily as being responsible for Met regeneration [5,6,7]. However, a subsequent study found that Gram-positive and archaeal organisms did not contain any aminotransferases which belonged to subfamily Ia. In the case of *B. subtilis*, it was found that a branched-chain aminotransferase from family III catalysed Met regeneration [9]. This aminotransferase belonged to a subfamily designated as IIIa. In this paper, the homologous aminotransferase activities were identified and characterised in *B. cereus* and *B. anthracis*.

As was the case with *B. subtilis*, both *B. cereus* and *B. anthracis* homogenates preferentially catalysed KMTB transamination using branched-chain amino acids. However, while this activity is catalysed by the ybgE gene product in *B. subtilis*, neither *B. cereus* nor *B. anthracis* was found to contain any aminotransferase with a high identity to ybgE. In fact, neither *B. cereus* nor *B. anthracis* had any putative enzyme sequence falling within subfamily IIIa. Both of these organisms were found to contain four family III aminotransferases, two with homology to BCATs and two with homology to DAATs. All eight of these sequences were members of subfamily IIIb. The sequence identity between ybgE and the *B. cereus* and *B. anthracis* aminotransferases is very low (approximately 17%). Given that the *B. cereus* complex and *B. subtilis* are quite closely related as judged by 16S rDNA sequences (Figure 2), this lack of homology between BCAT sequences is quite striking. It would appear quite likely that acquisition or loss of BCATs occurred after the split from a *Bacillus* progenitor to pre-subtilis and pre-cereus complexes. As *Bacillus halodurans* also contains a BCAT within subfamily IIIa, and is much more distantly related to *B. subtilis* and *B. cereus*, the most likely explanation is that the *B. cereus* complex lost any subfamily IIIa sequence(s) and evolved BCAT functionality from DAAT members of subfamily IIIb. These results highlight the perils of extrapolating biochemical findings from one bacterial species to another regardless of the appearance of a close evolutionary relationship.

With both *B. cereus* and *B. anthracis*, the sequence designated BCAT2 was found to readily catalyse the transamination of KG or KMTB using branched-chain amino acids as the amino donors. Kinetic analysis of the two enzymes showed that there was little difference in Km or Vmax when using KG or KMTB as the amino acceptor. Therefore, as was seen with the tyrosine aminotransferase in *K. pneumoniae*, BCAT2 catalyses Met regeneration as effectively as the "classical" activities normally associated with the enzyme. Moreover, with the exception of the use of alanine, the activity seen with recombinant BCAT2 accounts for all the activity seen in the *B. cereus* homogenates. As there was little difference in amino donor preference in homogenates made from cells grown in Nutrient broth (which contains numerous sulfur sources, including methionine) or minimal medium (where sulfate is the sole

sulfur source), BCAT2 is likely the primary catalyst of Met regeneration in both the presence and absence of exogenous methionine.

In our previous study, the aminooxy compound canaline was found to be an effective inhibitor of recombinant ybgE and also inhibited the growth of *B subtilis* in minimal medium. Canaline was found to be an efficient inhibitor of *B. cereus* growth in minimal medium, with an IC50 of 35 μM and an MIC of 100 μM. Unlike *B. subtilis*, where canaline had no effect on cell growth in Nutrient broth, *B. cereus* in Nutrient broth was inhibited by canaline. With an IC50 of 760 μM and an MIC of 5.0 mM. The basis for the differential sensitivity to canaline between rich and minimal media was examined from two potential mechanisms: exogenous methionine as an antagonist, and drug binding to exogenous protein. Addition of methionine to minimal medium had little effect on the IC50 of canaline, which suggested that methionine neither interefered with canaline transport nor rescued metabolically starved cells. The addition of 30 mg/ml BSA to the minimal medium also had little effect on the IC50 of canaline. Therefore, the compound does not bind well to BSA. As canaline is known to bind to pyridoxal phosphate dependent enzymes and pyridoxal phosphate itself, a logical step for the future would be the examination of Nutrient broth for the levels of pyridoxine, pyridoxal, and pyridoxal phosphate. Addition of a similar amount of cofactor to the minimal medium would then test for loss of canaline activity.

The effectiveness of canaline against *B. cereus* in in vitro growth inhibition tests suggests that the compound should be examined against *B. anthracis* in vitro and against *B. cereus* and *B. anthracis* in vivo. These experiments, and the screening of further aminooxy analogues against the bacilli are planned for the immediate future. In addition, potential synergy with other inhibitors of enzymes involved in polyamine biosynthesis and Met regeneration is to be examined.

References

1. Marton, L. J. and Pegg, A. E. (1995). Polyamines as targets for therapeutic intervention. *Annual Review of Pharmacology and Toxicology, 35*, p. 55-91.

2. Morgan, D. M. (1999). Polyamines. An overview. *Molecular Biotechnology, 11*, p. 229-50.

3. Igarashi, K. and Kashiwagi, K. (2000). Polyamines: mysterious modulators of cellular functions. *Biochemical and Biophysical Research Communications, 271*, p. 559-64.

4. Backlund, P. S. Jr, Chang, C. P., and Smith, R. A. (1982). Identification of 2-keto-4-methylthiobutyrate as an intermediate compound in methionine synthesis from 5'-methylthioadenosine. *Journal of Biological Chemistry, 257*, p. 4196-202.

5. Berger, L. C., Wilson, J., Wood, P., and Berger, B. J. (2001). Methionine regeneration and aspartate aminotransferase in parasitic protozoa. *Journal of Bacteriology, 183*, p. 4421-34.

6. Heilbronn, J., Wilson, J., and Berger, B. J. (1999). Tyrosine aminotransferase catalyzes the final step of methionine recycling in Klebsiella pneumoniae. *Journal of Bacteriology, 181*, p. 1739-47.

7. Berger, B. J., Dai, W. W., and Wilson, J. (1998). Methionine formation from alpha-ketomethiobutyrate in the trypanosomatid Crithidia fasciculata. *FEMS Microbiology Letters, 165*, p. 305-12.

8. Berger, B. J. (2000). Antimalarial activities of aminooxy compounds. *Antimicrobial Agents and Chemotherapy, 44*, p. 2540-2.

9. Berger, B. J. and Knodel, M. H. (2002) Characterisation of potential antimicrobial targets in *Bacillus spp.* I. Aminotransferases and methionine regeneration in *Bacillus subtilis*. (DRDC Suffield TR2002-048) Defence R&D Canada - Suffield

10. Helgason, E., Okstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I., and Kolsto (2000). Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis--one species on the basis of genetic evidence. *Applied and Environmental Microbiology, 66*, p. 2627-30.

11. Milner, J. L., Raffel, S. J., Lethbridge, B. J., and Handelsman, J. (1995). Culture conditions that influence accumulation of zwittermicin A by Bacillus cereus UW85. *Applied Microbiology and Biotechnology, 43*, p. 685-91.

12. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res, 25*, p. 3389-402.

13. Hall, T. A. (1999). Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser, 41*, p. 95-98.

14. Aslanidis, C. and de Jong, P. J. (1990). Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Res, 18*, p. 6069-74.

15. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res, 22*, p. 4673-80.

16. Felsenstein, J. (1989). PHYLIP - phylogeny inference package (version 3.2). *Cladistics, 5*, p. 164-166.

17. Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4*, p. 406-25.

18. Page, R. D. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences, 12*, p. 357-8.

19. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Danchin, A., and et, a. l. (1997). The complete genome sequence of the gram-positive bacterium Bacillus subtilis. *Nature, 390*, p. 249-56.

20. Kuramitsu, S., Ogawa, T., Ogawa, H., and Kagamiyama, H. (1985). Branched-chain amino acid aminotransferase of Escherichia coli: nucleotide sequence of the ilvE gene and the deduced amino acid sequence. *Journal of Biochemistry, 97*, p. 993-9.

21. Yonaha, K., Misono, H., Yamamoto, T., and Soda, K. (1975). D-amino acid aminotransferase of Bacillus sphaericus. Enzymologic and spectrometric properties. *Journal of Biological Chemistry, 250*, p. 6983-9.

22. Hutson, S. M., Bledsoe, R. K., Hall, T. R., and Dawson, P. A. (1995). Cloning and expression of the mammalian cytosolic branched chain aminotransferase isoenzyme. *Journal of Biological Chemistry, 270*, p. 30344-52.

23. Faure, M., Glomot, F., Bledsoe, R., Hutson, S., and Papet, I. (1999). Purification and cloning of the mitochondrial branched-chain amino acid aminotransferase from sheep placenta. *European Journal of Biochemistry, 259*, p. 104-11.

24. Chou, T. C. (1976). Derivation and properties of Michaelis-Menten type and Hill type equations for reference ligands. *Journal of Theoretical Biology, 59*, p. 253-76.

25. Sekowska, A., Mulard, L., Krogh, S., Tse, J. K., and Danchin, A. (2001). MtnK, methylthioribose kinase, is a starvation-induced protein in Bacillus subtilis. *BMC Microbiol, 1*, p. 15.

26. Sekowska, A. and Danchin, A. (1999). Identification of yrrU as the methylthioadenosine nucleosidase gene in Bacillus subtilis. *DNA Research, 6*, p. 255-64.

27. Sekowska, A., Coppee, J. Y., Le Caer, J. P., Martin-Verstraete, I., and Danchin, A. (2000). S-adenosylmethionine decarboxylase of Bacillus subtilis is closely related to archaebacterial counterparts. *Molecular Microbiology, 36*, p. 1135-47.

28. Sekowska, A. and Danchin, A. (2002). The methionine salvage pathway in Bacillus subtilis. *BMC Microbiol, 2*, p. 8.

29. Murphy, B. A., Grundy, F. J., and Henkin, T. M. (2002). Prediction of gene function in methylthioadenosine recycling from regulatory signals. *Journal of Bacteriology, 184* , p. 2314-8.

List of symbols/abbreviations/acronyms/initialisms

KMTB	ketomethiobutyrate
KG	ketoglutarate
BCAT	branched-chain amino acid aminotransferase
DAAT	D-amino acid aminotransferase
TyrAT	tyrosine aminotransferase
Met	methionine

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   Characterization of potential antimicrobial targets in *Bacillus spp.*
   II. Branched-chain aminotransferase and methionine regeneration in *B. cereus* and *B. anthracis.* (U)

4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.)

   Berger, Bradley J., English, Shane, Knodel, Marvin H. and Chan, G.

5. DATE OF PUBLICATION (month and year of publication of document)

   September 2002

6a. NO. OF PAGES (total containing information, include Annexes, Appendices, etc) 34

6b. NO. OF REFS (total cited in document) 29

7. DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

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   CBD-01-013

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   TR 2002-094

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The final step of methionine recycling from methylthioadenosine has been examined in the gram-positive bacteria *Bacillus cereus* and *B. anthracis*. Subcellular homogenates were able to convert ketomethiobutyrate to methionine using leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, and alanine as amino donors. Four putative family III aminotransferases, two with homology to branched-chain amino acid aminotransferases and two with homology to D-amino acid aminotransferases, were cloned from *B. cereus*. The two branched-chain aminotransferases were found to have a low sequence identity with the corresponding enzymes from *B. subtilis*, indicative of membership of a different subfamily. After expression of the *B. cereus* enzymes in *Escherichia coli* and subsequent purification, one branched chain aminotransferase, designated Bc-BCAT2, was found to catalyse methionine regeneration using leucine, isoleucine, valine, phenylalanine, tryosine, and tryptophan as amino donors. The homologue of Bc-BCAT2 was cloned from *B. anthracis* and designated Ba-BCAT2. Expression of the recombinant enzyme in *E. coli* and subsequent purification yielded a protein which catalysed methionine regeneration using branched-chain and aromatic amino acids as the amino donors. Kinetic analysis showed that the Km and Vmax values for the enzymes were similar for leucine, valine, and isoleucine as amino donors and ketomethiobutyrate and ketoglutarate as amino acceptors with the $K_m = 0.41 - 4.34$ mM and the $V_{max} = 0.13 - 1.44$ nmol/min/mg protein. Therefore, in both *B. cereus* and *B. anthracis*, BCAT2 would appear to be the primary catalyst of methionine production from ketomethiobutyrate. The aminotransferase inhibitor canaline was found to inhibit the growth of *B. cereus* with an IC50 of 35 μM in minimal medium and 760 μM in nutrient broth. The activity in minimal medium was only marginally antagonised by the addition of exogenous methionine or protein.

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Bacillus cereus
Bacillus anthracis
methionine recycling
aminotransferases
inhibition
canaline