

Induced Transcriptional Expression of *Bacillus subtilis* Amino Acid Permease *yvbW* in Response to Leucine Limitation

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Abstract

T box sequences have been identified upstream of a large number of uncharacterized genes such as transporters in bacterial genomes. Expression of each T box family gene is induced by limitation for a specific amino acid. T box family genes contain an untranslated leader region containing a factor-independent transcriptional terminator upstream of the structural genes. The anticodon of uncharged tRNA base-pairs with the leader mRNA at a codon referred to as the specifier sequence, inducing formation of an alternative antiterminator structure, allowing expression of the structural genes. There are several additional conserved primary sequence and secondary structural elements. Analysis of these elements can be used to predict the identity of the specifier codon and the amino acid signal. *Bacillus subtilis* hypothetical amino acid permease, *yvbW*, was analyzed as an example of this type of transcriptional regulatory prediction suggesting expression in response to leucine limitation. Expression was induced up to 130-fold in response to leucine limitation, utilizing a *yvbW-lacZ* transcriptional fusion. These data suggest that hypothetical amino acid permease YvbW may participate in leucine metabolism. A *yvbW* knockout strain was generated, although the substrate specificity for the putative amino acid permease was not identified.

Keywords

T Box, Antitermination, Riboswitch, Amino Acid Permease, YvbW

1. Introduction

The T box transcriptional antitermination system is widely used in Gram-positive bacteria for the regulation of aminoacyl-tRNA synthetase, amino acid biosynthesis and transporter genes [1] [2]. The expression of each gene

in the T box family is induced by limitation for a specific amino acid and not by general amino acid starvation. T box family genes are characterized by an untranslated leader region containing a factor-independent transcriptional terminator upstream of the structural genes. Uncharged tRNA, corresponding to the amino acid class of the response, interacts with the leader mRNA, thus inducing formation of an alternative antiterminator structure, allowing read through the leader region terminator and synthesis of the full-length message [3] [4] (see **Figure 1**). Charged tRNA is unable to stabilize the antiterminator, therefore the factor-independent terminator is formed, which aborts transcription before expression of the structural gene(s) [3] (see **Figure 1**).

Analysis of T box transcriptional leaders revealed two base-pairing interactions between the leader mRNA and the cognate tRNA that are required for antitermination. The leader mRNA contains a codon designated the “specifier sequence” which base-pairs with the anticodon of the cognate tRNA [3] [5]. Additionally, the conserved acceptor end sequence of tRNA, NCCA where N is referred to as the discriminator base, base-pairs with a bulged region of the antiterminator (UGGN) with covariance observed between the two variable bases (N) of these sequences [4]. This second base pairing putatively stabilizes the thermodynamically less favorable antiterminator structure. The acceptor end of charged tRNA is sterically blocked from inducing the antiterminator structure, thus allowing formation of the leader mRNA terminator. T box family leaders also contain a number of additional conserved primary sequence and secondary structural elements [3] [6] [7]. Four large stem-loop structures, designated as Stems I, II, IIA/B and III, are typically present. Several conserved primary sequence elements were also observed, including the AG box, GNUG, AGUA-box II, F box and CGUCCC. Additionally, the specifier codon typically ends with a C; this is referred to the C-rule.

For many T box family genes, the specificity of the amino acid signal has been determined experimentally [3]

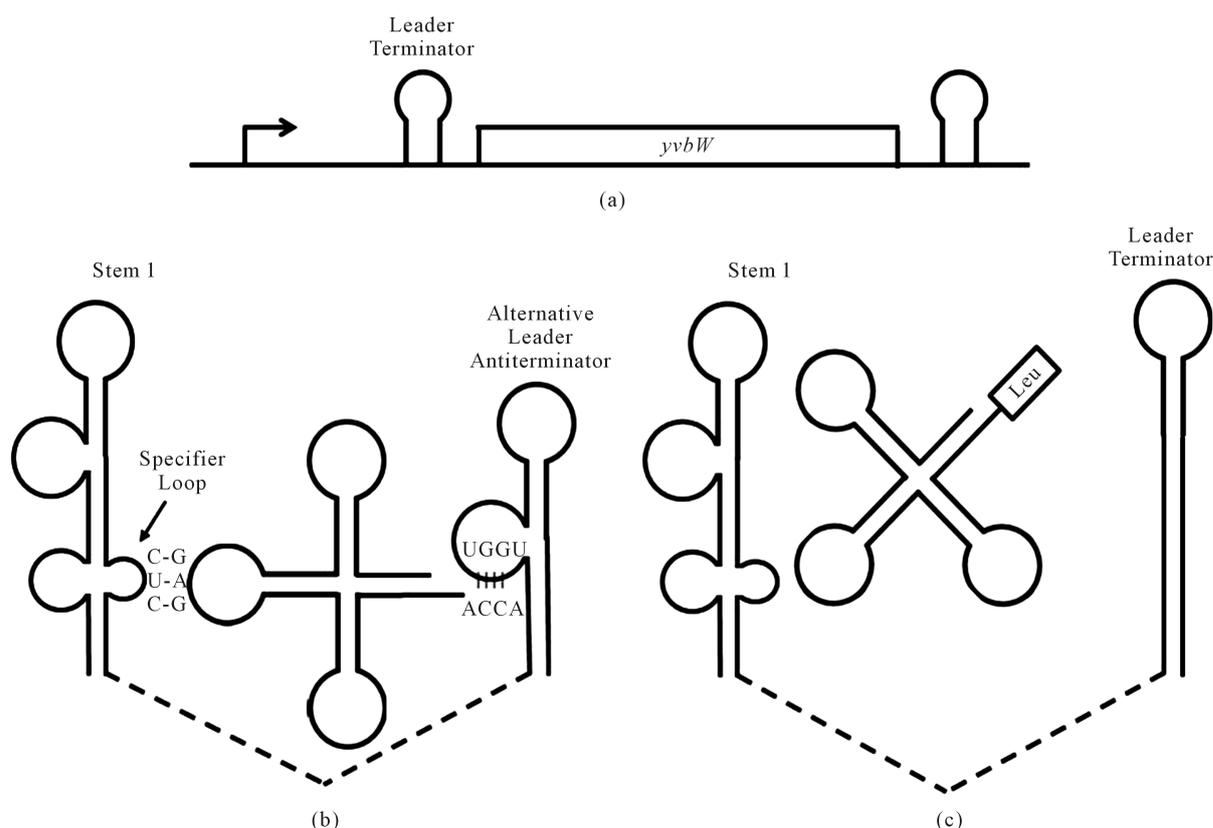


Figure 1. Model of *B. subtilis yvbW* expression. (a) An approximately 350 nt untranslated leader region, containing a factor independent transcriptional terminator, is upstream of the structural gene. Regulation of the leader region terminator controls *yvbW* expression; (b) Under limiting leucine conditions the leader region terminator is inactive. Uncharged tRNA^{Leu} stabilizes the antiterminator structure and *yvbW* expression is induced; (c) Under high leucine conditions, Charged tRNA^{Leu} does not stabilize the antiterminator and the leader region terminator is active and a low level of full-length transcript is synthesized.

[8]-[10] or has been predicted based on the physiology of the gene; for instance tRNA synthetase genes and amino acid biosynthesis genes are regulated by their cognate amino acid class. When the amino acid class can not be determined by the physiology of the T box gene (for instance with transporters or hypothetical ORFs), the amino acid specificity can be determined by the cumulative analysis of several factors. The position of primary sequence and secondary structural elements relative to the specifier loop allow for the predictive identification of the corresponding specifier sequence codon and hence, the identity of the amino acid limitation signal for induced expression. This determination can also be supported with covariance between the T box variable base and the cognate tRNA discriminator base. In many cases, the amino acid signal can be utilized to assist in the determination of the function of an uncharacterized gene such as *Bacillus subtilis* hypothetical amino acid permease, *yvbW*.

In 2008, Wels *et al.* and Vitreschak *et al.* used *in silico* analysis and alignments of T box genes that lacked a clear physiological correlation with an amino acid class, and compared these sequences with T box genes that had a correlation with an amino acid class [11] [12]. Using these data, *B. subtilis* hypothetical amino acid permease, *yvbW*, was predicted to be regulated by leucine limitation suggesting YvbW may participate in the transport of leucine [11] [12]. In a study by Irnov *et al.*, [13] *yvbW* was annotated as responding to tryptophan limitation. In this study, we will present the conserved primary sequence and secondary structural model of *yvbW*, supporting regulation by leucine limitation. Folding of the *yvbW* leader sequence suggested a CUC leucine specifier sequence. In a recent study by Saad *et al.* [10], the *Clostridium acetobutylicum* *gatCAB* operon was demonstrated to respond to two adjacent specifier sequence codons and two separate amino acid signals. Two additional specifier codon possibilities in the *yvbW* specifier loop were identified as a UGC serine codon or a GCG arginine codon. A *yvbW-lacZ* transcriptional fusion was generated [14]. The transcriptional fusion was moved into *B. subtilis* strains auxotrophic for leucine, serine and arginine. These auxotrophic strains grew on minimal media with limiting concentrations of leucine, serine and arginine, respectively. Induced expression was observed only under limiting leucine conditions and not for serine and arginine limiting conditions. The induction ratio varied between 89 and 130 folds under leucine limiting conditions, compared to growth with excess leucine. By identifying the amino acid specificity of the *yvbW* gene expression signal, the possible substrate specificity for YvbW transport can be hypothesized. The most logical substrate would be leucine or a biosynthetic precursor for leucine biosynthesis. A *yvbW* knockout strain was generated and tested under limiting leucine conditions. No growth phenotype was observed for the *yvbW* knockout strain.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Escherichia coli cloning strain DH5 α was maintained in at 37°C in Luria-Bertani media and *B. subtilis* strains were maintained at 37°C in tryptose blood agar base (Difco) or 2xYT broth. Ampicillin (50 μ g/ml) was used to select for the maintenance of plasmid pFG328 derivatives in *E. coli*. *B. subtilis* strains ZB307A (SP β c-2del2::Tn917::pSK10 Δ 6) [15] and ZB449 (*trpC2 pheA1 abrB703*; SP β cured) [16] were used for the introduction of *yvbW-lacZ* transcriptional fusions in single copy into the genomes of *B. subtilis* auxotrophic strains 1A9 (*ald-1 aroG932 leuB8 trpC2*), 1A622 [*arg(GH)95::Tn917::pSK10 Δ 6*] and 1A621 (*serC82::Tn917trpC2*) by standard procedures utilizing SP β transduction as previously described [17] [18]. Chloramphenicol (5 μ g/ml) was used to select for maintenance of the prophage.

The *yvbW-lacZ* transcriptional fusion was tested by streaking the corresponding auxotrophic strains on defined Spizizen minimal media plates containing X-gal (0.004%), required nutrients and limiting (2 μ g/ml) or non-limiting (20 μ g/ml) concentrations of the amino acid being tested [19]. Auxotrophic strains 1A9, 1A622 and 1A621 containing transcriptional fusions were tested for leucine, arginine and serine limitation, respectively.

Strain 1A9 SP β *yvbW-lacZ* was further tested for leucine limitation in defined Spizizen liquid media with limiting (5 μ g/ml) and non-limiting (50 μ g/ml) concentrations of leucine. Cultures were grown to early log (approximate A_{595} of 0.1) under non-limiting leucine conditions; cultures were split, and cells were collected by centrifugation. Cell pellets were resuspended under limiting and non-limiting leucine conditions. Culture samples were taken at the time of splitting and at 1 hr intervals for four hours. Toluene permeabilized cells were assayed for β -galactosidase activity as described by Miller [20]. Induction experiments were performed in duplicate with multiple 1A9 SP β *yvbW-lacZ* isolates.

2.2. Generation and Testing of the *Bacillus subtilis yvbW-lacZ* Transcriptional Fusion

The *B. subtilis yvbW* leader sequence was obtained from the SubtiList database [21] [22]. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs and Promega and were used as described by the manufacturer. Oligonucleotides were purchased from DNA International or Genosys Technologies. PCR was performed amplifying the *yvbW* leader region utilizing *B. subtilis* BR151 genomic DNA as a template. The oligonucleotides utilized were upstream-CTTTTGG|**GGATCC**|ATTCGAAAAGGGAC and downstream reverse complement-CCTGCCA|**TCTAGA**|TCGCTGAGCTGCTCCCC (boldface represents base substitutions required to incorporate *Bam*H I and *Xba* I sites and vertical lines encompass these sites). The PCR product was digested with *Bam*H I and *Xba* I and inserted into the plasmid pFG328 [3] generating a *lacZ* transcriptional fusion. Multiple transcriptional fusion clones were sequenced with a USB Sequenase kit confirming the SubtiList *yvbW* leader sequence [21] [22] and the integrity of the clones.

2.3. Construction of a *Bacillus subtilis yvbW* Knock-Out Strain

PCR was performed amplifying an internal fragment of the *yvbW* coding sequence utilizing *B. subtilis* BR151MA genomic DNA as a template [23]. The oligonucleotides utilized were upstream-ATGGCTGTTC|**GGATCC**|GTAACGCCAGAACCTTCCG and downstream reverse complement-AACGGCAGCA|**GGTACC**|ACGCGACAATCCGTGTCAG (boldface represents base substitutions required to incorporate *Bam*H I and *Kpn* I sites and vertical lines encompass these sites). The PCR fragment was digested with *Bam*H I and *Kpn* I and inserted into plasmid pIC156, which lacks a *B. subtilis* origin of replication [24]. Strains 1A9, 1A490 (*gltB1 leuB8 metB10*) and BR151MA (*lys-3 trpC2*) were transformed with the plasmid constructs selecting for spectinomycin resistance (100 µg/ml). The recombination event was confirmed by PCR analysis utilizing oligonucleotides TATAGAGAGAAGAAGTGC (from the *yvbW* coding sequence, downstream of the homologous fragment) and AGATGTCGCTGCAGAATGGG (from within the spectinomycin resistance gene). Several isolates from each strain were used for growth analysis. Strains 1A9, 1A490 and BR151MA were also transduced with SPβ*yitJ-lacZ* (*spcR* control; Murphy and Henkin, unpublished construct) to provide spectinomycin-resistant control strains.

3. Results

Determination of *yvbW* Secondary Structural Model and Specifier Sequence

The *yvbW* leader mRNA contains all of the conserved primary sequence and secondary structural elements of the prototypical T box leader (See **Figure 2**) [1] [2]. Stem I contains consensus CAGAGA and GGUGNRA sequences, surrounded by consensus secondary structural folding with a 13 base-pair helix containing a side bulge, which can be formed directly above the conserved AGUA and GAA sequences of the specifier internal loop. A consensus GA motif is also located below the specifier region [7]. Within the specifier loop is a conserved GAA sequence that contributes to the formation of a common loop E RNA structural motif [25]. Downstream of the GAA, is the sequence CUCGC. A CUC leucine codon is located directly downstream of the GAA, placing the specifier at the consensus position, a single base from the lower helix. The work of Saad *et al.* [10] demonstrated that the specifier sequence can shift allowing more than one codon to serve as the specifier sequence, thereby two different tRNA amino acid species are able to induce antitermination. Additionally, two alternative *yvbW* specifier codons were tested. Allowing a single nucleotide between the conserved GAA and the specifier sequence would generate a UCG-serine specifier. This specifier would not follow the specifier C rule. Allowing two nucleotides between the conserved GAA and the specifier sequence would generate a C rule CGC-arginine specifier codon but would require one less base-pair in the lower helix than represented in **Figure 2**.

An amino acid designation can also be supported by the ability of the T box variable base to pair with the discriminator base of the cognate tRNA [4]. Likewise, the identity of the T box variable base may allow specific tRNAs to be excluded from consideration. tRNA^{Leu} GAG contains an A discriminator base which can pair with the *yvbW* U T box variable base, although tRNA^{Ser} and tRNA^{Arg} amino acid species would allow G-U pairs [21] [22].

The cumulative folding, alignment and tRNA compatibility data suggest that *yvbW* is regulated by leucine limitation. Arginine and serine were also considered as secondary possible amino acid signals. These predictions were tested by incorporating a *yvbW-lacZ* transcriptional fusion into strains auxotrophic for the candidate amino acid specificities and testing expression during growth in defined minimal media limiting for the respective

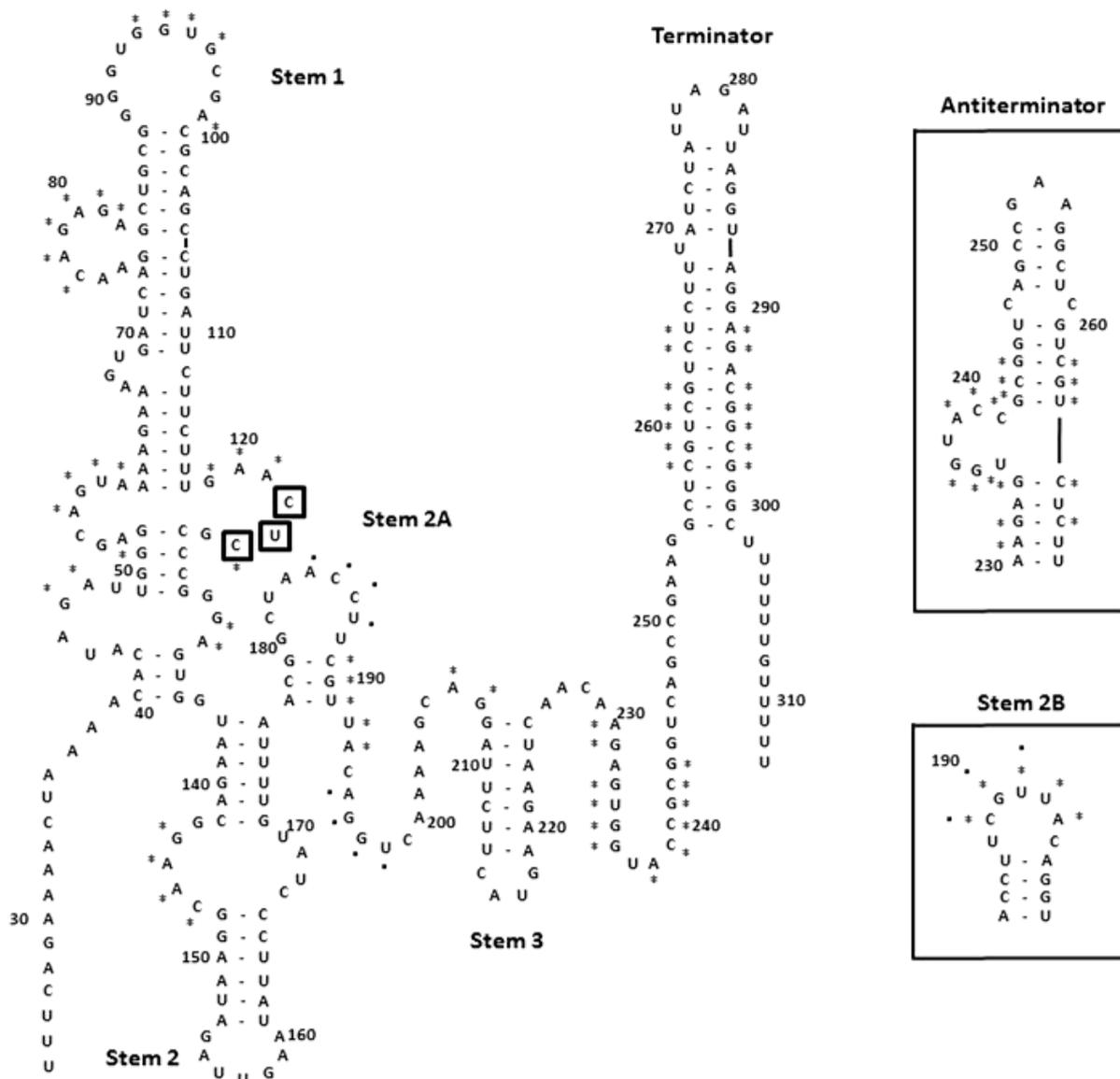


Figure 2. Structural model of the *B. subtilis* *yvbW* leader region. The *B. subtilis* *yvbW* leader folds into a structure that strongly resembles the consensus model. A CUC-leucine codon is positioned at the consensus specifier location and indicated by boxes (positions 122 - 124). Numbering is relative to the predicted *yvbW* transcription start site downstream of a TGGACA-17N-TATATT sigma-A promoter. Stems I, II, IIA and III and the T box precede the leader region terminator. A portion of the T box may alternatively base-pair with a conserved primary sequence on the 5' side of the terminator to form the antiterminator (shown in the top box on the right). A putative pseudoknot is denoted by Stems 2A and 2B [6]. Antiterminator formation allows synthesis of the full-length transcript containing the *yvbW* coding sequence. Asterisks indicate primary sequence elements conserved in T box family member genes. These elements include GA-I (positions 46 and 47), A (position 53), AGUA-I (positions 56 - 59), CAGAGA (positions 77 - 82), GGUGNRA (positions 93 - 99), GAA (positions 119 - 121), C (third position of the specifier sequence; position 124), GA-II (positions 131 and 132), GAAC (positions 144 - 147), AGUA-II (*yvbW* lacks this element), F box (CCGUUA, positions 189 - 194), AG (positions 206 - 207), T box (AGGGUGGNACCGCG, positions 230 - 243; *yvbW* lacks the G at position 232, and the anti-T box (UCGUCCCU, positions 258 - 265; *yvbW* lacks the consensus C at position 263 but covaries with the T box variant), AGGCGGCG, positions 291 - 298 (*yvbW* lacks the consensus G at position 293 but covaries with the anti-T box variant).

amino acids [19]. Transcriptional fusions were initially tested by streaking strains auxotrophic for arginine, leucine and serine on defined minimal media plates containing the full complement of amino acids or limited for the respective amino acids. The fusions tested in serine and arginine auxotrophic strains exhibited no induction

of expression via β -galactosidase activity. Only the leucine auxotroph exhibited induced expression under conditions limiting for leucine. Expression was quantified by β -galactosidase assays of the leucine auxotroph grown in defined liquid minimal media with limiting and non-limiting concentrations of leucine (Figure 3 and Table 1). These data indicate *yvbW* expression is induced 110-fold after 1 hr of leucine limitation. Induction ratios ranged from 89 to 130 fold over a four hour period. These data suggest YvbW may transport leucine or a compound related to leucine metabolism.

To further test the possible function of YvbW, a strain in which the *yvbW* gene was inactivated by insertion of a *spc* cassette was generated. Strains 1A9 *yvbW::spc* and 1A9 SP β *yitJ-lacZ* (*spcR* control) were grown on defined minimal media containing the required amino acids at 50 μ g/ml and leucine at 5 μ g/ml to induce expression

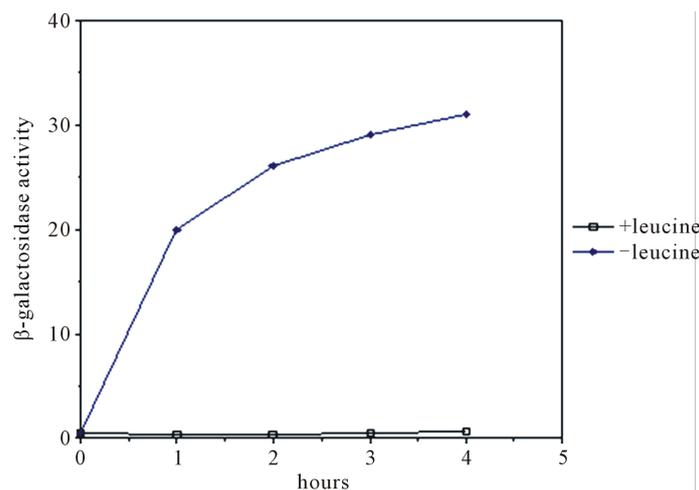


Figure 3. Expression of a *B. subtilis yvbW-lacZ* transcriptional fusion in a leucine auxotroph grown under excess or limiting concentrations of leucine. A *B. subtilis yvbW-lacZ* transcriptional fusion was tested in leucine auxotroph 1A9. Cultures were grown in defined Spizizen minimal media [19] containing 50 μ g/ml leucine and split at early logarithmic growth. Subsequently, cultures were grown with 5 μ g/ml (–leucine) or 50 μ g/ml leucine (+leucine). Samples were taken at 1 hr intervals and assayed for β -galactosidase activity [20].

Table 1. Expression of a *B. subtilis yvbW-lacZ* transcriptional fusion under excess or limiting leucine conditions. A *yvbW-lacZ* transcriptional fusion in *B. subtilis* leucine auxotrophic strain 1A9 was grown in defined Spizizen minimal media [19] containing 50 μ g/ml leucine and split during early logarithmic growth. Subsequently, cultures were grown with 5 μ g/ml (limiting leucine) or 50 μ g/ml leucine (excess leucine). Culture samples were taken at 1 hr intervals and assayed for β -galactosidase activity [20]. Data recorded in Miller β -galactosidase units [20]. The induction ratio is the β -galactosidase activity value under induced conditions divided by the uninduced condition value. Multiple isolates of *yvbW-lacZ* 1A9 were tested in 3 independent trials. Data was analyzed utilizing a paired student's T-test. Comparisons at each tested time point produced p-values below 0.001.

Time	Limiting leucine	Excess leucine	Induction Ratio
1 hr	0.18	20	110
2 hr	0.20	26	130
3 hr	0.25	29	116
4 hr	0.35	31	89

of the *yvbW* gene in the control strain. No difference in growth was observed between the *yvbW* knockout strain and the control strain. These strains were also tested for resistance to the leucine analog 4-aza-leucine (Sigma) [26]. Spizizen minimal media (leucine at 5 µg/ml) was supplemented with 2, 20 and 200 µg/ml 4-aza-leucine and growth curves were monitored. Again, no difference in growth was noted.

These results are inconclusive for the substrate specificity of YvbW. Alternatively, glutamate is utilized as an amine donor in the final step of leucine, isoleucine and valine biosynthesis; therefore glutamate is also a potential substrate for YvbW transport. Several additional transporters with putative substrate specificity for leucine and glutamate have also been identified in *B. subtilis*, suggesting a functional redundancy [21] [22] [26]. It is also possible that YvbW may transport valine, isoleucine or one of their precursors, which could be converted to leucine.

4. Discussion

Many uncharacterized genes and operons have been identified as downstream of T box leaders. These ORFs include a number of proteins likely to participate in tRNA aminoacylation, amino acid biosynthesis and amino acid transport. Transporters are the most prevalent class of proteins identified within the *B. subtilis* genome, representing approximately 2% of the ORFs [21] [22]. Despite their abundance, this class of proteins remains one of the least understood one. Amino acid biosynthesis genes also represent a widespread group of genes that present many questions. In many cases, some characteristics of the enzyme may be known but the full function is not understood. For example an enzyme may interconvert two amino acids or represent a biosynthetic branch point but the general direction of the catalyzed reaction may not be known. The T box system allows regulatory predictions to be made based on the specificity of the leader region sequence. These data can be used to identify potential transporter substrates or the physiological role of an amino acid biosynthesis gene.

yvbW has been demonstrated to be transcribed as a monocistronic mRNA that contains a prototypical T box antitermination leader [13] [27] [28] (Figure 1 and Figure 2). YvbW is annotated as a hypothetical amino acid permease, although the amino acid specificity was not defined [29]. The protein with the highest identity alignment (32%) is gamma-aminobutyrate permease (GabP) from *B. subtilis*. Expression of *gabP* was shown to be induced by nitrogen limitation and complemented an *E. coli gabP* deficient strain [30]. Despite the complementation results, a difference in substrate specificity between the two proteins was identified. Unlike *E. coli* GabP, the *B. subtilis* protein was shown to transport β-alanine (3 carbon) and gamma-amino butyrate (4 carbon) with similar efficiencies and was resistant to larger analogs that bind and inhibit GABA transporters of the brain [31].

These results are inconclusive for the substrate specificity of YvbW. Alternatively, glutamate is utilized as an amine donor in the final step of leucine, isoleucine and valine biosynthesis; therefore it is also a potential substrate for YvbW transport. Several additional transporters with putative substrate specificity for leucine and glutamate have also been identified in *B. subtilis*, suggesting a functional redundancy [21] [22] [26]. It is also possible that YvbW may transport a precursor for leucine, isoleucine, valine or glutamate biosynthesis.

With the proliferation of bacterial genomic analysis, the study of gene expression and regulation has become a logical extension of this research. T box leaders allow for *in silico* predictions of gene regulation in response to environmental conditions. Regulatory predictions can be made suggesting gene expression in response to specific amino acid limitation. As regulatory sequences become incorporated into the annotation of genomes, the analysis of T box regulatory sequences may contribute to the study of uncharacterized amino acid biosynthesis pathways and identifying transporter substrates.

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