Regulation of the *rplY* gene encoding 5S rRNA binding protein L25 in *Escherichia coli* and related bacteria

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ABSTRACT

Ribosomal protein (r-protein) L25 is one of the three r-proteins (L25, L5, L18) that interact with 5S rRNA in eubacteria. Specific binding of L25 with a certain domain of 5S r-RNA, a so-called loop E, has been studied in detail, but information about regulation of L25 synthesis has remained totally lacking. In contrast to the *rplE* (L5) and *rplR* (L18) genes that belong to the polycistronic *spc*-operon and are regulated at the translation level by r-protein S8, the *rplY* (L25) gene forms an independent transcription unit. The main goal of this work was to study the regulation of the *rplY* expression in vivo. We show that the *rplY* promoter is down-regulated by ppGpp and its cofactor DksA in response to amino acid starvation. At the level of translation, the *rplY* expression is subjected to the negative feedback control. The 5'-untranslated region of the *rplY* mRNA comprises specific sequence/structure features, including an atypical SD-like sequence, which are highly conserved in a subset of gamma-proteobacterial families. Despite the lack of a canonical SD element, the *rplY*'-*lacZ* single-copy reporter showed unusually high translation efficiency. Expression of the *rplY* gene in *trans* decreased the translation yield, indicating the mechanism of autogenous repression. Site-directed mutagenesis of the *rplY* 5' UTR revealed an important role of the conserved elements in the translation control. Thus, the *rplY* expression regulation represents one more example of regulatory pathways that control ribosome biogenesis in *Escherichia coli* and related bacteria.

Keywords: ribosomal protein L25; *rplY* gene; transcription; translation; autogenous control; phylogenetic conservation

INTRODUCTION

5S rRNA is a universally conserved component of the large ribosomal subunit where it forms a part of the central protuberance (CP), but its exact role in ribosome functioning is still debated (for review, see Bogdanov et al. 1995; Szymanski et al. 2003; Gongadze 2011). Bacterial 5S RNA interacts directly with three ribosomal proteins (r-proteins)—L18, L25, and L5 (Chen-Schmeisser and Garret 1977), two of which, L5 and L18, are essential for viability (Korepanov et al. 2007). Although the *rplY* gene encoding L25 is not absolutely required for survival, *Escherichia coli* cells deficient in L25 reveal a slow growth phenotype even in rich media under normal growth conditions, indicating importance of L25 for ribosome functioning (Korepanov et al. 2007). As shown recently (Korepanov et al. 2012), L5 plays a crucial role in the CP formation during assembly of the 50S subunit, but precise roles for L18 and L25 still remain to be elucidated.

Unlike L5 and L18 which are universal r-proteins, L25 is bacteria-specific (Wilson and Nierhaus 2005). It belongs to the CTC protein family (catabolite controlled) which embraces L25 homologs across the bacterial kingdom (for

review, see Gongadze et al. 2008). The best studied representatives are L25 from *E. coli* (*Eco*), TL5 from *Thermus thermophilus* (*Tth*), and a general stress protein CTC from *Bacillus subtilis* (*Bsu*) (Gongadze et al. 2008). *Eco* L25 and TL5 are bona fide ribosomal proteins that are permanently bound to 5S rRNA within the 50S subunit, whereas *Bsu* CTC is induced only under stress conditions (Schmalisch et al. 2002). Most of the CTC proteins, including TL5 and *Bsu* CTC, consist of two domains, with the N-terminal domain being homologous to *Eco* L25 (94 amino acids) and capable of 5S rRNA binding (Schmalisch et al. 2002, Korobeinikova et al. 2008). Even within γ -proteobacteria there exists a difference in length of the L25 homologs, so that only several families (e.g., Enterobacteriaceae, Pasteurellaceae, Vibrionaceae, Shewanellaceae) produce a short, *E. coli*-like L25 variant, while a long (~200 residues) protein is typical of most other species (NCBI Protein database). Why this diversity exists and what is the function of the C-terminal extension remains unclear.

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Binding of L25 to the 5S rRNA has been examined by different approaches. The L25 binding site was located in a so-called loop E of 5S rRNA which is highly conserved in bacteria but distinct in structure from the eukaryotic 5S rRNA loop E. The bacterial loop E is symmetrical, highly structured and characterized by a specific set of noncanonical base pairs (Dallas and Moore 1997; Leontis and Westhof 1998). The structure of the complex between *E. coli* L25 and the 5S RNA loop E-containing fragment has been solved at high resolution both in solution (Stoldt et al. 1999) and in a crystal (Lu and Steitz 2000). At the same time, little if any is known about regulation of the L25 synthesis. In all eubacteria with completed genomes (NCBI Gene database), the *rplY* gene (L25) constitutes an independent transcription unit, while the *rplE* (L5) and *rplR* (L18) genes belong to the polycistronic *spc*-operon which is negatively regulated at the translation level by the r-protein S8 (for review, see Zengel and Lindahl 1994, Aseev and Boni 2011).

The main goal of this work was to study the *rplY* expression regulation in *E. coli* in vivo. We show here that the *rplY* transcription is under negative stringent control and that at the translation level, the *rplY* expression is subjected to autogenous regulation. Phylogenetic comparison revealed a conservation of several sequence/structure features of the *rplY* translation initiation region in a number of γ -proteobacterial families. Among the conserved features, the absence of a canonical Shine–Dalgarno sequence is specifically remarkable. By using site-directed mutagenesis, we determined the conserved RNA elements which are required for the high translation efficiency of the *rplY* mRNA and its regulation by L25 in *trans*. The stringent control and autogenous repression of the L25 synthesis found in this work represent one more example of the regulatory loops that control r-protein synthesis in *E. coli* and its relatives.

RESULTS

The *rplY* promoter is negatively regulated by ppGpp and transcription factor DksA

Analysis of the *rplY* upstream regions in sequenced genomes of *E. coli* and related

bacteria (NCBI Gene database) revealed that the presumable *rplY* promoter possesses features typical of the σ^{70} -dependent promoters (Harley and Reynolds 1987; Schultzaberger et al. 2007), including a “good” putative -10 element (TAg/tAAT) separated from a -35 hexamer (bearing a universally conserved TTG triplet) by an optimal spacer region of 17 bp (Fig. 1A). In addition, there is a GC-rich “discriminator” downstream from the -10 element, suggesting that the *rplY* transcription could be negatively regulated by ppGpp during stringent response (Travers 1984; Zacharias et al. 1989;

FIGURE 1. Features of the *rplY* promoter. (A) Conservation of the *rplY* promoter pattern in γ -proteobacteria. Sequence logo analysis of the *rplY* promoter regions from representatives of Enterobacteriaceae, Pasteurellaceae, Vibrionaceae, and Shewanellaceae. The 40-nt sequences from the nontemplate DNA strand upstream of the presumable transcription start (that was assumed to be located 7 bp downstream from the -10 hexamer) were taken for the alignment. (B) The *rplY* and *rrnBP1* promoters are coordinately regulated by ppGpp and its cofactor DksA in response to serine starvation. RT-PCR analyses of *rplY*, *rrnBP1*, and *thrL* transcripts in total mRNA preparations isolated from wild-type (WT), *relA::kan* and *dksA::tet* strains before (–), 15 min and 30 min after addition of serine hydroxamate (SHX). RT-PCR fragments were analyzed in 2% agarose gels. 23S—the fragment for loading normalization was obtained by RT-PCR on the same total mRNA preparations with primers specific for a mature 23S rRNA. (C) Quantification of the *rplY* transcripts in WT, *relA::kan* and *dksA::tet* strains before and 15 min after SHX addition by RT-qPCR with an external standard using the LightCycler software. (Left panel) The calibration curve generated using the *rplY* RNA standard. (Right panel) Changes in the level of the *rplY* transcript (in molecules per 1 μ g total RNA) after SHX addition; average of two independent RT-qPCR experiments (each probe in triplicate), and standard deviations are shown.

Haugen et al. 2008; Lemke et al. 2011). To validate this prediction, we analyzed the changes in the *rplY* promoter activity after induction of the stringent response by serine-hydroxamate (SHX). The treatment with SHX has been widely applied for triggering the RelA-dependent ppGpp synthesis in studies of individual promoters (e.g., Mallik et al. 2006; Lemke et al. 2011) as well as of the global effect of ppGpp on gene expression (Durfee et al. 2008). The changes in the transcription level were first evaluated by RT-PCR analysis of individual transcripts in total RNA isolated from the wild-type (*relA*⁺, *dksA*⁺) strain and its derivatives deficient in ppGpp (*relA::kan*) or its cofactor DksA (*dksA::tet*) (Fig. 1B). The *rrnB* P1 and the *thrL* transcripts served as internal controls whose response to the increased ppGpp level is opposing, DksA-dependent and well characterized (Paul et al. 2004, 2005). The results demonstrated the synchronous down-regulation of the *rplY* and the *rrnB* transcripts by ppGpp/DksA under serine starvation, whereas the *thrL* promoter showed an expected increase in activity (Fig. 1B).

Quantitative real-time RT-PCR with an external standard (Tobish et al. 2003; Fey et al. 2004) confirmed the substantial decrease in the *rplY* transcript concentration (in molecules per 1 µg total RNA) after induction of serine starvation in wild-type cells, while in the same conditions no decrease was observed in strains deficient in RelA or DksA (Fig. 1C). It should be mentioned that in *relA::kan* cells after SHX treatment a modest yet reliable increase in the *rplY* transcript level was observed (Fig. 1C, right panel), the nature of this effect remaining unknown. Taken the data obtained into account, we concluded that the *rplY* gene expression is stringently regulated at the transcription level. The negative stringent control was recently shown for a number of other *E. coli* r-protein operons including the *spc*-operon that encodes 5S rRNA-binding proteins L5 and L18 (Lemke et al. 2011). Thus, genes of all three 5S rRNA-binding proteins are coordinately regulated during stringent response.

L25 is a negative regulator of the *rplY* gene expression at the translation level

Many r-protein operons in *E. coli* are regulated by the mechanism of autogenous repression (for review, see Zengel and Lindahl 1994; Aseev and Boni 2011). That is, one of the r-proteins encoded in the polycistronic operon or the product of the monocistronic operon, e.g., S15, has a dual functionality in translation by acting as both a structural component of the ribosome and an operon-specific repressor when produced in excess over its target on the rRNA (Nomura et al. 1984). However, for a half of r-protein operons (10 out of 21) information about expression regulation is still lacking, including *rplY*. L25 can be regarded as a promising candidate for regulating its own synthesis by binding to the *rplY* mRNA, since it is an RNA-binding protein capable of recognizing a specific fragment of 5S rRNA (see Introduction).

To test a possibility of the *rplY* autogenous regulation, we used an approach previously developed for dissecting *rpsA* and *rpsB* regulatory circuits (Boni et al. 2000, 2001; Tchufistova et al. 2003; Aseev et al. 2008, 2009). To this end, we created a specialized strain (LABrplY::lacZ) bearing a single-copy (chromosomal) *rplY*'-*lacZ* translational fusion and a plasmid (pL25) expressing the *rplY* gene as a source of L25 in *trans* (Fig. 2A). The *rplY* moiety of the *rplY*'-*lacZ* reporter comprised the *rplY* promoter and the *rplY* translation initiation region (TIR). The plasmid pL25 provided the synthesis of a biologically active L25 protein in a cell, as it completely suppressed the slow-growth phenotype of the *rplY::kan* mutant deficient in L25 (Fig. 2B).

The strain LABrplY::lacZ was then transformed by an empty vector pACYC184 and its derivatives that expressed L25, S1, and an RNA-chaperon Hfq. The plasmids pS1 and pHfq encoding well-studied RNA-binding proteins (Hajnsdorf and Boni 2012) provided specificity controls for the pL25 impact on the β-galactosidase yield. The β-galactosidase assays showed that expression of the *rpsA* (pS1) and *hfq* (pHfq) genes from plasmids did not significantly affect the *rplY*'-*lacZ* translation level. At the same time, the presence of pL25 decreased the translation yield by about threefold (Fig. 2C, left panel). No effect of pL25 on the unrelated *rpsO*'-*lacZ* reporter was observed (Fig. 2C, right panel). Taken together, the results suggest that L25 is able to specifically down-regulate the *rplY* gene expression.

In *E. coli*, most r-proteins involved in autogenous regulation of its own mRNA block translation initiation; only L4 represents an exception since it affects both translation and transcription of the *S10* operon (Zengel and Lindahl 1992, 1994). Transcription regulation was found to result from the L4-stimulated premature termination within the *S10* mRNA leader (Zengel and Lindahl 1992). To test such a possibility for the L25 autogenous control, we determined whether the level of the *rplY* moiety in the *rplY*'-*lacZ* transcript could change in the presence of pL25 compared with an empty vector. No alteration in the transcript amount has been revealed by the RT-PCR analysis (Fig. 2D), indicating that the L25-mediated autogenous repression occurs at the translation level. The *rpsO* transcript served as an internal control, because it was not expected to respond to the L25 overexpression (Fig. 2D). We conclude that L25 represents one more autogenous regulator that acts at the translation level to control the ribosome biosynthesis in *E. coli*, in addition to 10 previously defined r-protein repressors, viz S1, S2, S4, S7, S8, S15, L1, L4, L10/L12₄, and L20 (for review, see Zengel and Lindahl 1994; Aseev and Boni 2011).

Phylogenetic conservation of the *rplY* mRNA 5' UTR in γ-proteobacteria

Regulatory RNA *cis*-elements involved in autogenous control of r-protein operons usually are narrowly distributed to γ-proteobacteria, although more wide distribution across

FIGURE 2. Regulation of the *rplY* expression at the translation level. (A) Structure of the *rplY* operon; (P) promoter; (T) terminator. The flanking genes (*yejH* and *yejK*) and directions of their transcription are also shown. Regions amplified for constructing the plasmid pL25 and the *rplY*'-lacZ fusion are designated by lines. (Below) The sequence of the *rplY* region on the *E. coli* chromosome. Transcription and translation regulatory elements are in bold and boxed; stop codons are in bold; the ρ -independent terminator is underlined. (B) Full complementation of the slow-growth phenotype of the *rplY*::kan strain in the presence of pL25. Growth curves were obtained by monitoring OD₆₀₀ for cell cultures growing in LB at 37°C. (C) Specific effect of L25 in trans on the *rplY*-lacZ expression. An empty vector pACYC184 and its derivatives pL25, pS1, pHfq, and pS15 were used. Average of at least three independent assays and standard deviations are shown. (D) RT-PCR analysis of the *rplY*-lacZ transcript level in cells bearing pACYC184 and pL25. The *rpsO* transcript level served as an internal control. The promoter proximal portions of the in vivo transcripts were analyzed; RT-PCR products were separated in 2% agarose gels; (M) 100-bp DNA ladder (Fermentas).

bacterial phyla has been found in a few cases (Fu et al. 2013). To study the conservation of the *rplY* regulatory region, we predicted its structural organization by computer modeling (Zuker 2003). Phylogenetic comparison of the *rplY* leader structures from completed genomes revealed conservation of a number of sequence/structure features in several γ -proteobacterial families (Enterobacteriaceae, Pasteurellaceae, Vibrionaceae, and Shewanellaceae), but not outside this group (Table 1; Fig. 3). The similar phylogenetic distribution has been previously found for the 5' UTRs of the *S10* (Allen

et al. 1999) and *rpsA* operons (Boni et al. 2001; Tchufistova et al. 2003), while the *rpsB*-tsf regulatory RNA showed more wide conservation including Pseudomonadaceae (Aseev et al. 2009; Fu et al. 2013). The found phylogenetic similarity implies that the predicted folding of the *rplY* 5' UTR might have biological relevance (Fig. 3A,B).

Conserved features within the *rplY* mRNA leaders include 5'-proximal (HI) and central (HII) imperfect hairpins followed by an extended AU-rich sequence and an atypical Shine-Dalgarno-like (SD-like) element (Table 1; Fig. 3A,B). While the 5'-proximal stem-loop HI shows significant sequence divergence (see Fig. 3A for *E. coli* and *Y. pestis* *rplY* 5' UTR), the central hairpin HII, especially its upper part, is highly conserved (Fig. 3B), which may be suggestive of its functional importance for the *rplY* expression regulation. In support of this view, we found that the *rplY*'-lacZ reporter bearing the *rplY* TIR from *Y. pestis*, where the central stem-loop is identical to the *E. coli* HII, was down-regulated by *Eco* L25 in trans (Fig. 3A). Given that the overall sequences of the *rplY* 5' UTRs from *Y. pestis* and *E. coli* are rather dissimilar outside the hairpin II, these results argue in favor of the important role of HII in the *rplY* autogenous control, which was further confirmed by site-directed mutagenesis (see below).

The remarkable conserved feature is the presence of the unusual SD-like sequence in front of the start codon (Table 1). In place of a classic SD-element which forms at least four contiguous base pairs with the 3'-terminus of 16S rRNA, the *rplY* mRNAs from a number of γ -proteobacterial families comprise an SD-like sequence (GAG) capable of forming only 3 bp (Table 1). This feature is not

inherent to species beyond this group; for example, representatives of Pseudomonadaceae and Legionellaceae bear a canonical GGAG element in front of the *rplY* start codon. Despite the presence of a *prima facie* weak SD sequence, the *E. coli* *rplY* TIRs appeared to provide a high level of translation (Fig. 3C). This is reminiscent of the *fis* (Nafissi et al. 2012) and *rpsA* (Boni et al. 2000, 2001; Skorski et al. 2006) mRNAs that lack true SD-elements; nevertheless, they are translated very efficiently due to the presence of the AU-rich translational enhancers and specific secondary structures within their

TABLE 1. Conservation of the unusual ribosome binding site (RBS) of the *rplY* mRNA in several γ -proteobacterial families

Bacteria	Sequence ^a	Start codon
Enterobacteriaceae		
<i>E. coli</i> and <i>Shigella</i>	AUUAAUUUAA <u>GAGAG</u> AAAGAA <u>AUG</u>	
<i>Salmonella enterica</i>	AAUUAAUUAA <u>GAGAG</u> AUUUAA <u>AUG</u>	
<i>Klebsiella pneumoniae</i>	CUUUAAUAAUAGAGAGACAUA <u>AUG</u>	
<i>Citrobacter rodentium</i>	AAUUAAUUAA <u>GAGAG</u> AUUUAA <u>AUG</u>	
<i>Enterobacter cloacae</i>	AAUUAAUUAA <u>GAGAG</u> AUUUAA <u>AUG</u>	
<i>Yersenia pestis</i>	UAAAUUUUUCUUUAA <u>GAGAAA</u> CAAA <u>AUG</u>	
<i>Erwinia amylovora</i>	AAUAAAACCUGA <u>GAGAC</u> UUA <u>AUG</u>	
<i>Edwardsiella tarda</i>	CUUCAUUUUUUAA <u>GAGAU</u> AGAUA <u>AUG</u>	
<i>Proteus mirabilis</i>	AUUUUCUUUUUCAUAA <u>GAGAAAA</u> GA <u>AUG</u>	
<i>Pantoea vagans</i>	AUUAAAUA <u>GAGAU</u> AUAUA <u>AUG</u>	
<i>Xenorhabdus bovienii</i>	AUUUAUUUUCUUCUUUAA <u>GAGAAAA</u> UA <u>AUG</u>	
<i>Photorhabdus luminescens</i>	AUUUAUUUUCUUCUAA <u>GAGAAAA</u> UA <u>AUG</u>	
<i>Dickeya dadantii</i>	UUCAUUUUAUCAAGUA <u>GAGAA</u> GC <u>AUG</u>	
<i>Pectobacterium atrosepticum</i>	AUUACUUUAUAUAAGUA <u>GAGAA</u> GA <u>AUG</u>	
<i>Serratia marcescens</i>	UAAAUUUUUCUUUAA <u>GAGAAAA</u> CA <u>AUG</u>	
Pasteurellaceae		
<i>Actinobacillus pleuropneumoniae</i>	UUUUUACUUUAA <u>GAGAA</u> UCAAC <u>AUG</u>	
<i>Mannheimia succiniciproducens</i>	UAUUAAUUAAUUAAUUAA <u>GAGAA</u> CAUUAA <u>AUG</u>	
<i>Haemophilus parasuis</i>	UUUUUAUUUAU <u>GAGAA</u> UUUAAU <u>AUG</u>	
<i>Haemophilus influenzae</i>	UUUUAAUAUUAAUUUAA <u>GAGAA</u> CAUUAA <u>AUG</u>	
<i>Haemophilus ducreyi</i>	UUCCUUUUUAUUUAA <u>GAGAA</u> UCAAC <u>AUG</u>	
<i>Haemophilus somnus</i>	UUUUUUUAUUUUUAUCAA <u>GAGAA</u> CAUUAA <u>AUG</u>	
<i>Pasteurella multocida</i>	UUUUACUAUUAA <u>GAGAU</u> AGCGAA <u>AUG</u>	
Vibrionaceae		
<i>Vibrio cholerae</i>	UUUUUUACUAAUUU <u>GAGAG</u> UAAUAUA <u>AUG</u>	
<i>Vibrio fischeri</i>	AUUUAUUUAUUUUAUAUUU <u>GAGAG</u> UAAAGACUA <u>AUG</u>	
<i>Aliivibrio salmonicida</i>	AUUUAUUUAUUUUAUAUUU <u>GAGAG</u> UAAAGACUA <u>AUG</u>	
Aeromonadaceae		
<i>Aeromonas salmonicida</i>	AUAAAUUCAUCUAUAU <u>GAGAU</u> AACCA <u>AUG</u>	
Shewanellaceae		
<i>Shewanella violacea</i>	UAAUUUAUUUUUAA <u>GUGAG</u> AAAA <u>CAUG</u>	
<i>Shewanella oneidensis</i>	AUUUUUUACUCUAAAUUUAA <u>GUGAG</u> AAAC <u>CAUG</u>	

^aSequences of RBSs include extended A/U-rich sequences (underlined) followed by the atypical SD-like elements (GAGAG, GAGA, or GUGAG, in bold), spacer regions of slightly various length (depending on genera) and start codons, universally AUG (in bold).

5' UTRs. Many data provide evidence that AU-rich sequences within the mRNA leaders served as targets for the r-protein S1 facilitating ribosome recruitment during initiation (Boni et al. 1991, 2001; Tzareva et al 1994; Komarova et al. 2002, 2005; Hook-Barnard et al. 2007; Vimberg et al. 2007; Nafissi et al. 2012). Most likely, the extended AU-rich sequence of the *rplY* leader functions analogously.

Site-directed mutagenesis of the *rplY* 5' UTR

To identify the conserved elements involved in translational control of the *rplY* expression, we created LABrplY::lacZ derivatives bearing diverse mutated variants of the *rplY* 5' UTR (Fig. 4). Removal of the AU-element which separates the central stem-loop and the "atypical" SD-like sequence completely abolished translation activity (not shown). In contrast, a deletion of the central stem-loop structure (HII) together with the adjacent AU-rich sequence increased translation yield by about twofold, eliminating simultaneously the autogenous control (Δ HII+AU on Fig. 4).

To determine the minimal length of the *rplY* 5' UTR required for the autogenous regulation, we removed the 5' proximal stem-loop HI and its adjacent sequence up to the stem-loop HII. This truncation diminished the translation yield but still allowed the L25-mediated repression (Δ HI plus on Fig. 4), indicating that the 5' proximal part of the *rplY* 5' UTR does not comprise important determinants for the *rplY* autogenous control, although it somehow contributes to the translation efficiency. However, the further abridgement of the 5' UTR up to position -40 restored the high translation efficiency but eliminated the L25-mediated control, which may be explained by disrupting the operator structure (Δ HIplus_HIImut1 on Fig. 4A,B). We concluded that the intactness of the hairpin HII is important for the *rplY* autogenous regulation. A high conservation of the upper part of HII (Fig. 3A,B) prompted us to remove it from the context of the full-length 5' UTR. While elevating the translation efficiency, this mutation decreased the extent of the autogenous repression (Fig. 4B). This suggests the negative effect of the upper part of HII on the *rplY* TIR efficiency.

FIGURE 3. Conservation of the *rplY* 5' UTR in γ-proteobacteria. (A) Predicted secondary structures of the *rplY* 5' UTRs from *E. coli* and *Yersinia pestis* as distant representatives of Enterobacteriaceae. The "atypical" SD-elements and the initiator codons are in bold and underlined. Only conserved stem-loops HI and HII (shadowed) are depicted. (Below) *Eco*L25-mediated regulation of the *rplY-lacZ* fusions bearing the *rplY* sequences from *E. coli* and *Y. pestis*. (B) Secondary structure conservation in a central part of the *rplY* 5' UTRs from representatives of Enterobacteriaceae, Vibrionaceae, Pasteurellaceae, and Shewanellaceae as revealed by LocARNA algorithm (Smith et al. 2010). Sequences (30 nt) comprising a universally conserved GUCG loop (in bold) were taken for analysis. Nucleotides forming base pairs are shown by gray gradations.

and emphasizes the role of the hairpin II conservation in evolution of the *rplY* autogenous control.

To study the role of the GAGAG SD-like sequence in the *rplY* translation, we replaced it with a canonical GGAGG-element. Surprisingly, this change did not augment the translation yield, rather decreased it by ~2.5-fold and, in addition,

abolished the autogenous control (SD+ on Fig. 4C). However, when GAGAG was replaced with CACUC, a sequence irrelevant to the SD-element, translation of the *rplY-lacZ* reporter dropped by ~20-fold, indicating an important contribution of GAGAG to translation efficiency. At the same time, pL25 repressed translation of the *rplY-lacZ* gene lacking the GAGAG-element (SD- on Fig. 4C), suggesting that though important for translation activity the GAGAG sequence per se is not essential for the *rplY* autogenous repression.

DISCUSSION

In this work, we have studied for the first time the in vivo regulation of the *E. coli rplY* gene encoding a 5S rRNA binding protein L25. The *rplY* expression was found to be regulated at both transcriptional and translational levels.

Transcriptional control

We show that the *rplY* promoter activity is down-regulated by ppGpp and its cofactor DksA in response to amino acid starvation, presumably due to the presence of the GC-rich discriminator separating the -10 hexamer and the transcription start (Fig. 1A,B). Although for *E. coli* r-proteins it is the translational autogenous regulation that has been considered as the main strategy to modulate their expression (Zengel and Lindhal 1994), it is becoming apparent that the transcriptional control is also very important (Lemke et al. 2011). Simultaneous down-regulation of transcription of rRNA and r-protein operons upon nutrient starvation certainly ensures more rapid reallocation of the scarce resources from energy consuming ribosome biogenesis in favor of processes involved in stress resistance and amino acid synthesis. Transcriptional regulation of the r-protein expression appears not to be

limited to stringent response. Recently, several promoters of r-protein operons (e.g., *rpmI-rplT*, *rplM-rpsI*, *rpsF-priB-rpsR-rplI*) have been reported to be under the control of cAMP-CRP (cyclic AMP receptor protein), the effector usually implicated in modulating carbon source utilization (Shimada et al. 2013). Mechanisms for transcription regulation

FIGURE 4. Site-directed mutagenesis of the *rplY* 5' UTR. (A) Inferred secondary structures of the *rplY* 5'-UTR mutant variants. (B,C) Impacts of the 5'-truncations, internal deletions, and changes in the atypical SD-like element (SD+ and SD-) on the translation activity of the *rplY* TIR and its repression by L25 in *trans*.

involving other transcriptional factors (e.g., FNR, see Salmon et al. 2003) can also be anticipated.

Autogenous control of the *rplY* mRNA

At the translation level, the *rplY* expression was found to be controlled by the mechanism of autogenous repression. This regulatory strategy has been previously demonstrated for many r-protein operons from *E. coli* and, in some cases, from other bacterial species (Zengel and Lindahl 1994; Allen et al. 1999, 2004; Boni et al. 2001; Tchufistova et al. 2003; Guillier et al. 2005; Aseev et al. 2008, 2009; Fu et al. 2013, 2014). However, the overall picture remains incomplete, as information is available only for a half of *E. coli* r-protein operons. Whether every operon encoding r-protein(s) is regulated by feedback inhibition or, in some cases, the adequate level of r-protein synthesis could be achieved by other means (e.g., rapid turnover of proteins) cannot be predicted with a certainty, and each case should be experimentally validated. In this regard, our results on the *rplY* in vivo regulation (this paper) together with the recent in vitro data suggesting the S6:S18-mediated control of the *rpsF-priB*-

rpsR-rplI operon (Matelska et al. 2013; Fu et al. 2014) expand our knowledge of the regulatory circuits controlling ribosome biogenesis in bacteria.

Regulation of r-protein operons is provided by specific *cis*-regulatory RNA elements which, as a rule, are more or less widely conserved in γ -proteobacteria (Fu et al. 2013, 2014). Phylogenetic analysis of the *rplY* mRNA 5' UTR revealed conservation of several regulatory RNA motifs including an atypical SD-sequence in a subset of γ -proteobacterial families, viz. Enterobacteriaceae, Pasteurellaceae, Shewanellaceae, and Vibrionaceae. Given that L25 homologs in γ -proteobacteria can be divided in two groups—one-domain (short) *E. coli*-like L25 and two-domain (long) proteins (Introduction), it is of interest that all the species with the “atypical” *rplY* RBSs (Table 1) produce a short, *E. coli*-like L25 while the *rplY* mRNAs in species producing long L25 proteins bear classic SD sequences. One may speculate that it is not a mere coincidence, and that the process of the L25 shortening proceeded concurrently with the loss of the SD sequence and evolution of the *rplY* autogenous control.

By using site-directed mutagenesis, we identified the *E. coli* *rplY* mRNA features that are essential for the autogenous reg-

ulation (Fig. 4). The data obtained demonstrate a key role of the conserved hairpin HII that represents a 5'-border of the *rplY* autogenous operator. This hairpin seems to be required only for the L25-mediated control but not for translation efficiency, since its deletions increased the translation yield, simultaneously decreasing autogenous repression. At first glance, a set of base pairings within HII does not resemble the L25 target on 5S rRNA, the loop E, so that at present it is hard to suggest (or to rule out) that binding of L25 to its mRNA mimics its interaction with 5S rRNA, following the principle of “molecular mimicry” earlier proposed for a number of r-protein operons (Nomura et al. 1980; Stelzl et al. 2003; Mathy et al. 2004; Guillier et al. 2005; Fu et al. 2014).

A well-conserved SD-like sequence GAGA(G) (Table 1) appeared to be required for both translation efficiency and autogenous repression. In *E. coli*, mRNAs capable of providing a high level of expression in the absence of a canonical SD element are rare: As yet, only *rpsA* (Boni et al. 2001; Skorski et al. 2006), *fis* (Nafissi et al. 2012), and *rplY* (this work) mRNAs can be attributed to this category. The replacement of the *rplY* GAGA(G) SD-like sequence by a classic GGAGG-element abolished L25-mediated control and reduced rather

than increased the translation yield. This reduction (Fig. 4) is consistent with the idea that the formation of a more stable initiation complex does not necessarily lead to higher translation efficiency, because it may retard the RBS liberation and transition to the elongation step of protein synthesis (see Komarova et al. 2002; Milón and Rodnina 2012). An analogous situation exists in transcription where too tight binding of RNA-polymerase to the consensus promoter elements retards the enzyme on the promoter, hampering promoter clearance and increasing the risk of abortive transcription (Ellinger et al. 1994; Vo et al. 2003; Goldman et al. 2009).

To explain the simultaneous elimination of the *rplY* autocontrol, two plausible reasons may be considered. First, GAGAG may be a part of the autogenous operator hence its mutation could eliminate the L25-mediated repression. However, the replacement of GAGAG by CACUC had only a marginal effect on the extent of the L25 autoregulation but dramatically decreased the translation yield (Fig. 4), suggesting that the GAGAG-sequence is necessary for translation efficiency but not crucial for the operator formation. Another possibility is that the L25-mediated repression may occur via the mechanism of “displacement,” rather than “entrapment.” According to the “entrapment” mechanism, the repressor protein and the 30S subparticle can bind to the mRNA simultaneously but the resulting complex is invalid in translation. The “displacement” model stipulates that the repressor and the 30S ribosomal subunit are mutually exclusive on the mRNA and compete for its binding (Zengel and Lindahl 1994). If L25 in excess competed with the 30S ribosome for binding to the *rplY* TIR, the presence of the classic GGAGG element (which in fact is even longer—UAAGG AGG, see Fig. 4) would work in favor of the 30S binding, stabilizing the initiation complex and abolishing the L25-mediated repression. This is reminiscent of the *rpsA* regulatory loop, where the change of a weak SD-like sequence GAAG for a canonical GGAG eliminated autogenous control (Boni et al. 2001). Thus, a weak SD within a particular context may ensure both efficient translation and its control.

At present, the results obtained do not yet allow us to describe the mechanistic details underlying the *rplY* autogenous regulation. In vitro, an isolated L25 did not form an obvious band-shift with the *rplY* mRNA fragment comprising TIR, while in the same conditions, L25 from the same preparation completely shifted 5S rRNA in a native gel (our data, not shown). A possible explanation is that the in vitro mRNA structure does not conform to the in vivo cotranscriptional mRNA folding. A contribution of yet-unknown accessory factors cannot

be excluded either. These aspects represent an interesting problem to be resolved in the future.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli strains and plasmids used in this study are listed in Table 2. The plasmid pL25 was created by cloning the whole *rplY* gene flanked with its own promoter and terminator into BamHI/HindIII sites of pACYC184. The corresponding region was amplified by PCR on the *E. coli* genomic DNA by using *PfuUltra* II fusion HS DNA polymerase (Stratagene) and a couple of primers, L25-for 5'-CACGGATCCGATTACGAAGGTCG (BamHI site is italicized) and L25-rev 5'-GGTAAGCTTGATTGATGTATAAGGCG (HindIII site is italicized). Expression of the wild-type L25 from the resulting plasmid pL25 was checked by its ability to suppress the slow growth phenotype of the *rplY::kan* strain (Baba et al. 2006).

To create a strain bearing the *rplY*'-*lacZ* single-copy (chromosomal) reporter, a DNA fragment that comprises the *rplY* regulatory regions including the *rplY* promoter and translation initiation region (positions from −188 to +92 relative to A+1 in the initiator ATG) was amplified using a pair of *rplY*-specific primers: L25-for (see above) and L25TIR-rev 5'-GCTAAGCTTCCGTAGATGATTGCC (complementary to the *rplY* coding sequence from +77 to +92, HindIII site is italicized). The DNA fragment was digested with BamHI and HindIII, ligated into pEMBLΔ46BamHI/HindIII and cloned in DH5α. The resulting blue colonies were used for plasmid isolation. The plasmids were sequenced and subsequently used for transformation of the ENS0 (Lac[−]) strain to transfer the *rplY*'-*lacZ* fusion onto the chromosome by homologous recombination. Recombinant colonies were selected on McConkey agar. The resulting strain LABrplY::lacZ produced β-galactosidase under the control of the *rplY* promoter hence no induction by IPTG was required. In a

TABLE 2. *Escherichia coli* strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Reference or source
Strains		
DH5α	Cloning host	Laboratory stock
ENS0	<i>his</i> , former HfrG6Δ12, the Lac [−] strain lacking the <i>lac</i> promoter and <i>lacZ</i> RBS	Dreyfus (1988)
LABrplY::lacZ	ENS0 derivative bearing chromosomal <i>rplY</i> '- <i>lacZ</i> fusion under the control of the <i>rplY</i> promoter	This work
IBrpsO188::lacZ	ENS0 <i>rpsO</i> '- <i>lacZ</i>	Le Derout et al. (2010)
JW2173	<i>rplY::kan</i>	Baba et al. (2006)
RLG8124	<i>dksA::tet</i>	R.L. Gourse
CF1693	MG1655 <i>relA251::kan</i> , <i>spoT207::Cm</i>	Xiao et al. (1991)
Plasmids		
pEMBLΔ46	pEMBL8 ⁺ derivative lacking <i>lacZ</i> RBS	Dreyfus (1988)
pEL25TIR-	pEMBLΔ46 derivative bearing the <i>rplY</i> '- <i>lacZ</i> reporter	This work
pACYC184	tet ^r , Cm ^r , cloning vector	Laboratory stock
pL25	pACYC184 derivative expressing <i>rplY</i>	This work
pSP261 (pS1)	pACYC184 derivative expressing <i>rpsA</i>	Skouv et al. (1990)
pTX381 (pHfq)	pACYC184 derivative expressing <i>hfq</i>	Tsui et al. (1994)
pS15	pACYC184 derivative expressing <i>rpsO</i>	Le Derout et al. (2010)

similar way we created a recombinant *E. coli* strain bearing the *rplY-lacZ* reporter in which the *rplY* TIR was amplified from the *Y. pestis* genomic DNA with the use of primers YpL25-for 5'-CATGGATCCAGAGATATTCGCAG and YpL25-rev 5'-AGCAAGCTTGAAACACCGTAAACG.

Mutagenesis of the *rplY* 5' UTR

The plasmid bearing the wild-type *E. coli rplY-lacZ* fusion was named as pEL25TIR. It served for generating its various mutated variants (pEL25TIRmut) to create the corresponding recombinant strains LABrplY::lacZmut. Mutations were introduced into the *rplY*-TIR within pEL25TIR by PCR techniques. For deletion analysis, PCR amplification was performed by using pEL25TIR as a template, *PfuUltra* II fusion HS DNA polymerase (Stratagene) and primers flanking the site to be deleted (to amplify the whole plasmid except for the region chosen for deleting).

To introduce a canonical SD sequence, GGAGG, in place of the unusual GAGAG element, the two-step PCR variant was used (Komarova et al. 2002). Briefly, at the first step, two PCR fragments were obtained on pL25TIR with two pairs of primers: SDplus-for–DSLac and SDplus-rev–UPlac. Overlapping SDplus primers comprised a desirable GGAGG sequence (SDplus-for) or its complement (SDplus-rev); UPlac and DSLac were described (Komarova et al. 2002). At the second step, the two purified PCR fragments were mixed and amplified in the presence of DSLac and UPlac. Finally, the resulting PCR product was digested with BamHI and HindIII and cloned into pEMBLΔ46/BamHI, HindIII to create pL25TIR(+SD). The same strategy was used to change GAGAG for the sequence unrelated to SD (CACUC) in order to obtain pL25TIR(-SD). The corresponding recombinant strains were designated as LABrplY::lacZ(+SD) and LABrplY::lacZ(-SD).

Cell growth and β-galactosidase assay

The strains were routinely grown at 37°C in Luria-Bertani (LB) medium supplemented with antibiotics if necessary: kanamycin (kan) 30 μg/mL, tetracycline (tet) 12 μg/mL, chloramphenicol (Cm) 34 μg/mL. The cells were harvested in exponential phase at optical density (OD₆₀₀) ~0.4–0.5 and used for preparing clarified cell lysates. The cells were broken by a repeated thawing–freezing procedure as described (Komarova et al. 2002; Tchufistova et al. 2003). Protein concentration in a fraction of soluble proteins was determined by Bradford assay (Bio-Rad). The specific β-galactosidase activities in the same fraction were expressed in nanomole ONPG (*o*-nitrophenyl-β-D-galactopyranoside) hydrolyzed per minute per milligram of total soluble cell proteins.

Induction of stringent response and isolation of total RNA

Strains were grown as described above. At OD₆₀₀ ~0.4–0.5, 2 mL aliquots of the cell cultures were harvested and mixed with 4 mL RNAlprotect bacterial reagent (Qiagen). To induce stringent response, serine hydroxamate (SHX) (Sigma) was added to the residual volume of the culture (0.5 mg/mL final concentration), and cultivation continued for additional 15 and 30 min. Aliquots of 2 mL were withdrawn at each time point and mixed with 4 mL RNAlprotect bacterial reagent. Total RNA was isolated from treated

and untreated probes using the RNeasy Mini Kit (Qiagen) according to recommendations of the manufacturer. RNase-Free DNase (Qiagen) was added to the columns during RNA extraction for 15 min to ensure the absence of DNA contaminations in RNA samples. The amount of total RNA in preparations was estimated by OD₂₆₀ measuring.

RT-PCR analysis

RT-PCR analysis was used to examine the stringent response of the studied *rpsY* promoter in comparison with the well-characterized ppGpp-regulated promoter *rrnB* P1. One microgram total RNA was reverse transcribed with AMV reverse transcriptase (Promega) in the presence of two reverse primers specific for the *rplY* and *rrnBP1* transcripts: L25TIR-rev shown above and *rrnBP1*-rev 5'-ACTGACACGGAACAACGGCAACAC complementary to the region +196 to +220 downstream from the transcription start. Then, 1/10 (2 μL) of the RT mix was used as a template in 25 μL PCR mix supplemented with two pairs of transcript-specific primers—L25TIR-for 5'-GCATACGCAAAGCAGATCACTTACC and *rrnBP1*-for 5'-ACTGACACGGAACAACGGCAACAC (corresponding respectively to the beginning of the *rplY* and *rrnBP1* transcripts) together with the abovementioned reverse primers. The “–RT” control PCR probes were prepared for each total RNA preparation to ascertain the absence of DNA contaminations. Similarly, the *thrL* transcript was analyzed in the same total RNA preparations as an additional internal control; specific primers used were *thrL*-for 5'-GCGCACAGACAGATAAAAATTACAG and *thrL*-rev 5'-CTGTGTTTCCTGTACGCGTCAGC. The PCR products were analyzed in 2% agarose gels. Usually, 5 μL aliquots of 25 μL PCR mix were loaded on a gel. For each RNA sample, loading was normalized by visualization of the RT-PCR product for the 23S rRNA internal region, given that the concentration of the mature ribosomal RNAs within the ribosome remains invariable after cessation of cell growth upon induction of amino acid starvation.

To evaluate the effect of pL25 on the transcription of the 5'-part of the *rplY-lacZ* gene, total RNA was prepared from exponential cultures of LABrplY::lacZ bearing pACYC184 or pL25 and reverse transcribed (1 μg in 20 μL reaction mix) in the presence of two reverse primers—DSLac (specific for the *rplY-lacZ* transcript) and RpsO-rev 5'-CAGAAGCTTGCGTCACGACCAAACCTC (corresponding to the *rpsO* transcript, positions +40 to +58 relatively A+1 of the initiation codon). The subsequent PCR (18 cycles) was performed on 2 μL of RT-mix with two pairs of primers—L25TIR-for/DSLac (specific for the *rplY-lacZ* fusion, see above), and RpsO-for/RpsO-rev, where RpsO-for (5'-CACGGATCCTGGGATCGCTGAATTAG) corresponded to the beginning of the *rpsO* transcript.

Quantification of the *rplY* in vivo transcript by RT-qPCR using the LightCycler software

To quantify the concentration of the *rplY* transcript before and after SHX treatment of the wild-type, *relA::kan* and *dksA::tet* cells, we chose the real-time RT-PCR approach based on using the external RNA standard (Tobish et al. 2003; Fey et al. 2004). This method enables the quantification of the number of copies of a given transcript in a total RNA pool. To generate the *rplY* RNA standard for the calibration curve, we first prepared the DNA template by PCR on pL25 using the *rplY*-specific primers. The forward primer (T7rplY-for)

comprised the T7 promoter sequence (underlined) fused to the beginning of the *rplY* transcript (5'-AGTAATACGACTCACTATA GGGCTTTTGCATACGC), and the reverse primer (rplY-RTrev) was complemented to the *rplY* coding region (from +66 to +89 relatively the initiation codon): 5'-ATGATTGCCGGGAAGCTTGTT AGCG. The purified PCR product was transcribed in vitro with T7 RNA polymerase by using the Riboprobe System-T7 (Promega), the transcription was followed by DNase RQ digestion. The resulting *rplY* RNA product was purified according to Promega protocol. RNA concentration was estimated by measuring OD₂₆₀. Serial dilutions of the *rplY* RNA standard (from 10 ng to 100 fg per 1 µL) were then prepared in RNase-free H₂O mixed with MS2 RNA (0.5 µg/µL final concentration). These serial dilutions (2 µL each) served in 20 µL RT reaction with the primer rplY-RTrev. At the same time, 1 µg total RNA isolated from each strain before and 15 min after SHX treatment was reverse transcribed. RT reactions were performed in final volume 20 µL for 1 h at 42°C. Real-time PCR was run with the use of LightCycler 480 II (Roche), each 25 µL reaction contained 5 µL 5×-qPCRmix HS SYBR (Evrogen), primers rplY-RTrev, and L25TIR-for (1 µL of 5 µM solution), 2 µL of RT mix. The amplified products in all cases had identical properties since they were generated with the same primer pair. The amount of the *rplY* transcript was determined using the external *rplY* standard curve for quantification with the second derivative maximum method (Tobish et al. 2003). The *rplY* RNA concentrations were calculated in molecules per 1 µg total RNA, assuming that molar mass of single-stranded RNA = length × 340. Thus, for 159-nt *rplY* RNA, 1 ng is equivalent to $\sim 1.1 \times 10^{10}$ molecules (see Tobish et al. 2003 for calculation details).

Bioinformatic tools

Nucleotide sequences of the *rplY* regions from bacterial genomes were taken from NCBI Gene database. A graphical representation of the patterns within multiple *rplY* promoter sequences from a subset of γ-proteobacterial families was generated by using WebLogo (Crooks et al. 2004). The genomes analyzed are listed in Table 1. RNA structures of the *rplY* 5' UTRs were predicted by using Mfold program (Zuker 2003). To demonstrate the conservation of the secondary structure in the central part of the *rplY* 5' UTR, we used the LocARNA algorithm (Smith et al. 2010).

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