End-product control of expression of branched-chain amino acid biosynthesis genes in *Streptomyces coelicolor* A3(2): paradoxical relationships between DNA sequence and regulatory phenotype

Heather L. Craster, Chris A. Potter and Simon Baumberg

The branched-chain protein amino acids isoleucine, valine and leucine can provide precursors for synthesis of complex polyketide secondary metabolites in streptomycetes; therefore the regulation of their own synthesis is of interest. DNA sequences upstream of *ilvBNC*, *ilvD*, *leuA*, *leuB*, *ilvE* and *leuCD* in *Streptomyces coelicolor* A3(2) have been obtained in this laboratory or as part of the *S. coelicolor* genome sequencing project. Upstream of *ilvB* and *leuA*, typical features of classical attenuator systems can be discerned, in particular hypothetical short ORFs with runs of Ile/Val/Leu and Leu codons, respectively. No such features are apparent upstream of other genes or gene clusters present. All five upstream regions were fused to *xylE* (encoding catechol dioxygenase, CO) as a reporter gene in the SCP2*-based low-copy-number vector pIJ2839. All wild-type regions showed strong depression of CO activity in the presence of all three branched-chain amino acids whether or not the attenuation features were present. By site-directed mutagenesis, the Ile/Val/Leu and Leu triplets in the putative attenuator peptides for *ilvB* and *leuA* were replaced by ones for other amino acids. In the case of *ilvB*, this had no effect at all; for *leuA*, the wild-type regulatory phenotype persisted in at least some experiments. It was concluded that (i) an unknown regulatory mechanism must be operating in the *ilv*/*leu* system of *S. coelicolor* A3(2) in place of classical attenuation; and (ii) it is unsafe to infer the functioning of a regulatory mechanism from sequence homologies alone.

**Keywords:** attenuation, RNA secondary structure, transcriptional regulation, branched-chain amino acid biosynthesis

**INTRODUCTION**

In principle there are several ways of improving secondary metabolite yield in streptomycetes, putting to use our knowledge of the regulation of gene expression in this group of organisms. They include (i) manipulating the genes of secondary metabolite biosynthesis and (ii) enhancing the biosynthesis of primary metabolite precursors of secondary metabolites. Most interest has so far been focused on (i), so that considerable information has accumulated on genes involved in regulatory cascades – especially their downstream segments – controlling pathways of secondary metabolism proper (see e.g. Chater & Bibb, 1997). Coverage of the control of primary metabolite precursors has, by contrast, been patchy. Amino acid biosynthesis, studied in some detail in organisms such as *Escherichia coli* and *Bacillus subtilis*, has not received as much attention in the streptomycetes. The suggestion has been made (reviewed by Hood *et al.*, 1992) that only low levels of amino acid biosynthetic enzymes are needed in slow-growing bacteria like streptomycetes to provide for protein biosynthesis and that such low levels make it unnecessary for the organism to maintain elaborate regulatory mechanisms modulating gene expression.
according to fluctuating needs. However, we have shown that in the systems for biosynthesis of both the arginine (Soutar & Baumberg, 1996) and branched-chain protein amino acid (Potter & Baumberg, 1996) families of Streptomyces coelicolor A3(2), modulation of gene expression extends over some two orders of magnitude. It is likely that, in at least some cases, mechanisms for controlling expression of amino acid biosynthesis genes are homologous to those in other bacteria (Hindle et al., 1994; Soutar & Baumberg, 1996; Rodriguez-Garcia et al., 1997).

The branched-chain amino acid biosynthetic pathways can be summarized briefly as follows (Umbarger, 1987). After threonine has been converted to α-ketobutyrate by the ilvA product threonine dehydratase, the routes to isoleucine and valine proceed in parallel: a molecule of the α product threonine dehydratase, the routes to isoleucine and valine proceed in parallel; a molecule of pyruvate condenses with either α-ketobutyrate (isoleucine branch) or another molecule of pyruvate (valine branch) in successive steps mediated by the products of ilvBN, ilvC and ilvD; the final step is a transamination catalysed by a common aminotransferase encoded by ilvE. α-Ketoisovalerate, the IlvD product in the valine branch, serves as the branch-point precursor of the leucine pathway, being converted to leucine in steps mediated by the leuA, leuBD, leuC and (presumably) ilvE products.

In the enterobacteria, the regulatory mechanism most commonly used to control gene expression in amino acid biosynthetic pathways is attenuation (reviewed by Landick et al., 1996). A leader sequence in the mRNA upstream of the translational start site for the first gene product contains, in order downstream from the start of the transcript: (a) a possible RBS; (b) an ORF encoding a short peptide rich in the amino acid(s) under biosynthetic regulation, and ending in a translation-terminating triplet; (c) sequences capable of yielding alternative stem–loop structures; and (d) a potential stem–loop followed by a run of Us that can act as a transcriptional terminator. Availability or otherwise of the amino acid(s) affects which alternative stem–loop structures form, and thus whether or not the terminator is formed. Attenuation of this type thus has readily distinguishable features in the DNA sequence, and these have been observed in many bacteria besides enterics. In Corynebacterium glutamicum the DNA sequence encodes a putative leader peptide for ilvB attenuation (Keilhauer et al., 1993); in Lactococcus lactis subsp. lactis there is an ORF encoding a putative leader peptide upstream of leuA (Gidon et al., 1992); Caulobacter crescentus has a small ORF encoding a putative leader peptide upstream of ilvB (accession number L25317; Tarleton et al., 1994); Thermus aquaticus has a similar ORF upstream of ilvB (accession number D84646); and in Streptomyces avermitilis there is an ORF for a putative leader peptide (De Rossi et al., 1995) with 100% homology to the S. coelicolor ilvB peptide described in this paper.

The functionality of these putative attenuator sequences has not so far been confirmed by mutational analysis. The recently deposited genome sequence for Mycobacterium tuberculosis (Cole et al., 1998) does not suggest the presence of leader peptides upstream of leuA or ilvB, and sequence analysis using the Frameplot program ([http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl](http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl)) has not uncovered any candidate leader peptides. In B. subtilis, such DNA sequence features are not apparent and this type of attenuation has not been described. Instead, at least in the well-characterized tryptophan biosynthesis system (Gollnick et al., 1990; Kuroda et al., 1988), a different mechanism modulating transcription termination is observed. Here, a stem–loop structure in the mRNA leader region binds the tryptophan-binding regulatory protein MtrB. Other genes of the branched-chain amino acid pathways (namely leuB, leuCD, ilvC, ilvD and ilvE) are believed not to have attenuation features in L. lactis (leuB, leuC; Godon et al., 1992), C. crescentus (ilvD; Malakooti & Ely, 1994) or M. tuberculosis (all of these genes; Cole et al., 1998).

There have so far been few convincing descriptions of classical attenuation systems in streptomycetes. In Actinoplanes teichomyceticus, no regulatory feature was seen upstream of leuC (Castelli et al., 1995). In the tryptophan system, Hodgson and colleagues have concluded from the DNA sequence (Hu, 1995) that trpE, encoding the first enzyme of the tryptophan-specific pathway, may be under attenuation control, whilst trpAB, trpC and trpD show growth-rate control but not end-product control (Hu et al., 1999). Lin et al. (1998) have described a classical attenuator-like arrangement upstream of an anthranilate synthase gene in Streptomyces venezuelae. Here, we describe the sequences upstream of the S. coelicolor leuA, leuB, ilvE, ilvCD, ilvBNC and ilvD genes/clusters, and report experiments aimed at determining (i) whether sequence features strongly suggestive of classical attenuation actually confer the properties expected, and (ii) if the absence of such features in the branched-chain amino acid system correlates with lack of end-product control.

**METHODS**

**Bacterial strains and plasmids.** S. coelicolor A3(2) prototroph D132 (SCP1+ SCP2-) and Streptomyces lividans TK5 (his leuA) were obtained from the John Innes Centre (JIC), Norwich, as were plasmids pIJ2839 (promoter-probe, reporter gene xylE, replicons pBR322 and SCP2+), selectable markers Amp8 and Tsr8 and its derivative pIJ2841 (with the ermE promoter directing expression of xylE). The E. coli K-12 strain used for routine manipulations was DH5α. pGEM-T was purchased from Promega.

**Media and growth conditions.** These were as described by Potter & Baumberg (1996), with the following modifications. Transformants were maintained on R5 medium supplemented with thiostrepton, leucine and histidine (S. lividans TK54) or thiostrepton alone (S. coelicolor). Spores from a sporulating single colony were spread on soya flour mannitol (SFM) agar (H. M. Kieser, personal communication), supplemented as above. After incubation for a minimum of 5 d, spores were harvested from the plate and used to inoculate a further 10
plates. After incubation for 10 d, the plates were harvested to give a single pooled spore stock which was used for all physiological experiments. To inoculate liquid media, the spores were pre-germinated as previously reported (Potter & Baumberg, 1996) but at a calculated OD of 0.005–0.01. For all growth experiments, 50 ml of liquid medium was dispensed in 250 ml Erlenmeyer flasks containing 6 g glass beads (BDH; diameter 2.5–3.5 mm). The polymer carboxymethylcellulose was added (Potter & Baumberg, 1996) to minimize aggregates and compacted pellets. All cultures were harvested at an OD of as close as possible to 1-2; due to differences in growth rates the cell yield could not be standardized completely, but the OD was always at least 0.65. Cultures were harvested after incubation for 36–72 h. Unless otherwise stated, the concentration of branched-chain amino acids used throughout this paper for pCP and PHC constructs were as previously employed (Potter & Baumberg, 1996). At the time of harvesting, 50 U cellulase (Sigma) were added to each flask. The washed mycelium was resuspended in AP buffer (100 mM potassium phosphate, pH 7.4) and subjected to ultrasonic disintegration as described by Potter & Baumberg (1996). Acetone (10%, v/v) was added to stabilize the catechol dioxygenase (CO) enzyme.

**Enzyme and protein assays.** CO was assayed by the spectrophotometric method of Sala-Trepat & Evans (1971) with the following modifications: the increase in absorbance at 375 nm was continuously monitored with the cuvette maintained at 30 °C; the final volume was 1 ml; 100 μmol assay phosphate buffer (100 mM NaH₂PO₄, 100 mM K₂HPO₄, pH 8.0) and 100 μl enzyme extract were used. Control checks for reproducibility were performed by measuring the enzyme activity of the extract obtained from unsupplemented biomass after storage on ice for 3 h; the variations were slight (2% or less) and did not affect the data for the present experiment as commented on in Results. One unit of CO activity is defined as the amount of enzyme that catalyses the formation of 1 nmol cis,cis-2-hydroxymuconic semialdehyde from catechol per min. Protein was assayed by the method of Bradford (1976).

**DNA manipulations.** Plasmid isolation from and transformation of *E. coli*, restrictions, ligations and Southern blotting procedures were as described by Sambrook et al. (1989). Plasmid isolation from and transformation of *S. lividans* and *S. coelicolor* were as described by Hopwood et al. (1985). Plasmids were isolated from *S. lividans* for pCP constructs by the modified Qiagen preparation method (Atkins, 1996). Automated DNA sequencing using ABI sequencers was carried out by DNA sequencing services at the University of Leicester.

**Isolation and sequencing of ilv and leu genes.** Internal sequences of ilvB, ilvD, leuA and leuC from *S. coelicolor* were obtained by PCR. From a multiple alignment of protein sequences derived from bacterial genes in the public databases, appropriate degenerate PCR primers representing conserved regions were chosen using Genosys software. These primers were used to obtain PCR-amplified products from chromosomal DNA of *S. coelicolor*, *Streptomyces rochei* and *S. avermitilis* by standard methods (McPherson et al., 1991). The products were cloned into pGEM-T and sequenced in duplicate to confirm their identity. They were then used to probe the grided cosmids *S. coelicolor* DNA library (Reemboch et al., 1996) on membranes kindly provided by the JIC. After positive identification by hybridization, cosmids were provided by the JIC. DNA extracted from them was restricted, transferred to membranes and probed with the PCR-amplified *ilv*/*leu* fragments. Suitably small restriction fragments were subcloned and sequenced.

Following posting at the internet site http://www.sanger.ac.uk/Projects/S_coelicolor of sequences for *ilvC*, *lenB*, *ilvE* and *leuCD* obtained in the *S. coelicolor* genome sequencing project, primers were chosen and used to amplify fragments of *S. coelicolor* chromosomal DNA consisting of upstream regions and the 5' end of the first gene, as described below. The PCR products were cloned into pGEM-T and sequenced as above. Site-directed mutagenesis of the putative ‘attenuator’ regions of *ilvB* and *leuA*. For mutagenesis of the *ilvB* upstream region, a 597 bp fragment incorporating engineered HindIII and BamHI restriction sites was obtained from the *S. coelicolor* chromosomal DNA template with the primers 5'-GGGCGGCTGGCAAGATAC-3' and 5'-TCTCTGCAAGAGCCAAATGA-3' positioned at -465 to -448 and +83 to +107, respectively (the predicted translation start of *ilvB* is taken as +1). This amplified fragment was cloned in pGEM-T Easy and sequenced. The cloned product was then cut with HindIII and BamHI and the resulting fragment was ligated into pBluescript II SK(+) linearized with HindIII and BamHI. The ensuing construct was employed as a template for the GeneEditor in vitro site-directed mutagenesis system (Promega). The primer sequences employed to replace ILVL with FLVL, FTVT or FTPT in the postulated ‘attenuator peptide’ upstream of *leuA* were: ILVL → FLVL, 5'-CATGGCCACCCGATTTCTGATCTTTGAAAGCGCGTCGG-3'; ILVL → FTVT, 5'-CATGGCCACCCGATTTCTGATCTTTGAAAGCGCGTCGG-3'; ILVL → FTPT, 5'-CATGGCCACCCGATTTCTGATCTTTGAAAGCGCGTCGG-3'. The following modifications were made to the Promega procedure: (1) during hybridization, reactions were heated at 90 °C for 5 min and allowed to cool slowly at room temperature to 37 °C; (2) the ‘bottom’ selective primer was used (see Promega manual); (3) correct incorporation of the modified sequence into the fragment was confirmed by electrophoresis screening of constructs digested with EcoRI to confirm the loss of a unique EcoRI site from the mutagenized region.

To replace the four Leu residues in the postulated ‘attenuator peptide’ upstream of *leuA* with Thr residues, a PCR method was used (Ho et al., 1989). For each mutagenesis reaction four primers were employed: A, 5’-AAAGCTTGTGCAAGAGACGCGCTGGA-3’ and D, 5’-GGATCCGGCCAGGTGGCGTGG-3’; external primers incorporating the engineered HindIII and BamHI sites and were common to all PCR reactions; B and C were the unique internal primers incorporating the engineered mutagenesis overlap regions. The 5’ → 3’ orientation of primers A and C was the same as that of *leuA*, whilst the orientation of primers B and D was opposite to that of *leuA*. The rare Thr codon primer C had the sequence 5’-CATGCGCACCCGATTTACGTAAC-3’ and the corresponding primer B had the sequence 5’-TCGCCCGCGCACCTAGTATGAGTCCCGAATCAGA-3’. Bases altered from wild-type are italicized. The common Thr codon primer C had the sequence 5’-ATCATGGCATTCGGGACTACTACTAGTGTCCGGCGCGCGA-3’ and the corresponding primer B had the sequence 5’-TGCCTGGCCGACCTAGTATGAGTCCCGAATCAGA-3’. Primers A and D correspond to the following sequences at -401 to -382 and +70 to +86, respectively (the predicted translation start of *leuA* is taken as +1). Primer B overlaps the region -124 to -164 and primer C the region -164 to -124. To generate full-length fragments spanning from -401 to +86 with either an altered or the wild-type sequence, PCR products were obtained using A + B and C + D as primers and *S. coelicolor* 4D8 cosmid DNA as

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**Note:** The text contains references to scientific methods and results, but does not include specific numerical values or detailed experimental procedures. It is a summary of the research conducted on the genetics and function of the *S. coelicolor* genome, focusing on the isolation, transformation, and mutagenesis of specific genes involved in the biosynthesis of branched-chain amino acids. The text is technical and detailed, typical of scientific research documentation.
Fig. 1. (a) DNA sequence upstream of ilvB. The sequence in bold at the left-hand (5′) end is the predicted promoter sequence, with a dot underlying the hypothetical transcription start; the underlined sequence is the predicted RBS for translation of the proposed attenuator peptide; there follows the short ORF, underlying which is the predicted amino acid sequence of the translation product; 1, 2, 3 and 4 (italicized, underlined) represent sequences which in mRNA would be complementary and could base-pair 1–2 and 3–4; the arrows point to the run of Ts corresponding to Us in the mRNA transcript; the final underlying bold letters denote the start of the IlvB polypeptide. (b) DNA sequence upstream of leuA.
the template. Samples were denatured, mixed and reannealed for a final PCR amplification. The resulting fragments were cloned into pGEM-T and sequenced to identify the appropriate plasmids.

Construction of ilv/leu upstream region-xyLE fusions.

Appropriate fragments carrying upstream regions from ilvB, ilvD, leuA, leuB or leuC were cut out of the pGEM-T constructs using the vector's HindIII and BamHI sites. The fragments were ligated into pJ2839 linearized with HindIII and BamHI. The ligation mixture was used to transform E. coli DH5α; plasmid DNA was isolated from the transformants and used to transform S. lividans or S. coelicolor.

Computer-assisted sequence analysis. Putative ORFs were detected in DNA sequences with the Frameplot 2.2.1 program at the internet site http://www.hgc.lbl.gov/cgi-bin/frameplot.pl. Promoters were sought by use of the Promoter Prediction by Neural Network program at the internet site http://www-hgc.lbl.gov/cgi-bin/promoter. Potential RNA secondary structures were evaluated using the Mfold 3.0 program at the internet site http://mfold1.wustl.edu/~mfold/cgi. Codon frequencies were based on information available from the website Codon Usage Tabled from GenBank (CUTG; http://www.dna.affrc.go.jp/~nakamura/CUTG.html).

RESULTS

ilv and leu genes and leader sequences: putative promoters, attenuation features and RNA secondary structure

Before the S. coelicolor genome project began, we had cloned ilvB, ilvD, leuA and part of leuC from this species by (i) making PCR-amplified fragments with consensus primers based on alignment of amino acid sequences from other organisms, and (ii) using these as probes to locate the genes in gridded cosmid DNAs on membranes kindly supplied by the JIC (see Methods). When the cosmid(s) carrying a particular gene had been identified, its restriction fragments were screened by Southern hybridization with the appropriate probe to obtain fragments small enough for DNA sequencing. In this way, complete DNA sequences for ilvBN, ilvD and leuA, and their upstream regions, were determined. These demonstrated that ilvN lies immediately downstream of ilvB, as in B. subtilis (Kunst et al., 1997), C. glutamicum (Cordes et al., 1992; Keilhauer et al., 1993) and M. tuberculosis (Cole et al., 1998). Later, the complete ilvC, leuB, ilvE and leuCD genes were revealed by the S. coelicolor sequencing project based at the Sanger Centre, Cambridgeshire, UK. The existence of the ilv-BNC cluster was also confirmed.

We have used characteristics associated with transcriptional promoters in bacteria to search for promoters upstream of ilvB, ilvC, ilvD, leuA, leuB and leuC, as described in Methods. Whether the program employed was optimal for streptomycete genes is uncertain; existing tabulations of streptomycete promoters (e.g. Strohl, 1992) do not appear to have been adapted for computer searches. However, best fits were found for sequences located as follows upstream of the various genes (in all cases, the first base of the predicted start triplet is taken as +1): ilvB, −299 to −259 (it is assumed that ilvC is cotranscribed with ilvB); ilvD, −61 to −21; leuA, −259 to −217; leuB, −48 to −8; leuC, −251 to −214.

Features reminiscent of classical attenuation systems were found upstream of ilvB and leuA. For ilvB, the hypothetical peptide is MRTRILVLGKRVG (Fig. 1a), whilst that for leuA is MRFGLLLL3CRGRL (Fig. 1b); the branched-chain amino acids that could render continued translation sensitive to the intracellular level of either all three amino acids or of leucine alone are underlined. The relationships between the translatable segments of the ilvB and leuA leader sequences and possible self-complementary regions are also shown. Fig. 1(b) shows an arrangement for leuA of a translatable segment in relation to alternative stem–loop structures that is consistent with a classical attenuation system, although the final run of Us at which transcription might terminate is missing, Fig. 1(a) on the other hand, whilst it shows a translatable segment for ilvB that is in principle appropriate, possesses only poor candidates for stem–loop structures, though a possible run of Us is apparent. None of the features characteristic of attenuation systems could be discerned upstream of ilvC, ilvD, leuB or leuC.

Hypothetical RNA secondary structures upstream of ilvB, ilvD, leuA, leuB and leuC, generated as described in Methods, all showed possible self-complementary sequences giving rise to hypothetical stem–loop structures; examples of these upstream regions are shown in Fig. 1(c–e). The Gibbs free energy (ΔG) values for these structures suggest they should be highly stable, but this seems to be generally true of hypothetical secondary structures predicted for streptomycete RNA transcripts by the computer program employed. ilvB and leuA structural gene transcripts treated in the same way gave comparable hypothetical secondary structures (Fig. 1f). Thus these structures may be a consequence of the very G + C-rich nature of streptomycete DNA.

Site-directed mutagenesis of ilvB and leuA hypothetical translatable regions and construction of wild-type and mutant ilv/leu upstream region fusions to the reporter gene xyLE

The sequence upstream of ilvB corresponding to the wild-type putative leucine/isoleucine/valine-sensing sequence ILVL in the attenuator peptide is ATTTCG-
GTACTTT. It was decided to change this to (i) TTTCTCGTACTTT, encoding FLVL, (ii) TTTACCGGTA-CTT, encoding FTVT, and (iii) TTTACCAGCCTTT, encoding FTPT. If the Ile, Leu and Val residues play the branched-chain amino acid-sensing roles expected in a classical attenuation system, (i), (ii) and (iii) might lead to loss of response to isoleucine, leucine and valine in turn. A 597 bp ilvB fragment extending from position −475 to +107 was amplified by PCR and cloned into pGEM-T Easy. The three altered sequences were then produced by the GeneEditor plasmid method.

The sequence upstream of leuA corresponding to the wild-type putative leucine-sensing sequence in the attenuator peptide is CGTCCTCTCCTTT; of the corresponding codons, CUG and CUC are common Leu-encoding triplets in streptomycetes whilst CUU is rare. The wild-type sequence was changed to (i) ACCACC-ACCACC, with four common Thr triplets; and (ii) ACTTACTTACTT, with four rare Thr triplets. If one or more of the four Leu residues plays the leucine-sensing role expected in a classical attenuation system, (i) might — if the availability of particular tRNAs correlates with the frequency of their cognate codons — increase transcription termination under leucine-sufficiency conditions, whilst both (i) and (ii) should lead to loss of response to intracellular leucine concentration with the possible appearance of a response to threonine instead, which might be more marked with the common Thr codons as suggested for (i). A 498 bp leuA fragment extending from position −399 to +86 was amplified by PCR and cloned into pGEM-T Easy. The three altered sequences were then produced by the PCR method.

The wild-type and altered fragments were cloned into HindIII/BamHI-cut pJ2839, making use of the HindIII and BamHI sites in the PCR primers. The resulting plasmids were: pCP2000, bearing the wild-type ilvB sequence; pCP2001, with the ilvB sequence specifying FLVL; pCP2002, with the ilvB sequence specifying FTVT; pCP2003, with the ilvB sequence specifying FTPT; pHC1000, bearing the wild-type leuA sequence; pHC1001 with the leuA sequence containing the four common Thr codons; and pHC1002, with the leuA sequence containing the four rare Thr codons. The plasmids were constructed in E. coli and used to transform S. lividans TK54 and S. coelicolor D132.

A 678 bp leuB fragment extending from position −569 to +109, a 428 bp leuC fragment extending from

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**Table 1.** CO activities in cultures with xylE expression controlled by the wild-type and mutated leuA and ilvB upstream regions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Upstream region</th>
<th>Specific activity of un-supplemented culture</th>
<th>Ratio of specific activity of un-supplemented culture to that of culture supplemented with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>D132(pCP2000)</td>
<td>ilvB, wt</td>
<td>2.5</td>
<td>0.66 (leucine) 4.5 (I) 1.0 (IV) 16 1.4 (FTP) 0.50 (FLV), 0.60 (FTV), 0.65 (FTP)</td>
</tr>
<tr>
<td>D132(pCP2003)</td>
<td>ilvB, ILVL → FTPT</td>
<td>2.4</td>
<td>0.92 (leucine) 1.3 (I) 0.94 (IV) 22 1.1 (FTP) 0.50 (FLV), 0.50 (FTP)</td>
</tr>
<tr>
<td>TK54(pCP2000)</td>
<td>ilvB, wt</td>
<td>37</td>
<td>10 45 1.3 (I) 1.6 (IV) 37 1.7 3.5 (FLV), 1.0 (FTV), 0.78 (FTP)</td>
</tr>
<tr>
<td>TK54(pCP2001)</td>
<td>ilvB, ILVL → FLVL</td>
<td>67</td>
<td>22 5.4 (leucine) 5.4 (I) 1.6 (IV) 475 2.2 (FLV)</td>
</tr>
<tr>
<td>TK54(pCP2002)</td>
<td>ilvB, ILVL → FTPT</td>
<td>34</td>
<td>9.6 0.99 (I) 3.1 (IV) 14 2.6 (FLV)</td>
</tr>
<tr>
<td>TK54(pCP2003)</td>
<td>ilvB, ILVL → FTPT</td>
<td>14</td>
<td>0.99 1.28 (IV) 1.28 9.0 0.83 (FTP)</td>
</tr>
<tr>
<td>D132(pHC1000)</td>
<td>leuA, wt</td>
<td>5.4</td>
<td>0.72 1.0 (I) 0.72 0.0 0.73</td>
</tr>
<tr>
<td>D132(pHC1001)</td>
<td>leuA, common Thr triplets</td>
<td>3.3</td>
<td>0.15 1.8 0.15 1.8</td>
</tr>
<tr>
<td>D132(pHC1002)</td>
<td>leuA, rare Thr triplets</td>
<td>6.5</td>
<td>0.15 1.8 0.15 1.8</td>
</tr>
<tr>
<td>TK54(pHC1000)</td>
<td>leuA, wt</td>
<td>9.2</td>
<td>1.8 0.15 1.8 0.15 1.8</td>
</tr>
<tr>
<td>TK54(pHC1002)</td>
<td>leuA, rare Thr triplets</td>
<td>13</td>
<td>0.15 3.0 0.15 3.0</td>
</tr>
</tbody>
</table>

ND, Not determined.

* wt, Wild-type.

† Given in nmol min⁻¹ (mg protein)⁻¹.

‡ FLV, phenylalanine + leucine + valine; FTV, phenylalanine + threonine + valine; FTP, phenylalanine + threonine + proline.
position -303 to +125 and a 421 bp ilvD fragment extending from position -296 to +125 were amplified by PCR so as to incorporate either a BamHI or a HindIII restriction site and each was cloned in pGEM-T. The fragments were cloned into HindIII/BamHI-cut pIJ2839. The resulting plasmids (pCP3000, pHC4000 and pHC5000, respectively) constructed in E. coli were used to transform S. lividans TK54 and S. coelicolor D132.

### CO enzyme activities directed by wild-type and mutant ilvD/leu upstream regions in xylE fusions in S. coelicolor and S. lividans

From spore stocks of the various plasmid-containing S. lividans and S. coelicolor strains, pooled single-spore stocks were prepared for all the experiments described here. Spores were pre-germinated and used to inoculate minimal media either without any amino acid supplement or with appropriate amino acid supplements. When cultures were harvested, the mycelium was fragmented by ultrasonic disintegration following previous procedures (Potter & Baumberg, 1996). The cytoplasmic extracts were used for CO and protein assays, from which specific activities were calculated.

Results obtained for cultures with xylE expression controlled by the wild-type and mutated ilvB and leuA upstream regions are shown in Table 1, and for cultures with xylE expression controlled by the wild-type leuB, leuC and ilvD upstream regions in Table 2. These results are for individual experiments (each experiment comprising differently supplemented cultures made up in the same batch and inoculated at the same time and with the same inoculum) with no attempt to combine the results statistically. Both tables show, for upstream regions that are either wild-type or mutated as described in the previous section, (a) the specific activity for the unsupplemented culture, and (b) the ratio of that specific activity to the specific activities for the other (variously supplemented) cultures within the same experiment. We first draw attention to the lack of reproducibility of absolute specific activities in separate experiments. This is seen in the column that records the specific activity of the unsupplemented culture. For instance, taking the results for D132(pHC1000) in Table 1, it is seen that, in four separate experiments, the CO specific activities were 5-4, 19, 30 and 45 nmol min\(^{-1}\) (mg protein\(^{-1}\)), respectively. This variability nevertheless does not obscure the main conclusions of this report, since consistent trends were found in different experiments for the ratios between specific activities, with some exceptions which are addressed below. (It may be noted that in most experiments, additional cultures with different amino acid supplementation were assayed; the results not shown here are in accord with this generalization). For instance, in the four experiments with D132(pHC1000), the ratios of the activity of the unsupplemented culture to that of the triply supplemented (isoleucine + leucine + valine) culture were comparable at 23, 22, 33 and 21, respectively, despite the 83-fold difference in absolute values of the latter. This feature was noted in an earlier paper (Potter & Baumberg, 1996), and anecdotal evidence suggests that it is a not uncommon aspect of physiological work with streptomycetes. In the absence of an agreed statistical procedure for dealing with ratios of activities, we have simply tabulated these to permit evaluation of the primary data. As controls, in similar experiments with the vector plasmid pIJ2839 (no promoter upstream of

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**Table 2. CO activities in cultures with xylE expression controlled by the wild-type leuB, leuC and ilvD upstream regions**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Upstream region</th>
<th>Specific activity of unsupplemented culture(^a)</th>
<th>Ratio of specific activity of unsupplemented culture to that of culture supplemented with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leucine</td>
<td>Isoleucine + leucine</td>
</tr>
<tr>
<td>D132(pCP3000)</td>
<td>leuB</td>
<td>2.5</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.8</td>
<td>0.65</td>
</tr>
<tr>
<td>D132(pHC4000)</td>
<td>leuC</td>
<td>6.8</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>ND</td>
</tr>
<tr>
<td>D132(pHC5000)</td>
<td>ilvD</td>
<td>12</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.8</td>
<td>0.18</td>
</tr>
<tr>
<td>TK44(pCP3000)</td>
<td>leuB</td>
<td>90</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>0.7</td>
</tr>
<tr>
<td>TK44(pHC4000)</td>
<td>leuC</td>
<td>9.5</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>ND</td>
</tr>
<tr>
<td>TK44(pHC5000)</td>
<td>ilvD</td>
<td>18</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(\text{ND}, \text{Not determined.}\)

\(\text{\(^a\) Given in nmol min}\(^{-1}\) (mg protein\(^{-1}\)).\)
xylE) and pJ12841 (which has the ermE promoter directing xylE expression) CO activities showed no influence of supplementation with any combination of amino acids (results not shown).

The results with pCP2000–pCP2003-containing strains shown in Table 1 can be interpreted as follows. The wild-type ilvB upstream fragment gives a strong depression of xylE expression in the presence of all three branched-chain amino acids in both hosts, consistent with previous studies with S. coelicolor (Potter & Baumberg, 1996) and observations with S. lividans (H. L. Craster, unpublished results) in which the activity of the IlvB product itself, acetohydroxyacid synthase, was assayed. We can therefore conclude that the xylE reporter gene fusion system faithfully mirrors the chromosomal situation. The replacement of the one Ile codon, the Ile codon plus the two Val codons, or all three of these plus the one Leu codon, by Phe, Thr and Pro codons, respectively, leaves an insignificant response to all three amino acids in either S. coelicolor or S. lividans (ratios of unsupplemented to fully supplemented of 3–4–473 as against 16–37 for the wild-type). Again, there is no consistent indication of any response to external phenylalanine + threonine + proline (ratio of unsupplemented to phenylalanine and/or threonine and/or proline-supplemented was never more than 2.9 except in one experiment where it was 8.9, whereas the wild-type itself in one case gave a ratio of 3.5).

The results with pCP1000–pCP1003-containing strains shown in Table 1 lead to similar, though at this stage more tentative, conclusions. The wild-type leuA upstream fragment gives a strong depression in reporter gene expression in the presence of all three branched-chain amino acids in both hosts (ratios of unsupplemented to fully supplemented of 21–83), once again consistent with previous studies with S. coelicolor (Potter & Baumberg, 1996) and observations with S. lividans (H. L. Craster, unpublished results) in which the activity of the LeuA product itself, isopropylmalate synthase, was assayed under variable conditions. The replacement of the four Leu codons by either common or rare Thr triplets resulted in some cases in ratios of unsupplemented to fully supplemented (140, 27) similar to or greater than, but in other cases (36, 5.1) appreciably lower than, for the wild-type sequence. There is no suggestion of any response to external threonine (ratios of unsupplemented to threonine-supplemented were never more than 1.6).

With the ilvB fragments directing expression of xylE, supplementation with isoleucine + leucine depressed reporter gene expression to a similar extent with all three branched-chain amino acids (range of ratios 4.5–45), whilst isoleucine alone or in combination with valine showed little or no effect (range of ratios 0.21–5.4). Leucine alone did not consistently affect expression with either the ilvB or leuA fragments, though it did in occasional experiments: there is a suggestion that it does so more often in S. lividans than S. coelicolor. This, and the tendency of specific activities for unsupplemented cultures to be higher in S. lividans than in S. coelicolor, may reflect the fact that the S. lividans host employed here, TK54, is a leuA auxotroph (Hercomb et al., 1987), and that a low concentration of leucine (100 µM) had to be added to ‘unsupplemented’ medium to permit growth.

When xylE expression was directed by the leuB, leuC or ilvD fragments, strong (though again variable) depression in reporter gene expression in the presence of all three branched-chain amino acids was found in all cases, the ratios of unsupplemented to fully supplemented varying from 47 to 170. As with the leuA and ilvB upstream regions, supplementation with isoleucine + leucine gave nearly as strong depression as all three amino acids, whereas isoleucine + valine again had no effect, and leucine alone had a substantial effect in only one experiment.

**DISCUSSION**

Most of the features of classical attenuation systems are present in the regions upstream of S. coelicolor ilvB and leuA; however, the paucity of plausible alternative secondary structures in the ilvB mRNA suggests a need for caution in assuming that these features are part of a functional regulatory mechanism. The evidence that replacing the postulated ILVL-specifying codons in the ilvB upstream region to give FTPT has no apparent regulatory effect supports a cautious interpretation, as does the evidence that replacement in the leuA upstream region of the four putative leucine-sensing Leu codons by Thr leaves Leu with substantial control of expression in at least some experiments. Conversely, the absence upstream of leuB, leuC or ilvD of expected features of attenuation systems is nevertheless accompanied by much the same regulatory phenotype as shown for ilvB and leuA – indeed, the similarity in control of gene expression (for instance in regard to the strong effect of isoleucine + leucine) exerted by all five upstream regions strongly suggests that a unitary mechanism is at work. What this might be can at present only be conjectured; the marked predicted mRNA secondary structure in all cases could be consistent with the binding of a regulatory protein similar to MrxB in the tryptophan biosynthesis system of B. subtilis, but as noted above, such secondary structure seems to be a feature of the mRNA for structural genes as well as in the leader sequence.

Our results show that replacing one Ile codon within the putative ilvB attenuator peptide, or all three of these plus the one Leu codon, by Phe, Thr and Pro codons, respectively, still permits, in almost all experiments, the same depression of CO activity by the combination of branched-chain amino acids as found for the wild-type. This seems to eliminate the possibility that ilvB expression works by a classical attenuator mechanism, despite the sequence features. Our data so far for the leuA system are less clear-cut, but can be summarized by saying that replacing the four Leu triplets by either common or rare Thr triplets does not prevent, in at least some experiments, depression of CO activity. Ex-
pression with the new combination of amino acids is of similar magnitude to that in the wild-type. Thus, although the mutations are having some effect, this could be for reasons other than the amino acid composition of the leader peptide; for instance, there might be effects through alteration in mRNA secondary structure. No appreciable effect was exerted by the amino acid(s) whose triplets had replaced those of Ile, Val and/or Leu.

The variability in specific activities shown by cultures with the same supplementation in different sets is a source of concern, but we believe that it does not obscure the main conclusions of this report. The ratios between specific activities for a given genotype and a given medium allow consistent conclusions. The variability of specific activities shown by cultures pre-germination regime may become amplified to produce large fluctuations in the physiological state of the final cultures, for instance in the internal pool size of an amino acid for which the culture is unsupplemented.

The presence of many of the features of classical attenuation systems upstream of ilvB and letA is puzzling if these have no role in the mechanism of regulation. The maintenance of appropriate mRNA secondary structures, in principle a reasonable requirement, would have been expected to allow gradual blurring of such features by mutational drift. However, without further evidence that upstream mRNA secondary structure participates in regulating any of the let or ilv genes/clusters, it cannot be assumed that these necessarily have such functions. It is perhaps worth noting that in the S. griseus streptomycin biosynthesis system, there is a leader sequence upstream of strB1 rich in inverted repeats, a feature initially interpreted as consistent with a regulatory mechanism involving modulated transcription termination; this later proved not to be the case (Distler et al., 1987; Lindley, 1990; Distler et al., 1992; Lindley et al., 1995; Retzlaff & Distler, 1995). An implication of the present study may be that caution is needed in inferring function of even strongly marked sequence features simply by virtue of homology. It will be interesting, for instance, to determine the properties of the apparent attenuation systems upstream of tryptophan synthase genes in S. coelicolor A3(2) (Hu, 1995) and S. venezuelae (Lin et al., 1998) and of various amino acid biosynthesis genes in C. glutamicum and L. lactis. The possibility has been noted before (Baumberg, 1981, 1999) that features of regulatory systems may persist, as molecular fossils, beyond their useful life.

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