AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

GENETICS AND MOLECULAR BIOLOGY

The *ilvGMEDA* Operon Is Regulated by Transcription Attenuation in *Vibrio alginolyticus* ZJ-T

Yiqin Deng,[a,b,c] Xing Luo,[c] Mei Xie,[a] Philippe Bouloc,[c] Chang Chen,[a,d] Annick Jacq[c]

[a]Key Laboratory of Tropical Marine Bio-Resources and Ecology, Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China

[b]Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture and Rural Affairs, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China

[c]Institute for Integrative Biology of the Cell, CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, Gif-sur-Yvette, France

[d]Xisha/nansha Ocean Observation and Research Station, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China

ABSTRACT Bacteria synthesize amino acids according to their availability in the environment or, in the case of pathogens, within the host. We explored the regulation of the biosynthesis of branched-chain amino acids (BCAAs) (L-leucine, L-valine, and L-isoleucine) in *Vibrio alginolyticus*, a marine fish and shellfish pathogen and an emerging opportunistic human pathogen. In this species, the *ilvGMEDA* operon encodes the main pathway for biosynthesis of BCAAs. Its upstream regulatory region shows no sequence similarity to the corresponding region in *Escherichia coli* or other *Enterobacteriaceae*, and yet we show that this operon is regulated by transcription attenuation. The translation of a BCAA-rich peptide encoded upstream of the structural genes provides an adaptive response similar to the *E. coli* canonical model. This study of a nonmodel Gram-negative organism highlights the mechanistic conservation of transcription attenuation despite the absence of primary sequence conservation.

IMPORTANCE This study analyzes the regulation of the biosynthesis of branched-chain amino acids (leucine, valine, and isoleucine) in *Vibrio alginolyticus*, a marine bacterium that is pathogenic to fish and humans. The results highlight the conservation of the main regulatory mechanism with that of the enterobacterium *Escherichia coli*, suggesting that such a mechanism appeared early during the evolution of Gram-negative bacteria, allowing adaptation to a wide range of environments.

KEYWORDS *Vibrio alginolyticus*, acetolactate synthase (AHAS), branched-chain amino acids, *ilvGMEDA* operon, leader attenuator, transcription attenuation

Bacterial gene regulation has been extensively studied in model organisms such as *Escherichia coli* and *Bacillus subtilis*, uncovering many layers of exquisitely complex regulatory pathways at all levels of genome expression (transcriptional, translational, protein stability, and activity levels), involving regulatory proteins and noncoding small RNAs (sRNAs) as well as end products. In the case of carbon compound utilization, cells need to turn on the catabolic pathway only when the compound is present in their environment. In contrast, in the case of essential compounds such as amino acids, the genes encoding their biosynthetic pathways are expressed only when necessary, i.e., when these amino acids cannot be scavenged from the environment. The biosynthetic pathway for branched-chain amino acids (L-isoleucine, L-leucine, and L-valine [ILV]) (BCAAs) in *E. coli* was worked out in the 1950s by H. Edwin Umbarger, who went on to explore its regulation (reviewed in references 1 and 2). This biosynthetic pathway is presented in Fig. 1A. In *E. coli* and other *Enterobacteriaceae*, the 14 *ilv* structural genes are organized in five transcription units: *leuABCD*, *ilvGMEDA*, *ilvBN*, *ilvIH*, and *ilvC*. An

Citation Deng Y, Luo X, Xie M, Bouloc P, Chen C, Jacq A. 2019. The *ilvGMEDA* operon is regulated by transcription attenuation in *Vibrio alginolyticus* ZJ-T. Appl Environ Microbiol 85:e00880-19. https://doi.org/10.1128/AEM.00880-19.

Editor Robert M. Kelly, North Carolina State University

Address correspondence to Annick Jacq, annick.jacq@u-psud.fr.

Y.D. and X.L. contributed equally to this work. C.C. and A.J. contributed equally to this work.

Received 15 April 2019
Accepted 16 July 2019
Accepted manuscript posted online 19 July 2019
Published 17 September 2019

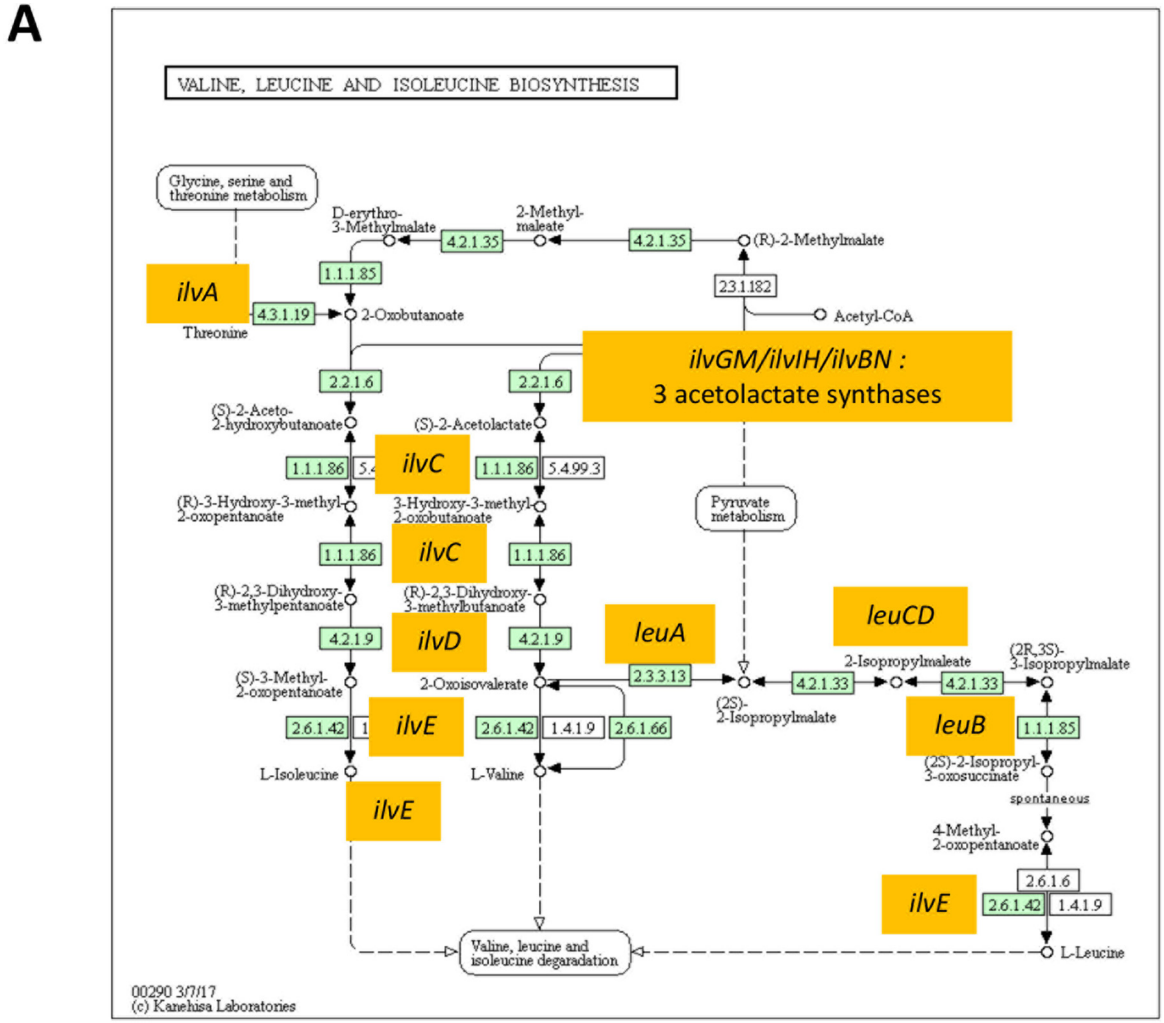

FIG 1 The branched-chain amino acid pathway. (A) Branched-chain amino acid (BCAA) biosynthesis pathways in *E. coli* drawn with permission of the Kanehisa laboratory according to KEGG (https://www.genome.jp/kegg-bin/show_pathway?org_name=eco&mapno=00290&mapscale=&show_description=hide). Green labels are for enzymatic activities present in *E. coli* (36). The names of genes encoding these activities are in gold boxes. The three pairs of genes encoding acetolactate synthase activity (*ilvGM*, *ilvIH*, and *ilvBN*) are indicated. (B) Genetic organization of the three operons encoding potential acetolactate synthase activity in *V. alginolyticus*.

additional gene, *ilvY*, upstream of *ilvC* and transcribed in the opposite direction, encodes a transcriptional regulator of *ilvC* (3).

A first level of regulation is feedback inhibition of the first pathway-specific enzyme by the end product of the pathway: L-leucine inhibits isopropylmalate synthase, encoded by *leuA*; L-valine inhibits the activity of acetolactate synthases (AHASs) encoded by *ilvIH* and *ilvBN*; and L-isoleucine inhibits the activity of threonine deaminase, encoded by *ilvA*. A second regulatory level is gene expression. At the transcriptional level, the global dual regulator leucine-responsive protein (Lrp) was found to regulate the *ilvBN* transcription unit (for a review, see reference 4), whereas the *leuABCD* and *ilvGMEDA* operons are essentially regulated by transcriptional attenuation (5–10), where a short leader peptide that contains a high number of the regulating amino acid (Ile, Val, or Leu) is encoded upstream of the operon between the promoter and the first structural gene. Each leader region is followed by a transcription terminator site, the attenuator. The short polypeptide sequence monitors the cellular supply of aminoacylated tRNAs for the regulatory codons. When the amino acids are in ample supply, the leader peptide is synthesized without interruption. Under these conditions, the terminator forms and transcription termination is maximum, preventing expression of the downstream genes in the operons.

If the amino acids are in short supply, translation of the leader peptide is impeded. In such a case, the untranslated leader transcript can form an alternative secondary structure that preempts the formation of the transcriptional terminator, allowing transcription to proceed through the operon (reviewed in reference 1).

More recently, attention has turned to regulation of BCAA biosynthesis in Gram-positive bacteria, such as *B. subtilis*, *Lactobacillus* spp., or *Staphylococcus aureus* (for a review, see reference 11). In these species, the *ilv* and *leu* genes are organized in three genetic loci, with *ilvBH* and *leuABCD* being part of a single transcription unit. Both transcription units are transcriptionally regulated by the CodY transcriptional repressor, which becomes active upon binding of either BCAA, at least *in vitro* (12). In the case of *S. aureus*, *in vivo* CodY seems to respond specifically to the presence of Ile (13). In addition, regulation at the level of transcription attenuation by an attenuator encoding a Leu-rich peptide has also been demonstrated (13). Hence, in *S. aureus*, there are two levels of regulation, each responding specifically to the presence of two different BCAAs.

CodY is also an important regulator of virulence (11), providing a link between BCAA biosynthesis and pathogenicity, reflecting the importance of dealing with possible scarcity of these amino acids in the host.

In Gram-negative bacteria, studies have concentrated on the model organisms *E. coli* and *Salmonella enterica* serovar Typhimurium. However, a comparative genomic analysis in proteobacteria suggested that regulation of BCAA synthesis by transcription attenuation is widely conserved in this phylum, including in three *Vibrio* species (14). In addition, it has been shown that Lrp from *Vibrio cholerae* could compensate partially for the absence of Lrp in *E. coli* (15).

In Gram-negative bacteria, no experimental studies on BCAA biosynthesis regulation have been carried out outside the *Enterobacteriaceae*. *Vibrio alginolyticus* is a Gram-negative halophilic bacterium that is ubiquitous in marine and estuarine environments. It is an opportunistic pathogen responsible for vibrioses in fish and shellfish and causes skin and ear infections in humans (16, 17).

In this report, we present a detailed analysis of BCAA biosynthesis regulation in a *Vibrio* species, showing that *V. alginolyticus* regulation in response to the presence or absence of environmental BCAAs is mostly through transcriptional attenuation.

RESULTS AND DISCUSSION

Two AHASs are involved in BCAA biosynthesis in *V. alginolyticus*. Acetolactate synthase is the first common enzyme in the pathways leading to L-isoleucine, L-leucine, and L-valine (18) (Fig. 1A). It is made of two polypeptides, a catalytic subunit and an activating subunit. In *E. coli*, three pairs of genes, *ilvBN*, *ilvGM*, and *ilvIH*, encode AHAS I, AHAS II, and AHAS III, respectively (reviewed in reference 1). *ilvB*, *ilvGM*, and *ilvIH* were detected on chromosome I of *V. alginolyticus* ZJ-T, while the downstream gene of *ilvB*

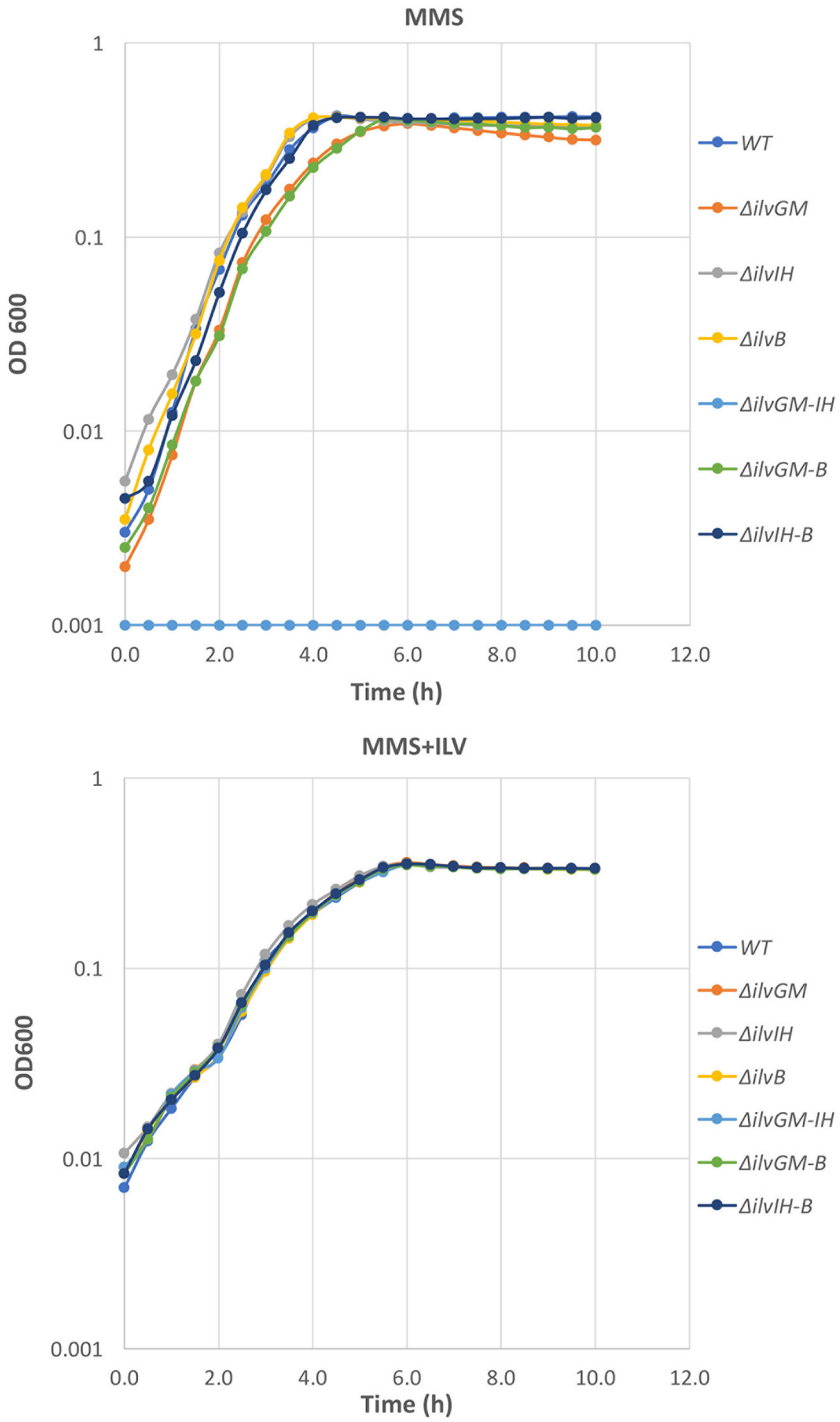

FIG 2 Growth of wild-type *V. alginolyticus* ZJ-T and *ilvGM*, *ilvIH*, and *ilvB* derivative mutants in various combinations. Cells from strains as indicated on the right were grown in MMS (upper panel) and in MMS supplemented with BCAAs (lower panel).

has no homology to *ilvN* but instead has high similarity to *fabG2*, which encodes a 3-oxoacyl-acyl carrier protein (ACP) reductase (Fig. 1A and B). To determine which acetolactate synthases were responsible for BCAA synthesis in *V. alginolyticus* ZJ-T, we constructed three deletion mutants, the Δ*ilvGM*, Δ*ilvIH*, and Δ*ilvB* mutants, as well as their various combination and examined the auxotrophy for ILV in these mutants (Fig. 2). In minimal medium supplemented with ILV, all mutants grew as well as the

wild-type (WT) strain, whereas in the absence of ILV, no growth was observed in the *ilvGM ilvIH* double mutant. Further, deleting only *ilvGM* had a significant effect on growth in the absence of ILV, whereas deleting *ilvIH* had no effect on auxotrophy. Deleting *ilvB* had no effect, either alone or in combination with other mutations. We concluded that IlvGM is the main acetolactate synthase in *V. alginolyticus* and that in its absence, IlvIH takes over, although not as efficiently. Finally, *ilvB* does not contribute to the cell acetolactate synthase activity.

Existence of an IlvL leader peptide encoded upstream of the *ilvGMEDA* operon in *V. alginolyticus*. In *E. coli*, the *ilvIH* operon is regulated at the transcriptional level mostly by the leucine-responsive protein (Lrp) in response to the presence of leucine and is repressed by the transcriptional silencer H-NS. In addition, it is subject to the stringent response mediated by ppGpp in response to amino acid starvation (2, 19, 20). In contrast, the *ilvGMEDA*, *ilvBN*, and *leuABCD* operons are regulated in response to the presence or absence of BCAAs by transcriptional attenuation (6, 8, 21). Transcriptional attenuation is mediated by coupling the translation of a small peptide rich in BCAAs with the formation of an attenuator, leading to transcription termination upstream of the structural genes (reviewed in reference 1). The BCAA-rich leader peptide is encoded between the operon promoter and the first structural gene of the operon. We investigated whether, in *V. alginolyticus*, the *ilvGMEDA* operon could also be regulated by a transcriptional attenuation mechanism. Alignments of the *ilvG* gene upstream region with the corresponding regions in *E. coli* and other *Enterobacteriaceae* showed no conservation at the primary sequence level. However, we identified a small open reading frame (ORF) encoding a putative 29-amino-acid peptide with 13 BCAAs (Fig. 3A), although again no conservation of the amino acid sequence with *E. coli* IlvL could be detected, other than the enrichment in BCAAs. The 5′ and 3′ ends of the putative transcript were determined by 5′ and 3′ rapid amplification of cDNA ends (RACE). In minimal medium with salt (MMS), a single 5′ end was identified 59 nucleotides (nt) upstream of the putative leader peptide start codon (Fig. 3A), as was a unique 3′ end upstream of a putative transcriptional attenuation structure (Fig. 3B). We concluded that this transcript had the potential to encode a BCAA-rich leader peptide, which we called IlvL.

To further localize the promoter of the *ilvLGMEDA* operon, and confirm the functionality of the terminator, we inserted in the multicopy plasmid pSCT32-gfp (Table 1) various DNA fragments from the upstream region of *ilvG* fused to a green fluorescent protein (GFP) reporter gene devoid of its own promoter but retaining 24 nt upstream of the ATG initiation codon, including the ribosome binding site (RBS), to generate transcriptional fusions. Plasmids carrying these fusions were introduced in *E. coli*, and relative fluorescence units (FU) were determined during growth (see Materials and Methods). As can be seen in Fig. 3C, GFP activity was maximal at the onset of growth. The region encompassing 53 nt upstream of the putative transcription start site (TSS) identified by 5′ RACE had a strong promoter activity (Fig. 3C), confirming the presence of the σ^{70} promoter predicted by BPROM (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) in this region. This activity further increased when 94 more-upstream nucleotides were included. Interestingly, this more-upstream region was predicted to contain an Lrp binding site (Fig. 3A). Lrp could contribute to the regulation of the operon at the transcriptional level, since its activity can be modulated by the binding of leucine, and it is known to regulate the *ilvBN* operon in *E. coli* (4).

Including the putative terminator positions −147 to +303 in the fusion led to a strong decrease of the transcription of the downstream GFP gene, confirming the presence of a transcriptional terminating activity upstream of the GFP gene. Deleting the predicted attenuator (*ilvL*Δatt) led to a 2-fold increase of transcription (Fig. 3C). However, the Δatt construct did not reach the GFP expression level observed in the fusion carrying only the promoter region, suggesting that more features in the *ilvL* coding region than just this terminator contribute to transcription attenuation. Finally, we deleted the *ilvL* initiation codon (*ilvL*ΔATG) to investigate the requirement for *ilvL*

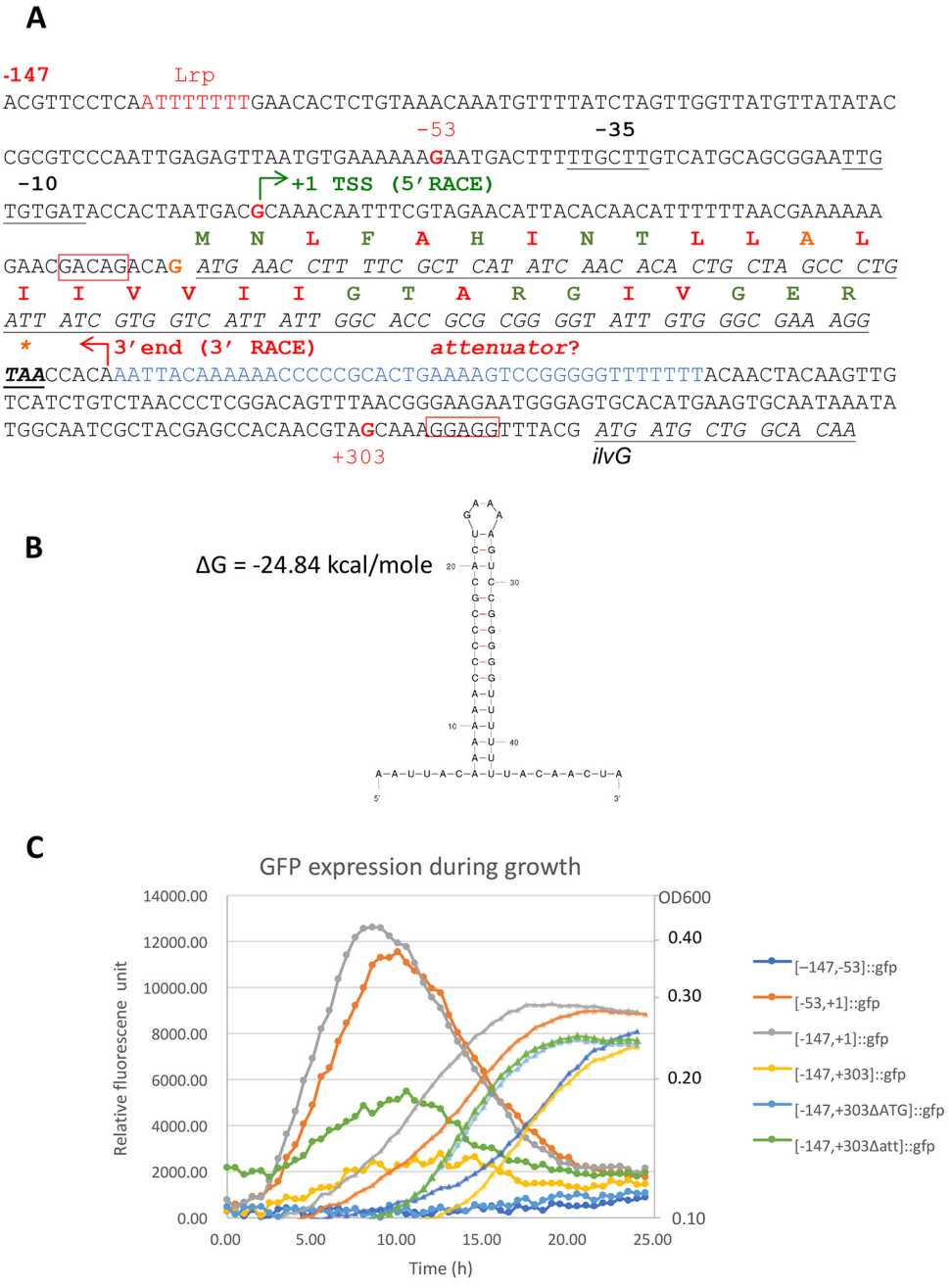

FIG 3 Structure of the *ilvG* upstream region and transcription termination in *V. alginolyticus*. (A) Sequence of the upstream region of the *ilvG* gene. A putative +1 transcription start site (TSS) as identified by 5′RACE is indicated and is numbered +1. The 3′ end of the leader transcript as determined by 3′ RACE is also indicated. The predicted −10 and −35 regions of a sigma 70 promoter are underlined, and an upstream Lrp binding site is indicated in red. The open reading frame for the IlvL leader peptide is underlined, and the peptide sequence is indicated above the nucleotide sequence. BCAAs are in red. The sequence corresponding to the potential transcription terminator/attenuator is in blue. The RBSs for *ilvL* and *ilvG* are boxed in red. The *ilvL* termination codon is underlined, boldface, and italic. (B) Secondary structure of the *ilvL* attenuator as predicted by RNA-fold (rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). (C) DNA fragments of various lengths from the upstream region of *ilvG* (coordinates corresponding to panel A, with +1 the *ilvL* RNA 5′ end) were fused to a promoterless GFP gene retaining its RBS. The resulting plasmids were introduced into *E. coli* BL21, and fluorescence was measured every 15 min during growth in M63. The results shown (primary axis, relative fluorescence units and circles; secondary axis, OD_{600} and triangles) are the means of three biological replicates.

translation and found a total absence of downstream transcription in the *ilvL*ΔATG mutant.

***ilvGMEDA* transcription attenuation is induced by BCAAs.** The results obtained using transcriptional fusions are consistent with a model where the structural *ilv* gene

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
V. alginolyticus strains		
ZJ-T	Ampicillin-resistant, translucent/smooth variant of wild strain ZJ51 (34); isolated from diseased *Epinephelus coioides* off the southern China coast	35
ZJ-T *ilvL*ΔATG	*ilvL*ΔATG	This study
ZJ-T Δ*ilvL*Δatt	*ilvL* with attenuator deleted	This study
ZJ-T Δ*ilvIH*	ZJ-T carrying a deletion of *ilvIH*	This study
ZJ-T Δ*ilvB*	ZJ-T carrying a deletion of *ilvB*	This study
ZJ-T Δ*ilvGM*	ZJ-T carrying a deletion of *ilvGM*	This study
ZJ-T Δ*ilvIH* Δ*ilvB*	ZJ-T carrying deletions of *ilvIH* and *ilvB*	This study
ZJ-T Δ*ilvIH* Δ*ilvGM*	ZJ-T carrying deletions of *ilvIH* and *ilvGM*	This study
ZJ-T Δ*ilvB* Δ*ilvGM*	ZJ-T carrying deletions of *ilvB* and *ilvGM*	This study
E. coli strains		
Π3813	K-12 *lacI*^{q} *thi-1 supE44 endA1 recA1 hsdR17 gyrA462 zei-298*::Tn*10*(Tc) Δ*thyA*::*erm-pir116*(Er); cloning host	30
GEB883	K-12 Δ*dapA*::*erm pir*(Er) RP4-2 Δ*recA gyrA462 zei-298*::Tn*10*(Tc); donor strain for conjugation	29
BL21(DE3)	F^{-} *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3)	Laboratory collection
Plasmids		
pSCT32-[−147, +1]::gfp	Cm^{r}; transcriptional fusion [−147, +1]::*gfp*	This study
pSCT32-[−147, −53]::gfp	Cm^{r}; transcriptional fusion [−147, −53]::*gfp*	This study
pSCT32-[−53, +1]::gfp	Cm^{r}; transcriptional fusion [−53, +1]::*gfp*	This study
pSCT32-[−147, +303]::gfp	Cm^{r}; transcriptional fusion [−147, +303]::*gfp*	This study
pSCT32-[−147, +303]::gfpΔilvLATG	Cm^{r}; transcriptional fusion [−147, +303 *ilvL*ΔATG]::*gfp*	This study
pSCT32-[−147, +303]::gfpΔilvLatt	Cm^{r}; transcriptional fusion [−147, +303 *ilvL*Δatt]::*gfp*	This study
pSCT32-gfp	Cm^{r}; *gfp* fusion vector	This study
pSCT32	Cm^{r}; pBR322 origin	32
pSW7848	Cm^{r}; suicide vector with an R6K origin, requiring the Pir protein for its replication, pBAD-*ccdB*	28
pSW7848-ΔilvLATG	Cm^{r}; pSW7848 carrying the mutant allele *ilvL*ΔATG	This study
pSW7848-ΔilvLatt	Cm^{r}; pSW7848 carrying the mutant allele *ilvL*Δatt	This study
pSW7848-ΔilvIH	Cm^{r}; pSW7848 carrying the *ilvIH* deletion	This study
pSW7848-ΔilvB	Cm^{r}; pSW7848 carrying the *ilvB* deletion	This study
pSW7848-ΔilvGM	Cm^{r}; pSW7848 carrying the *ilvGM* deletion	This study

transcription is regulated by transcription termination, which is itself coupled to *ilvL* translation. To confirm this model, we carried out Northern blot experiments in *V. alginolyticus* itself. Specifically, we tested the effect of adding all three BCAAs or any one of them on transcription of the *ilv* genes at 30 min after the addition (to prevent variation of internal concentration due to the cells synthesizing these amino acids). We also included *ilvI*, coding for the AHAS III large subunit, in our analysis because we wanted to know if, despite playing a minor role in BCAA synthesis, its expression was also repressed by the presence of BCAAs.

Probing *ilvL* showed that the *ilvL* transcript level was not affected by the BCAAs, confirming that the regulation by these amino acids is not at the level of the *ilvGMEDA* promoter. In contrast, *ilvG*, *ilvA*, and *ilvI* transcription was inhibited by the addition of all three BCAAs. In the case of the *ilvGMEDA* operon, valine alone seems to be the most efficient, whereas in the case of the *ilvIH* operon, leucine was the strongest inhibitor (Fig. 4B and C).

In the absence of externally added BCAAs, *V. alginolyticus* produces these amino acids to restore their internal concentration. This will contribute to downregulate expression of the biosynthetic genes even in the absence of ILV in the medium. Upon inactivation of the *ilvGM* genes, encoding the main acetolactate synthase activity, the cells are still able to grow in nonsupplemented minimal medium, thanks to the residual activity of IlvIH, but a reduction of the internal BCAA concentration could be expected. In keeping with this hypothesis, a strong accumulation of the *ilvEDA* mRNA was observed in the Δ*ilvGM* mutant (Fig. 4D). As expected, deleting *ilvGM* did not affect the overall functioning of the regulatory system, since adding ILV to the medium still repressed the expression of *ilvEDA* in the mutant.

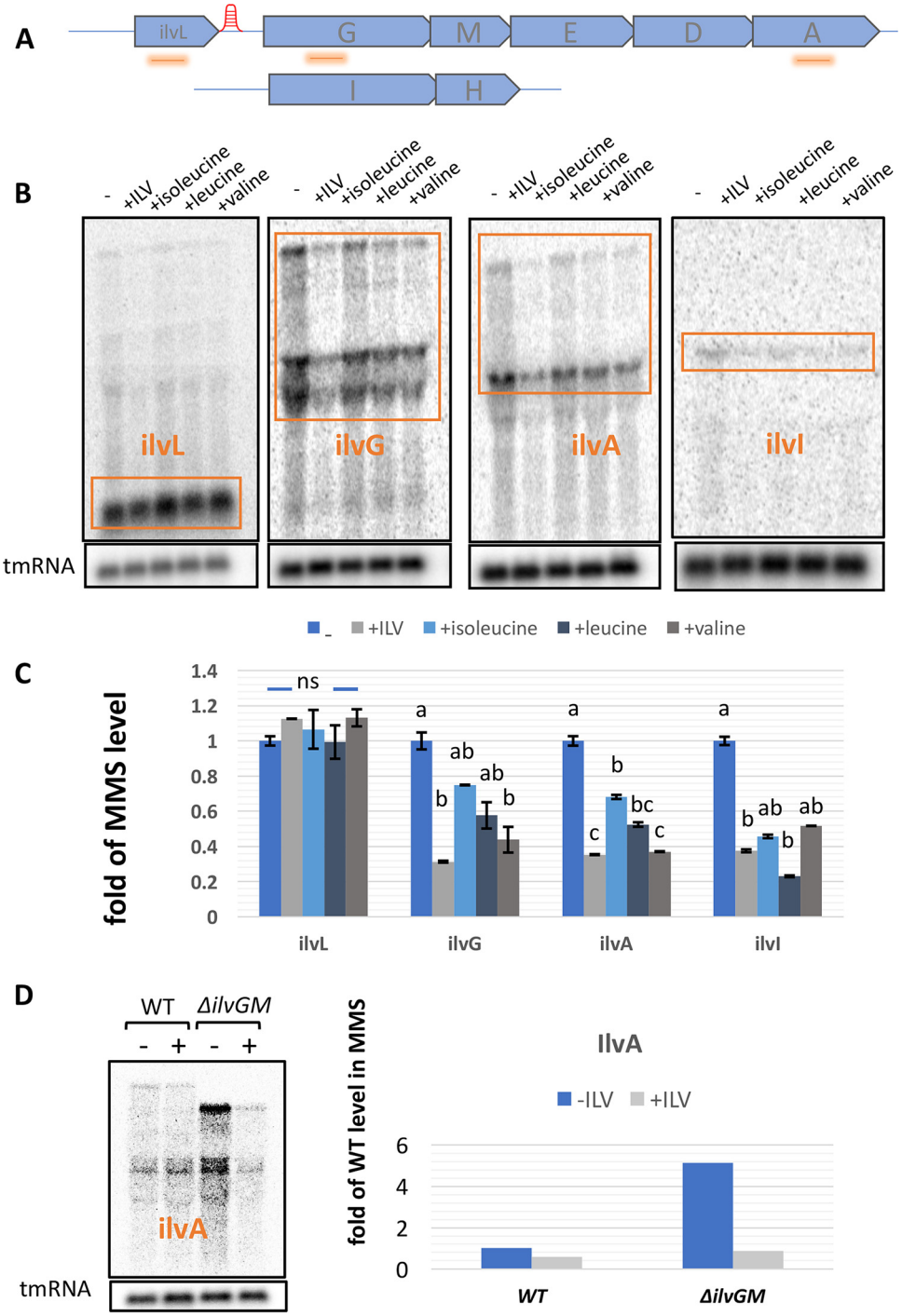

FIG 4 Effect of BCAAs on expression of *ilv* genes. *V. alginolyticus* ZJ-T was grown in MMS to an OD_{600} of ≈0.3 before incubation for 30 min in the same medium supplemented or not supplemented with BCAAs as indicated. Gene expression was then measured by Northern blotting, as described in Materials and Methods. (A) Approximate positions of the probes used. (B) Northern blots. The rectangles indicate the zones in the blot that were used for quantification. tmRNA was used to monitor loading. (C) Quantification of the results in panel B. The signal was first normalized to that of tmRNA, and then for each probe, expression levels were expressed relative to the level observed in the absence of BCAAs. Values represent averages of data from two independent experiments, with error bars corresponding to standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA) followed by pairwise *t* tests. Values considered to be significantly different in the test ($P < 0.05$) are indicated by different letters. (D) Effect of deleting the *ilvGM* genes on expression of the *ilvGMEDA* operon. Left, Northern blots as in panel B, probing for *ilvA*. Right, quantification was as in panel C.

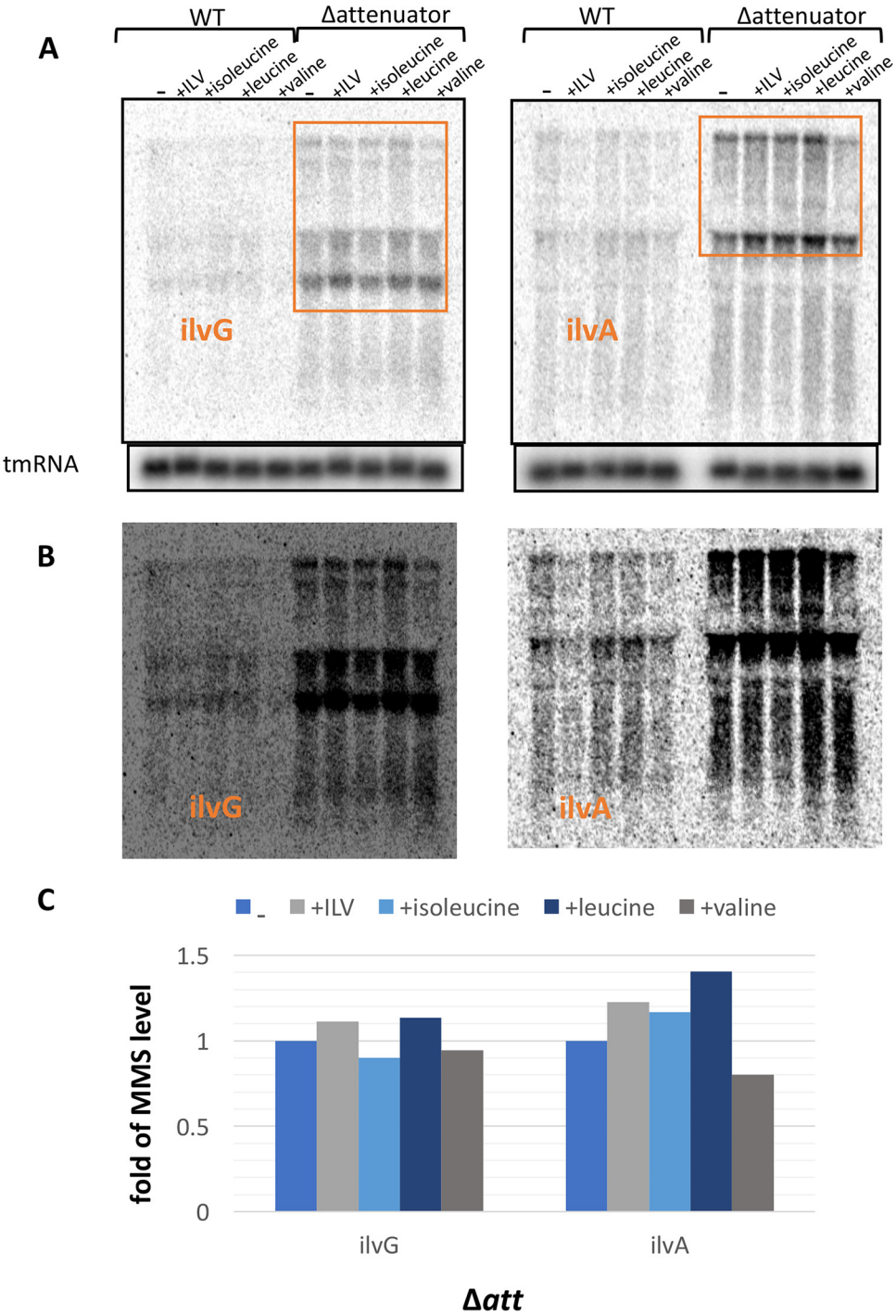

FIG 5 Effect of attenuator deletion on expression of the *ilvG* and *ilvA* genes. (A) Northern blots and their quantification were as in Fig. 4. (B) Overexposure of the blots presented in panel A, showing the effect of adding BCAAs to the medium on the WT strain. (C) Quantification (amounts relative to the level observed in the absence of BCAAs) is shown only for the Δatt strain.

***ilvL* translation mediates a BCAA-dependent inhibition of transcription through transcriptional attenuation.** We questioned the functionality of the *ilvL* transcription attenuator and the requirement of *ilvL* translation for regulation in *V. alginolyticus*. The chromosomal endogenous *ilvL* locus was replaced by the *ilvL*Δatt and the *ilvL*ΔATG mutations described above, and the expression of the *ilvGMEDA* operon in these mutants was monitored (Fig. 5A and B).

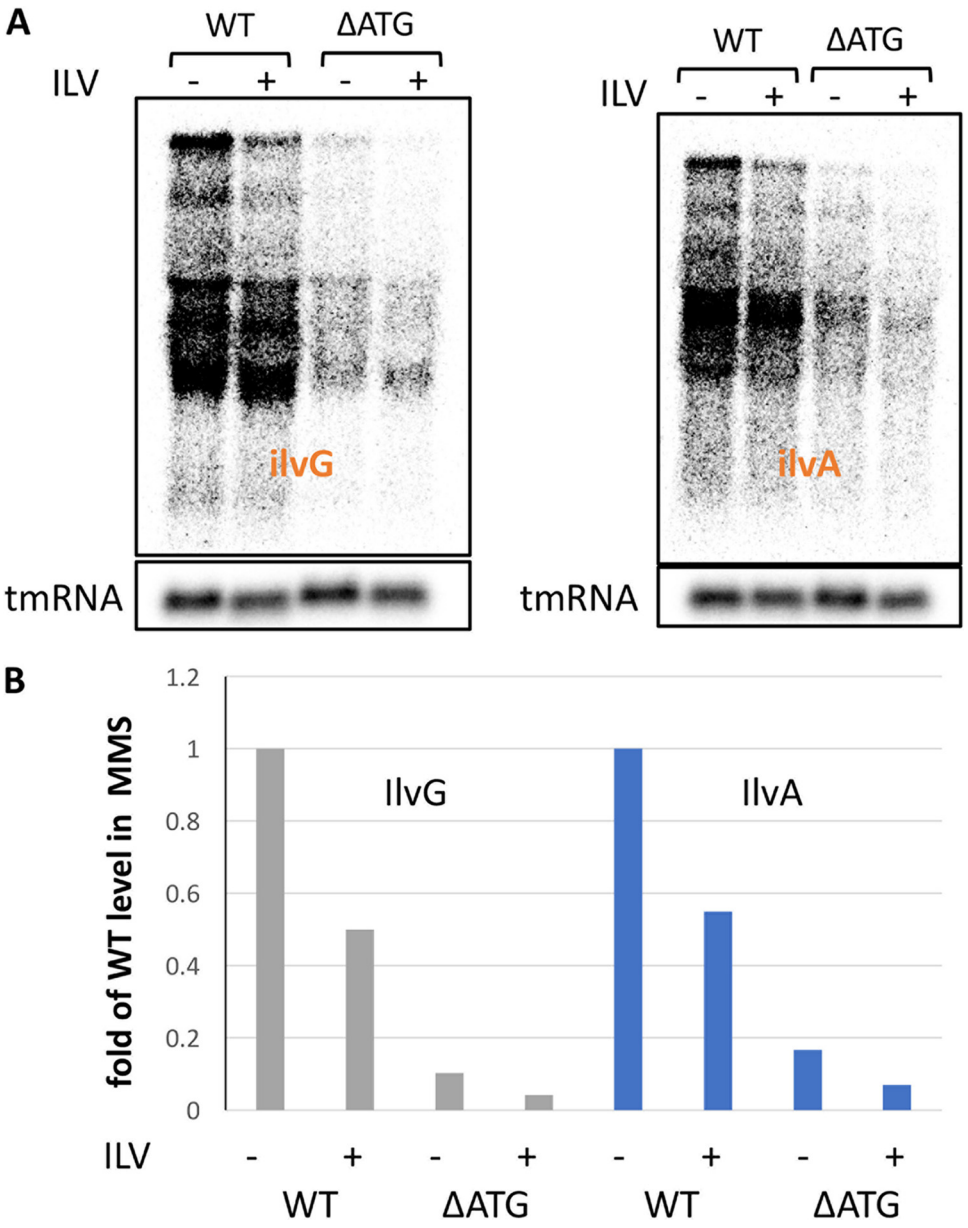

FIG 6 IlvL translation is required for the transcription of the *ilvG* and *ilvA* genes. (A) Northern blots in the absence (−) or presence (+) of all three BCAAs of *ilvG* (left panel) or *ilvA* (right panel) in the WT strain and the *ilvL*ΔATG mutant. (B) Quantification of Northern blots in panel A. Expression is expressed as amounts relative to the level observed in the WT strain in the absence of BCAAs (−ILV). Gray bars, IlvG probe; blue bars, IlvA probe.

First, the *ilvG* and *ilvA* transcript levels clearly showed a very strong increase of expression when the attenuator sequence had been removed. In addition, quantification of the transcripts relative to the level in the absence of any BCAAs showed that the presence of BCAAs in the environment could not repress *ilvGMEDA* expression in the absence of the attenuator (Fig. 5C), leading to the conclusion that its role is to block the downstream *ilv* gene expression in the presence of BCAAs.

In contrast, when the initiation codon ATG was deleted from the *ilvL* ORF, a very strong reduction of either *ilvG* or *ilvA* was observed even in the absence of BCAAs (Fig. 6), indicating that translation of *ilvL* is essential to overcome the action of the transcription terminator. However, in the absence of translation of the leader peptide (*ilvL*ΔATG strain), addition of ILV still had an inhibitory effect, suggesting that BCAAs could also control the expression of the *ilvGMEDA* through a mechanism other than the

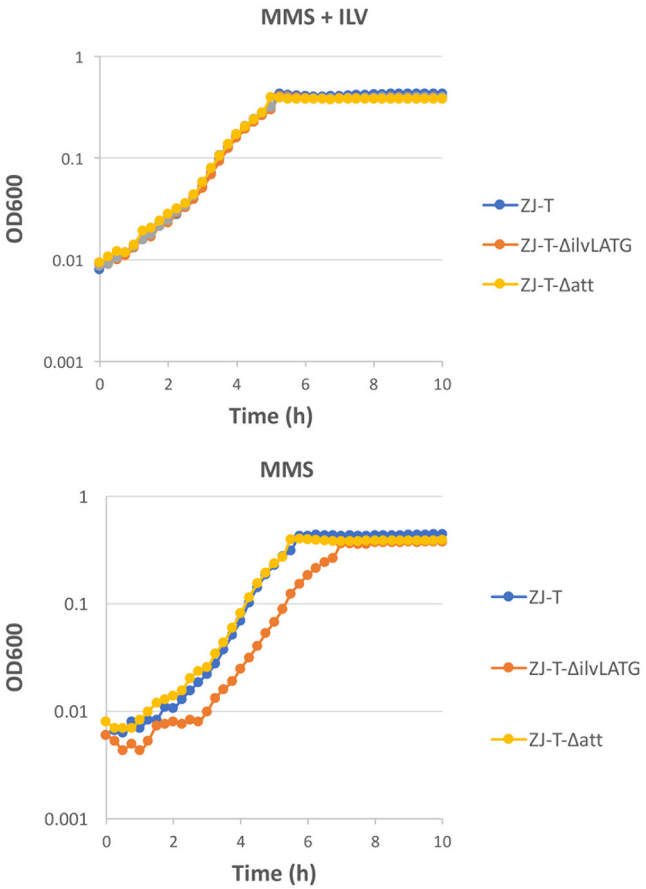

FIG 7 IlvL translation is required for optimal biosynthesis of BCAAs. Cells from various mutant strains as indicated on the right were grown in MMS (lower panel) and MMS supplemented with BCAAs (upper panel).

modulation of transcription termination through *ilvL* translation. One way could have been via an effect at the transcriptional level, for instance, mediated by Lrp. However, this seems to be excluded by the absence of an effect on *ilvL* transcription of adding BCAAs (Fig. 4B and C). Alternatively, posttranscriptional regulation could be indirect, through the action of an sRNA, for instance. To our best of knowledge, no sRNA that regulates the biosynthesis of branched-chain amino acids has been described. This was not explored further.

We also determined the effect of these mutations on BCAA auxotrophy. Deletion of the attenuator (Fig. 7, *ilvL*Δatt mutant) did not impair the ability of the strain to grow in the absence of BCAAs, as was expected since in this strain the expression of *ilvGMEDA* is constitutive. In contrast, the *ilvL*ΔATG mutant showed a significant decrease of growth rate in the absence of these amino acids, reflecting the decreased biosynthetic activity of the strain due to increased transcription termination in the absence of *ilvL* translation.

Conservation of the mechanism of transcription attenuation between *Enterobacteriaceae* and *Vibrionaceae*. In Gram-negative bacteria, transcriptional attenuation has been studied mostly in *E. coli* and *Salmonella* spp. (22), *Enterobacteriaceae* belonging to the class *Gammaproteobacteria*. A genomic study of proteobacteria predicted the presence of an attenuator in front of the *leu* and the *ilvGMEDA* operons in vibrios, suggesting a conserved mechanism of regulation of BCAA biosynthesis between *Vibrionaceae* and *Enterobacteriaceae* (14). As stated above, the 5′ untranslated region of the *ilvGMEDA* operon in *V. alginolyticus* does not show any primary sequence similarity to the analogous region in *E. coli*, whereas in the *ilvL* ORF, only the BCAA codons are

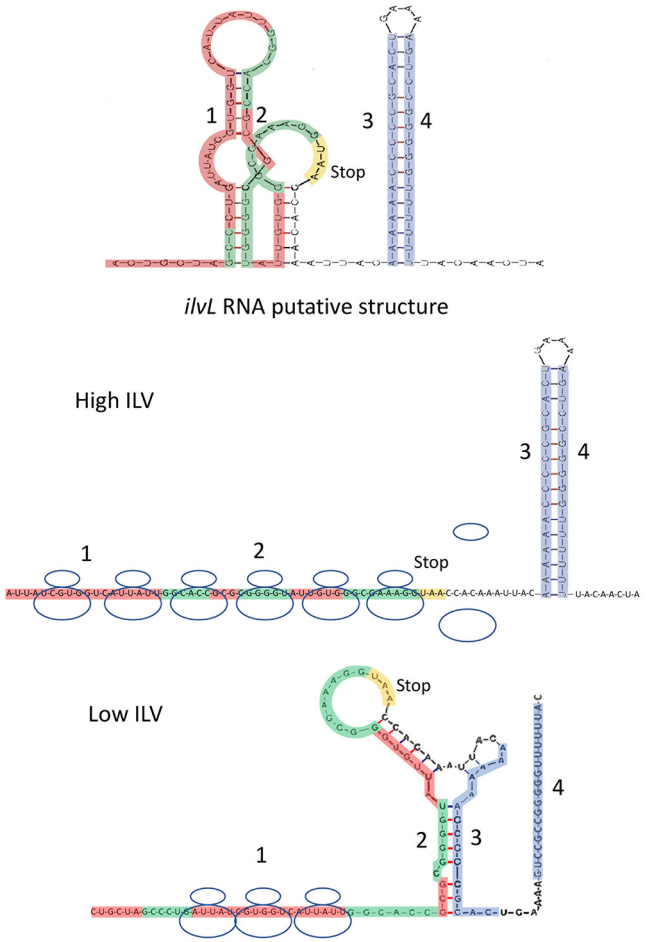

FIG 8 A model for transcription attenuation of the *ilvGMEDA* operon in *V. alginolyticus*. (Top) The *ilvL* RNA putative secondary structure was calculated using Mfold (version 2.3; http://unafold.rna.albany.edu/?q=mfold) (23), setting up the folding temperature at 30°C. The *ilvL* ORF is highlighted: red, BCAA codons; green, other codons; yellow, stop codon. The putative attenuator is highlighted in blue. Regions involved in secondary structures are numbered 1 to 4. (Middle) In the presence of a high BCAA (ILV) concentration, ribosomes can proceed with translation until the stop codon, where they are released. Region 2 is translated, allowing regions 3 and 4 to hybridize and form the attenuator. The downstream genes are not transcribed. (Bottom) When the BCAA concentration is low, ribosomes reach the BCAA codon-rich region and cannot proceed because of a lack of charged tRNAs. Region 2 is now free to hybridize with region 3, forming an alternative antitermination structure and preventing the formation of the terminator. See the text for more details on this model.

conserved. However, when analyzing the putative secondary structure of the *ilvL* transcript by using Mfold (23), we could identify all the features that have been described for the canonical *E. coli trp* leader transcript (Fig. 8) (reviewed in reference 22). A first secondary structure (1:2) can form after the RNA polymerase has initiated transcription, which acts as a pause site for transcription. This pause in transcription allows loading of the ribosome, which starts to translate the *ilvL* ORF and disrupts the pause site. Transcription resumes, with translation coupled to transcription. In the presence of sufficient amounts of Ile, Val, and Leu, translation can proceed to the stop codon, where the ribosome is released. In this case, a Rho-independent terminator (structure 3:4) can form, preventing transcription of the downstream genes. In contrast, in the presence of small amounts of Ile, Val, or Leu, ribosomes cannot proceed efficiently with the peptide translation due to the lack of charged tRNAs, and they remain stuck on the ILV codon-rich regions. This prevents the formation of a transcription pause site and allows the base pairing of RNA sequence 2 with RNA sequence 3, forming an alternative anti-transcription termination structure. Ribosome stalling, by

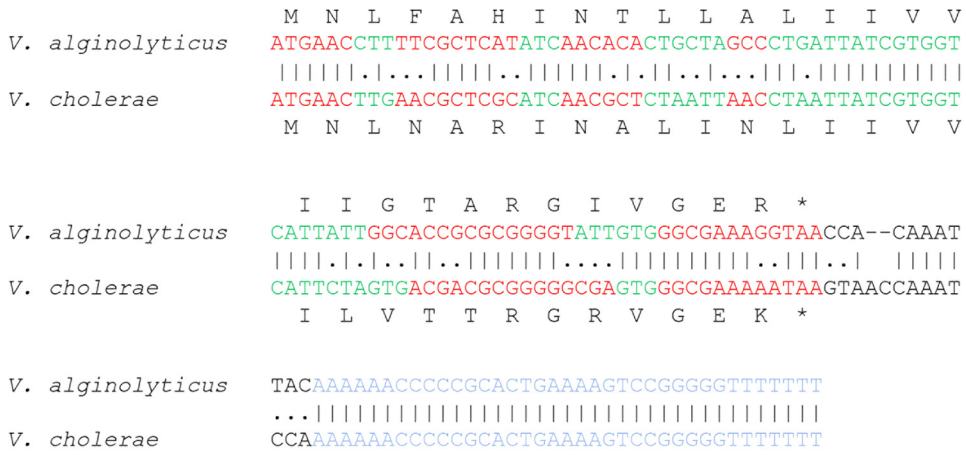

FIG 9 Structural conservation of the *ilvG* upstream region in *V. alginolyticus* ZJ-T and *V. cholerae*. The upstream regions of *ilvG* from *V. alginolyticus* ZJ-T and *V. cholerae* N16961 were aligned using EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). The BCAA codons are in green, other codons are in red, and the putative attenuator structure is in blue.

preventing the formation of a terminator, allows the RNA polymerase to transcribe the *ilvGMEDA* genes.

How well is such a mechanism conserved in the *Vibrio* genus? A blastn search using the *V. alginolyticus ilvL* mRNA sequence as a query did not retrieve similar sequences beyond the *Vibrio harveyi* clade, to which *V. alginolyticus* belongs (24). In particular, no hits were obtained in *Vibrio cholerae* genomes. However, the alignment of the upstream region of *ilvG* in the two species showed a strong conservation of features characteristic of transcription attenuation, i.e., the presence of an ORF rich in codons for BCAAs followed by a highly conserved attenuator structure, suggesting that all vibrios share this mechanism to regulate BCAA biosynthesis (Fig. 9).

Conclusion. Transcription attenuation is thought to be a widely used mechanism for gene regulation in bacteria. In Gram-negative bacteria, transcription attenuation of amino acid biosynthetic operons has been experimentally studied mostly in enteric bacteria such as *E. coli* and *Salmonella* spp. (22), and so far, to our best of knowledge, no experimental studies for other proteobacterial species have been reported. More recent studies focused on Gram-positive bacteria such as *B. subtilis* and *S. aureus* have shown that although transcription attenuation does exist, global transcriptional regulators such as CodY and TnrA play an essential role (13, 25, 26). In the case of regulation of the ILV genes, genomic studies have predicted the existence of attenuators linked to leader peptides in gamma-, alpha-, and betaproteobacteria as well as in some low-GC Gram-positive bacteria (i.e., *B. subtilis* and *S. aureus*) (14). In the gammaproteobacteria, attenuators were predicted in front of the BCAA operons *ilvGMEDA* and *leuABCD* in *Enterobacteriaceae* (with a few exceptions for *ilvGMEDA*) and in the three vibrios that were examined, suggesting that a similar mechanism was operating in the *Vibrio* genus (14). Our results confirm experimentally that this is indeed the case, illustrating a high degree of structural and mechanistic conservation beyond the lack of primary sequence conservation.

MATERIALS AND METHODS

Growth of bacterial strains. *V. alginolyticus* ZJ-T, a translucent variant of ZJ51 (27), was used in this study in preference to the original strain because its genome has been fully sequenced (accession numbers CP016224 and CP016225). ZJ-T was cultured in Luria-Bertani (LB) medium with 3% NaCl (LBS) at 30°C, with chloramphenicol (Cm) supplementation (5 μg · ml^{-1}) if needed. *E. coli* strains were cultured in LB medium at 37°C or in M63 medium [3 g · liter^{-1} KH_2PO_4, 7 g · liter^{-1} K_2HPO_4, 2 g · liter^{-1} $(NH_4)_2SO_4$, 0.5×10^{-3} g · liter^{-1} $FeSO_4$, 2×10^{-3} M $MgSO_4$] containing 10 g · liter^{-1} NaCl and supplemented with 4 g · liter^{-1} D-glucose and 5×10^{-3} g · liter^{-1} thiamine. If needed, Cm was added at 20 μg · ml^{-1}. For transconjugant selection, TCBS medium (Becton, Dickinson, USA) plus Cm (5 μg · ml^{-1}) was used. To select transconjugants having undergone plasmid excision and allelic exchange, expression of the *ccdB*

TABLE 2 Primers and probes used in this study

Function and name	Sequence (5′→3′)[a]	Target or goal
Northern blot probes		
NB-*ilvL*	AATGACCACGATAATCAGGGCTAGCAG	*ilvL*
NB-*ilvG*	GGTACCGAGGCCTTTTAATGTGCTAAC	*ilvG*
NB-*ilvA*	CTTGATGCCTAGCTTAGTACCAGAG	*ilvA*
NB-*ilvI*	CCGAAATAACGATCATTGGGATGG	*ilvI*
NB-tmRNA	GGAAGCTAGGGCGAGAGAGCTCTTAG	tmRNA
5′ and 3′ RACE		
RACE-UPML	TAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE product cloning
RACE-UPMS	CTAATACGACTCACTATAGGGC	RACE product cloning
RACE-ilvL-F	gattacgccaagcttACAGACAGATGAACCTTTTCGCTC	*ilvL* transcript
RACE-ilvL-R	gattacgccaagcttGCGCGGTGCCAATAATGACCA	*ilvL* transcript
RACE-ilvG-R	gattacgccaagcttGGGTAGCCAAAAACGGTTTCG	*ilvG* transcript
GFP transcriptional fusion		
pSCT32-GFP-TR-F	AGAATTAAAGAGGAGAAATTAAGCATGC	GFP gene
pSCT32-GFP-TR-R	AGATAGATCTTGCATGCGGG	GFP gene
[−147, +1]::GFP-TR-F	gcatgcaagatctatctACGTTCCTCAATTTTTTTGAACACTC	[−147, +1]
[−147, +1]::GFP-TR-R	atttctcctctttaattctCGTCATTAGTGGTATCACACAATT	[−147, +1]
[−147, −53]::GFP-TR-F	gcatgcaagatctatctACGTTCCTCAATTTTTTTGAACAC	[−147, +53]
[−147, −53]::GFP-TR-R	atttctcctctttaattctTTTTTCACATTAACTCTCAATTGGGA	[−147, +53]
[−53, +1]::GFP-TR-F	gcatgcaagatctatctAAGAATGACTTTTTGCTTGTCATG	[−53, +1]
[−53, +1]::GFP-TR-R	atttctcctctttaattctCGTCATTAGTGGTATCACACAATT	[−53, +1]
[−147, +303]::GFP-TR-F	gcatgcaagatctatctACGTTCCTCAATTTTTTTGAACACTC	[−147, +303], WT, *ilvL*ΔATG, *ilvL*Δatt
[−147, +303]::GFP-TR-R	atttctcctctttaattctCTACGTTGTGGCTCGTAGCG	[−147,+303], WT, *ilvL*ΔATG, *ilvL*Δatt
pSCT32::GFP-check-F	TGATGCCGCATAGTTAAGCCAG	Verification
pSCT32::GFP-check-R	CTTGGAGCCGTACTGGAACTGA	Verification
Mutagenesis		
pSW7848-F	GTCTGATTCGTTACCAATTATGACAAC	Vector
pSW7848-R	GAATTCGATATCAAGCTTATCGATAC	Vector
Del-check-pSW7848-F	TCACTGTCCCTTATTCGCACC	Verification
Del-check-pSW7848-R	CTGCTTTTGAGCACTACCCG	Verification
ilvLATG-UP-F	aagcttgatatcgaattcTGTTAGATAGTCTAGAGTGATGGG	*ilvL*ΔATG
ilvLATG-UP-R	gcgaaaaggttCTGTCTGTCGTTCTTTTTTC	*ilvL*ΔATG
ilvLATG-DOWN-F	acagacagAACCTTTTCGCTCATATCAACAC	*ilvL*ΔATG
ilvLATG-DOWN-R	ttggtaacgaatcagacGGTGCCATGCATACCCAAC	*ilvL*ΔATG
ilvLatt-UP-F	aagcttgatatcgaattcGAACTCATTAAGATTAGTGTTCAATATTG	*ilvL*Δatt
ilvLatt-UP-R	aacttgtagTTTGTGGTTACCTTTCGCCC	*ilvL*Δatt
ilvLatt-DOWN-F	aaccacaaaCTACAAGTTGTCATCTGTCTAACC	*ilvL*Δatt
ilvLatt-DOWN-R	ttggtaacgaatcagacGTGGCGCAGTTTATGAATCTC	*ilvL*Δatt
ilvB-UP_fwd	aagcttgatatcgaattcGCCATCGCTCGGGTAAGG	Δ*ilvB*
ilvB-UP_rev	tcaatgaattTACTTAAGCTGTTTTCGAGCTCG	Δ*ilvB*
ilvB-DOWN_fwd	gcttaagtaAATTCATTGAACGTACCTAAACCAAAATCCC	Δ*ilvB*
ilvB-DOWN_rev	ttggtaacgaatcagacGCGCCATGGCAATCGTCG	Δ*ilvB*
ilvIH-UP_fwd	aagcttgatatcgaattcTGCGTGGCTATTACAAAATG	Δ*ilvIH*
ilvIH-UP_rev	gcagtgacAATTGCTCTCCTTAGCCAG	Δ*ilvIH*
ilvIH-DOWN_fwd	agagcaattGTCACTGCTGCTCAATACACG	Δ*ilvIH*
ilvIH-DOWN_rev	ttggtaacgaatcagacGCCAAATGTTGGGGTACATC	Δ*ilvIH*
ilvGM-UP_fwd	aagcttgatatcgaattcGCGTTTGAAAAAGATTCAG	Δ*ilvGM*
ilvGM-UP_rev	ctactttgcCGTAAACCTCCTTTGCTAC	Δ*ilvGM*
ilvGM-DOWN_fwd	ggtttacgGCAAAGTAGCCAGCGTCG	Δ*ilvGM*
ilvGM-DOWN_rev	ttggtaacgaatcagacCCAGTGCCCACTTCAATC	Δ*ilvGM*
del ilvLATG-check-F	GTTATGTTATATACCGCGTCCC	Verification
del ilvLATG-check-R	GACAGATGACAACTTGTAGTTGT	Verification
del ilvLatt check-F	CGCAAACAATTTCGTAGAACATTAC	Verification
del ilvLatt check-R	GACAAGTTGTGCACCAGTCAT	Verification
del-ilvB-check-F	GTTATCAACCCACACAATATGGAC	Verification
del-ilvB-check-R	CTCTTCATTGCATAACTGGTCTTAC	Verification
del-ilvIH-check-F	GTGGCTGCTATTCCTACTAAC	Verification
del-ilvIH-check-R	CGTCTACTGCCTATAATGCTTAC	Verification
del-ilvGM-check-F	GTATTGTGGGCGAAAGGTAAC	Verification
del-ilvGM-check-R	CTTCCTTATGCACTTACGTGTT	Verification

[a]Nontarget bases complementary to the cloning vector are indicated by lowercase letters.

toxin-encoding gene carried by the pSW7848 suicide plasmid (28) was induced by adding 0.2% L-arabinose to the medium. Minimal medium with salt (MMS), used for *V. alginolyticus*, is an M63-based medium containing 30 g · liter^{-1} NaCl and supplemented with 4 g · liter^{-1} D-glucose and 5×10^{-3} g · liter^{-1} thiamine. When indicated, BCAAs were added at a final concentration of 20 mM each. Bacterial strains used in this study are listed in Table 1.

Measurement of bacterial growth. *V. alginolyticus* strains were grown overnight in LBS medium at 30°C with shaking at 200 rpm. Overnight cultures were collected by centrifugation, washed twice with MMS, and resuspended in the same medium to an optical density at 600 nm (OD_{600}) of ≈0.05. Cultures (3 replicates in each case) were then incubated in a microplate reader (EnSpire, Australia) at 30°C with shaking at 200 rpm in 96-well plates. The OD_{600} was measured at regular time intervals as indicated.

5′ and 3′ RACE. For 5′ and 3′ rapid amplification of cDNA ends (RACE), an overnight culture of strain ZJ-T in LBS was washed twice with MMS before being resuspended at an OD_{600} of ≈0.01 in either MMS or MMS plus ILV. The bacterial cells were collected after 3 h, 6 h, and 9 h of growth at 30°C. Total RNA was isolated using the RNAprep Pure Cell/Bacteria kit (Tiangen, China), and for each culture, RNA preparations from the different time points were pooled. 5′ and 3′ RACE experiments were then carried out using the SMARTer RACE 5′/3′ kit (Clontech, USA) according to the manufacturer's instructions. At least 12 resulting clones were sequenced to determine the ends of the *ilvL* and *ilvG* transcripts.

Plasmid construction and gene disruption. *V. alginolyticus* ZJ-T in-frame deletion mutants were constructed as described previously (29). Briefly, amplified linearized pSW7848 plasmid DNA (28) was assembled by isothermal assembly using the ClonExpress MultiS one-step cloning kit (Vazyme, China) with purified PCR products corresponding to the flanking regions of the desired deletion and were introduced into *E. coli* Π3813 (30) as an intermediate cloning strain, selecting recombinant cells on 20 μg/ml Cm and 0.3 mM thymidine. The recombinant plasmid was then introduced into the donor strain GEB883 (29) (Table 1) for transfer by conjugation to *V. alginolyticus* ZJ-T. pSW7848 requires the Pir^+ protein for its replication, which is present in the donor strain but absent in vibrios where the suicide plasmid inserts at the targeted locus by homologous recombination, generating a duplication. Plating out the exconjugants on arabinose induces expression of the *ccdB* gene present on pSW7848 and selects for bacteria that have lost the inserted plasmid and one copy of the targeted locus. The presence of the mutation was then confirmed by PCR amplification and sequencing.

Transcriptional fusions to GFP were constructed as described above by assembling the region to be assayed for promoter activity with the GFP gene amplified from pJBA113 (31) and a plasmid template derived from pSCT32 (32). The GFP encoded by pJBA113 has a half-life of approximately 110 min in *E. coli* (31). All plasmids are described in Table 1. All oligonucleotides used in this study are listed in Table 2.

GFP measurement. *E. coli* BL21 was transformed by the plasmids containing the GFP fusions to be assayed, and GFP activity was measured as follows. Cells from overnight cultures in LB supplemented with Cm were washed twice in M63 medium containing 10 g · liter^{-1} NaCl and D-glucose as a carbon source and resuspended to an OD_{600} of ≈0.05 in the same medium with 20 μg · ml^{-1} Cm. The diluted cultures were added to 96-well plates (100 μl/well) and incubated in a microplate reader (EnSpire, Australia) at 37°C with shaking. The OD_{600} and GFP fluorescence (FU) (excitation, 485 nm; emission, 535 nm) were monitored during growth. Background fluorescence measured with strain BL21/pSCT32-gfp (with no insert upstream of the promoterless GFP gene) was deducted from the measured FU. Relative fluorescence units (RFU) (FU/OD_{600} unit) were then calculated for each time point.

Northern blotting. *V. alginolyticus* WT and mutant cells were cultured overnight in MMS, diluted to an OD_{600} of ≈0.05 in the same medium, and grown at 30°C with continuous shaking at 180 rpm. When the OD_{600} reached 0.3, cells were centrifuged and resuspended at the same OD_{600} in MMS supplemented or not supplemented, as required, with all three BCAAs or any one of them as indicated. After 30 min of further incubation, bacterial cells were collected for total RNA extraction, which was carried out using DIrect-zol MiniPrep Plus (Zymo Research). After DNase treatment, 10 μg of purified RNA was used for Northern blotting as described previously (33). Oligonucleotide probes complementary to the gene to be assayed (Table 2) were labeled at their 3′ ends using terminal transferase (Fermentas, USA) and [α-^{32}P]dCTP. RiboRuler low- and high-range RNA ladders (Fermentas, USA) were run alongside the samples to allow an estimation of the transcript size. Transfer-messenger RNA (tmRNA) was used as a loading control. Transcripts were quantified by using ImageJ.

ACKNOWLEDGMENTS

This work was supported by grants from the Central Public-Interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute, CAFS (2017YB27), the Guangzhou Science and Technology Project (201803020018), and the National Natural Science Foundation of China (NSFC) (31872605). Y.D. benefited from a 1-year internship at the Institute for Integrative Biology of the Cell as part of an agreement between CNRS and the Chinese Academy of Science for a joint research training program. This work was also supported in part by funding from the ANR to A.J. (Vibriogen, ANR-11-BSV7-0023; Decipher, ANR-14-CE19-0023).

REFERENCES

1. Salmon K, Yang C, Hatfield G. 28 February 2006, posting date. Biosynthesis and regulation of the branched-chain amino acids. EcoSal Plus 2016 https://doi.org/10.1128/ecosalplus.3.6.1.5.
2. Willins DA, Calvo JM. 1992. In vitro transcription from the *Escherichia coli ilvIH* promoter. J Bacteriol 174:7648–7655. https://doi.org/10.1128/jb.174.23.7648-7655.1992.
3. Wek RC, Hatfield GW. 1986. Nucleotide sequence and in vivo expression of the *ilvY* and *ilvC* genes in *Escherichia coli* K12. Transcription from divergent overlapping promoters. J Biol Chem 261:2441–2450.
4. Newman EB, Lin R. 1995. Leucine-responsive regulatory protein: a global regulator of gene expression in *E. coli*. Annu Rev Microbiol 49:747–775. https://doi.org/10.1146/annurev.mi.49.100195.003531.
5. Searles LL, Wessler SR, Calvo JM. 1983. Transcription attenuation is the major mechanism by which the *leu* operon of *Salmonella typhimurium* is controlled. J Mol Biol 163:377–394. https://doi.org/10.1016/0022-2836(83)90064-5.
6. Wessler SR, Calvo JM. 1981. Control of *leu* operon expression in *Escherichia coli* by a transcription attenuation mechanism. J Mol Biol 149:579–597. https://doi.org/10.1016/0022-2836(81)90348-x.
7. Hauser CA, Hatfield GW. 1983. Nucleotide sequence of the *ilvB* multivalent attenuator region of *Escherichia coli* K12. Nucleic Acids Res 11:127–139. https://doi.org/10.1093/nar/11.1.127.
8. Lawther RP, Wek RC, Lopes JM, Pereira R, Taillon BE, Hatfield GW. 1987. The complete nucleotide sequence of the *ilvGMEDA* operon of *Escherichia coli* K-12. Nucleic Acids Res 15:2137–2155. https://doi.org/10.1093/nar/15.5.2137.
9. Hauser CA, Hatfield GW. 1984. Attenuation of the *ilvB* operon by amino acids reflecting substrates or products of the *ilvB* gene product. Proc Natl Acad Sci U S A 81:76–79. https://doi.org/10.1073/pnas.81.1.76.
10. Nargang FE, Subrahmanyam CS, Umbarger HE. 1980. Nucleotide sequence of *ilvGEDA* operon attenuator region of *Escherichia coli*. Proc Natl Acad Sci U S A 77:1823–1827. https://doi.org/10.1073/pnas.77.4.1823.
11. Brinsmade SR. 2017. CodY, a master integrator of metabolism and virulence in Gram-positive bacteria. Curr Genet 63:417–425. https://doi.org/10.1007/s00294-016-0656-5.
12. Guedon E, Serror P, Ehrlich SD, Renault P, Delorme C. 2001. Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis*. Mol Microbiol 40:1227–1239. https://doi.org/10.1046/j.1365-2958.2001.02470.x.
13. Kaiser JC, King AN, Grigg JC, Sheldon JR, Edgell DR, Murphy MEP, Brinsmade SR, Heinrichs DE. 2018. Repression of branched-chain amino acid synthesis in *Staphylococcus aureus* is mediated by isoleucine via CodY, and by a leucine-rich attenuator peptide. PLoS Genet 14:e1007159. https://doi.org/10.1371/journal.pgen.1007159.
14. Vitreschak AG, Lyubetskaya EV, Shirshin MA, Gelfand MS, Lyubetsky VA. 2004. Attenuation regulation of amino acid biosynthetic operons in proteobacteria: comparative genomics analysis. FEMS Microbiol Lett 234:357–370. https://doi.org/10.1016/j.femsle.2004.04.005.
15. Lintner RE, Mishra PK, Srivastava P, Martinez-Vaz BM, Khodursky AB, Blumenthal RM. 2008. Limited functional conservation of a global regulator among related bacterial genera: Lrp in *Escherichia*, *Proteus* and *Vibrio*. BMC Microbiol 8:60. https://doi.org/10.1186/1471-2180-8-60.
16. Citil BE, Derin S, Sankur F, Sahan M, Citil MU. 2015. *Vibrio alginolyticus* associated chronic cyringitis acquired in Mediterranean waters of Turkey. Case Rep Infect Dis 2015:187212. https://doi.org/10.1155/2015/187212.
17. Siddiqui R, Alam MM, Naser MN, Otomo Y, Yasmin M, Nessa J, Ahsan CR. 2016. Prevalence of *Vibrio alginolyticus* in sediment samples of river and coastal areas of Bangladesh. Bangla J Microbiol 29:1–6. https://doi.org/10.3329/bjm.v29i1.28421.
18. Wek RC, Hauser CA, Hatfield GW. 1985. The nucleotide sequence of the *ilvBN* operon of *Escherichia coli*: sequence homologies of the acetohydroxy acid synthase isozymes. Nucleic Acids Res 13:3995–4010. https://doi.org/10.1093/nar/13.11.3995.
19. Baccigalupi L, Marasco R, Ricca E, De Felice M, Sacco M. 1995. Control of *ilvIH* transcription during amino acid downshift in stringent and relaxed strains of *Escherichia coli*. FEMS Microbiol Lett 131:95–98. https://doi.org/10.1111/j.1574-6968.1995.tb07760.x.
20. Levinthal M, Lejeune P, Danchin A. 1994. The H-NS protein modulates the activation of the *ilvIH* operon of *Escherichia coli* K12 by Lrp, the leucine regulatory protein. Mol Gen Genet 242:736–743.
21. Friden P, Newman T, Freundlich M. 1982. Nucleotide sequence of the *ilvB* promoter-regulatory region: a biosynthetic operon controlled by attenuation and cyclic AMP. Proc Natl Acad Sci U S A 79:6156–6160. https://doi.org/10.1073/pnas.79.20.6156.
22. Gollnick P, Babitzke P. 2002. Transcription attenuation. Biochim Biophys Acta 1577:240–250. https://doi.org/10.1016/s0167-4781(02)00455-4.
23. Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406–3415. https://doi.org/10.1093/nar/gkg595.
24. Sawabe T, Ogura Y, Matsumura Y, Feng G, Amin AR, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T. 2013. Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. Front Microbiol 4:414. https://doi.org/10.3389/fmicb.2013.00414.
25. Tojo S, Satomura T, Morisaki K, Deutscher J, Hirooka K, Fujita Y. 2005. Elaborate transcription regulation of the *Bacillus subtilis ilv-leu* operon involved in the biosynthesis of branched-chain amino acids through global regulators of CcpA, CodY and TnrA. Mol Microbiol 56:1560–1573. https://doi.org/10.1111/j.1365-2958.2005.04635.x.
26. Tojo S, Satomura T, Morisaki K, Yoshida K-I, Hirooka K, Fujita Y. 2004. Negative transcriptional regulation of the *ilv-leu* operon for biosynthesis of branched-chain amino acids through the *Bacillus subtilis* global regulator TnrA. J Bacteriol 186:7971–7979. https://doi.org/10.1128/JB.186.23.7971-7979.2004.
27. Deng Y, Chen C, Zhao Z, Huang X, Yang Y, Ding X. 2016. Complete genome sequence of *Vibrio alginolyticus* ZJ-T. Genome Announc 4:e00912-16. https://doi.org/10.1128/genomeA.00912-16.
28. Val ME, Skovgaard O, Ducos-Galand M, Bland MJ, Mazel D. 2012. Genome engineering in *Vibrio cholerae:* a feasible approach to address biological issues. PLoS Genet 8:e1002472. https://doi.org/10.1371/journal.pgen.1002472.
29. Nguyen AN, Disconzi E, Charriere GM, Destoumieux-Garzon D, Bouloc P, Le Roux F, Jacq A. 2018. *csrB* gene duplication drives the evolution of redundant regulatory pathways controlling expression of the major toxic secreted metalloproteases in *Vibrio tasmaniensis* LGP32. mSphere 3:e00582-18. https://doi.org/10.1128/mSphere.00582-18.
30. Le Roux F, Binesse J, Saulnier D, Mazel D. 2007. Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. Appl Environ Microbiol 73:777–784. https://doi.org/10.1128/AEM.02147-06.
31. Andersen JB, Sternberg C, Poulsen LK, Bjorn SP, Givskov M, Molin S. 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl Environ Microbiol 64:2240–2246.
32. Zhang YJ, Chen G, Lin H, Wang P, Kuang B, Liu J, Chen S. 2017. Development of a regulatable expression system for the functional study of *Vibrio vulnificus* essential genes. Antonie Van Leeuwenhoek 110:607–614. https://doi.org/10.1007/s10482-016-0827-x.
33. Toffano-Nioche C, Nguyen AN, Kuchly C, Ott A, Gautheret D, Bouloc P, Jacq A. 2012. Transcriptomic profiling of the oyster pathogen *Vibrio splendidus* opens a window on the evolutionary dynamics of the small RNA repertoire in the *Vibrio* genus. RNA 18:2201–2219. https://doi.org/10.1261/rna.033324.112.
34. Xie ZY, Hu CQ, Chen C, Zhang LP, Ren CH. 2005. Investigation of seven *Vibrio* virulence genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains from the coastal mariculture systems in Guangdong. Lett Appl Microbiol 41:202–207. https://doi.org/10.1111/j.1472-765X.2005.01688.x.
35. Chang C, Jin X, Chaoqun H. 2009. Phenotypic and genetic differences between opaque and translucent colonies of *Vibrio alginolyticus*. Biofouling 25:525–531. https://doi.org/10.1080/08927010902964578.
36. Kanehisa M, Goto S. 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 28:27–30. https://doi.org/10.1093/nar/28.1.27.