Mechanism of translational coupling in the *nifLA* operon of *Klebsiella pneumoniae*

F.Govantes¹, E.Andújar and E.Santero²

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Ap. 1095, 41080-Sevilla, Spain

¹Present address: Department of Microbiology and Molecular Genetics, 1602 MSB, University of California, Los Angeles, CA 90095-1489, USA

²Corresponding author
e-mail: esantero@cica.es

The *nifLA* operon of *Klebsiella pneumoniae* encodes the sensor–activator pair involved in the regulation of other *nif* genes. Balanced synthesis of both proteins, which is required for correct regulation, is achieved by coupling translation of *nifA* to that of *nifL*. The mechanism of translational coupling at the *nifLA* operon was analysed using a specialized ribosome system, and the effect of substituting the natural Shine–Dalgarno of *nifL* or *nifA* for specialized Shine–Dalgarno sequences was determined. Our results indicate that translational coupling occurs in this operon by a reinitiation mechanism. Additionally, reinitiation at the *nifA* can happen even in the absence of good Shine–Dalgarno recognition by the reinitiating ribosome, although its efficiency is lower. The effect of a putative translational enhancer sequence (downstream box) on translational coupling efficiency was also determined. Mutations that reduce the homology of the putative downstream box to the consensus had only a minor effect on *nifA* translation by wild-type ribosomes. However, they had a significant effect on *nifA* translation by specialized ribosomes, suggesting that recognition of the downstream box may compensate inefficient ribosomal interactions with the Shine–Dalgarno sequence.

Keywords: downstream box/*nif* regulation/reinitiation

Introduction

Translational coupling has been described in an increasing number of bacterial operons as a phenomenon which allows the control of gene expression and the maintenance of a stoichiometry among gene products. Perhaps the most striking examples are the operons encoding ribosomal proteins, where such coupling is extensively used (Keener and Nomura, 1996). Translational coupling operates by making translation of a distal gene depend on the previous translation of the gene immediately upstream (McCarthy and Gualerzi, 1990; Draper, 1996). In such cases, independent translation of the distal gene is totally or partially inhibited, and translation of the proximal gene is able to overcome such inhibition. In some systems, low levels of independent translation are mainly due to an inherently

poor translation initiation region (TIR) (Adhin and van Duin, 1989; Ivey-Hoyle and Steege, 1992). However, in most cases, the distal gene presents a recognizable ribosome binding site (RBS). Inhibition of independent translation is then frequently achieved by occluding the RBSs of the distal genes within stable mRNA secondary structures, which make them inaccessible to the ribosomes (McCarthy and Gualerzi, 1990).

For systems in which a poor TIR does not allow efficient binding of ribosomes from solution, translation of the proximal gene may stimulate translation of the distal gene by a reinitiation mechanism. Upon terminating translation of the upstream gene, the ribosome would search for a near-located translation initiation signal and restart translation. Reinitiation was first reported to explain mutation-induced intragenic translation (Sarabhai and Brenner, 1967; Grodzicker and Zipser, 1968). It has also been proposed as the translational coupling mechanism at the overlapping coat and lysis genes of phages MS2 and fr (Adhin and van Duin, 1989, 1990). Since reinitiation may occur, this mechanism has often been assumed to explain translational coupling with no further evidence. However, an alternative mechanism of facilitated binding may be in operation for systems having a good, but occluded, RBS. By this mechanism, ribosomes translating the proximal gene would melt the inhibitory mRNA secondary structure, thus allowing new ribosomes to gain access from solution to the RBS of the distal gene. Facilitated binding has been suggested for other translationally coupled genes (Lesage *et al.*, 1992; Dallmann and Dunn, 1994), and it may be the only mechanism to explain the synthesis of the distal gene product in greater amounts than the proximal one, as is observed in some coupled systems (Yates and Nomura, 1981; Gerstel and McCarthy, 1989). However, unambiguous direct evidence regarding the involvement of the same or different ribosomes in the translation of a pair of translationally coupled genes has not yet been provided.

The *nifLA* operon of *Klebsiella pneumoniae* codes for the regulatory proteins which control expression of all other nitrogen fixation (*nif*) operons in this bacteria. NifA is a transcriptional activator which is synthesized in an active form. Its activity is inhibited by NifL in response to nitrogen availability and oxygen tension (Hill *et al.*, 1981; Merrick *et al.*, 1982). The two proteins are produced in stoichiometric amounts and inactivation of NifA by NifL appears to involve formation of a complex between both proteins (Henderson *et al.*, 1989). Additionally, we have previously shown that unbalancing the gene dosage ratio of *nifL–nifA* to 1:2 results in derepression of the synthesis of the nitrogenase under inappropriate conditions (Govantes *et al.*, 1996).

The *nifLA* operon displays a number of features frequently observed in translationally coupled operons. Trans-

Fig. 1. Representation of the *nifLA* operon, showing the features of the overlapping region. The Shine-Dalgarno (SD) of *nifA* is underlined. The overlapping stop and initiation codons are boxed. The putative downstream box is in bold face. Nucleotides involved in the secondary structure masking the *nifA* SD are covered by arrows.

lation of *nifL* proceeds across the *nifA* Shine-Dalgarno (SD) sequence and the *nifL* stop codon overlaps the *nifA* start codon. Based on its sequence, the *nifA* TIR is expected to be very efficient. The *nifA* SD is perfectly homologous to the consensus GGAGG, and is properly spaced to an AUG start codon. The three nucleotides immediately downstream of the SD provide extended complementarity to the 3' end of the 16S rRNA, which has been associated with efficient translation initiation (McCarthy and Brimacombe, 1994). Also, a sequence highly homologous to the bacterial translational enhancer downstream box (DB) (Sprengart *et al.*, 1990, 1996) is present at the 5' end of *nifA* (Figure 1). In spite of the sequence characteristics of the *nifA* TIR, independent translation of *nifA* is inefficient because a stable secondary structure prevents the binding of ribosomes when translation of *nifL* is not completed. Translation of *nifA* is therefore coupled to and limited by that of *nifL*, thus preventing the synthesis of an excess of NifA in relation to NifL (Govantes *et al.*, 1996).

Specialized ribosome systems usually consist of a mutant 16S rRNA gene with altered complementarity to the SD sequence. Ribosomes bearing this rRNA are then directed to a specific mRNA species displaying an altered SD that matches the mutant rRNA (Hui and de Boer, 1987). The use of specialized ribosomes has proven very useful to show that interaction of the SD in the mRNA and the complementary anti-SD sequence in the 16S rRNA provides the specificity in translation initiation (Hui and de Boer, 1987) and also to analyse functionally the 16S rRNA (Brink *et al.*, 1993, 1994). In this work we have designed a specialized ribosome system to address the mechanism of translational coupling at the *nifLA* operon. This approach has allowed us to determine that ribosomes translating *nifL* resume translation at the *nifA* TIR, and thus translational coupling in this operon occurs by a reinitiation mechanism. It has also provided us with some insights into the role of SD recognition in determining the efficiency of translational reinitiation and functionality of the putative DB of *nifA*. We believe that the use of specialized ribosome technology in a variety of translationally coupled gene pairs will lead to a better understanding of the mechanisms underlying the phenomenon of translational coupling.

Results

A specialized ribosome system to study translational coupling at the *nifLA* operon

An IPTG-inducible specialized ribosome system was constructed to study the mechanism of translational coupling

Fig. 2. Relevant characteristics of the plasmids used to analyse translational coupling of *nifLA* by normal or specialized ribosomes. (A) Plasmid overexpressing the specialized *rrnB* operon and a deletion derivative. (B) Reporter plasmids harbouring *nifL-lacZ* or *nifA-lacZ* protein fusions with different mutations in *nifL* or *nifA*, transcribed from the *lacUV5* promoter.

at the *nifLA* operon. The system comprises two sets of compatible plasmids. The first set consists of plasmids pIZ513 and pIZ514. pIZ513 contains a specialized *rrnB* operon whose expression is under control of the *tac* promoter and *lacI^q* (Figure 2A). This specialized ribosomal RNA operon contains two groups of mutations in the 16S rRNA gene. First, the anti-Shine-Dalgarno (ASD) sequence, responsible for the SD recognition, has been changed to CACAC (specialized anti-Shine-Dalgarno, ASDX). Ribosomes containing this 16S rRNA recognize an altered SD sequence GUGUG (specialized Shine-Dalgarno, SDX) (Hui and de Boer, 1987). Additionally, a C→T transition at position 1192 makes translation by ribosomes containing this 16S rRNA resistant to spectinomycin (Hui *et al.*, 1987; Brink *et al.*, 1993). As a negative control, plasmid pIZ514 is a derivative of pIZ513 which bears a deletion covering the whole 16S rRNA and *tRNA^{Glu}* genes, and also the 5' end of the 23S rRNA gene. The second set is a collection of plasmids harbouring *nifL-lacZ* or *nifA-lacZ* protein fusions transcribed from the *lacUV5* promoter (Figure 2B). Each type of fusion has different combinations of the natural SD or the specialized SDX in *nifL*, and the natural SD or the specialized SDX in *nifA*. As a negative control, two additional *nifA-lacZ* fusion plasmids were constructed. In these, *nifL* is translated from its own SD or the specialized

Fig. 3. Predicted most stable secondary structures of the mRNA region around the *nifA* TIR, bearing the different mutations used in this work. The structures and their ΔG_0 were displayed using the *mfold* 2.3 program, with the default settings and at 30°C.

SDX, while *nifA* carries a neutral SD called SDN, with the sequence GCUCG. The three central positions of this neutral SD are not complementary either to the wild-type ASD or the ASDX; therefore, poor recognition of SDN by both wild-type and specialized ribosomes is expected. Since independent translation of *nifA* is naturally inhibited by a stem-loop structure occluding its SD (Govantes *et al.*, 1996), plasmids bearing an altered SD sequence for *nifA* also carry compensatory mutations at the opposite side of the stem that restore a secondary structure with a similar stability to the wild-type (Figure 3).

When a pair of plasmids, one belonging to each set, were present in the same strain, addition of IPTG allowed simultaneous transcription of the modified *rrnB* operon and the *lacZ* fusion. Expression of *nifL* or *nifA* could then be monitored by production of β -galactosidase activity.

Translational coupling supported by wild-type or specialized ribosomes: effect of the Shine-Dalgarno recognition

Plasmids having different SD combinations in *nifL* and *nifA* were transformed into *Escherichia coli* ET8000 harbouring the plasmids pIZ513 or pIZ514, and differential rates of production of β -galactosidase activity were determined in the resulting strains. We have chosen *E. coli* for these experiments because the specialized ribosomal operon comes from this species. *Escherichia coli* and *K. pneumoniae* are closely related and translational coupling between *nifL* and *nifA* has been observed in both species (Govantes and Santero, 1996; Govantes *et al.*, 1996). An estimation of the apparent translational coupling efficiency was also calculated as the ratio between the differential rates of production of NifA- β -galactosidase

and its corresponding NifL- β -galactosidase for each SD sequence present in *nifA*. Differential rates of production were chosen because they provide a more accurate estimation of the rate of synthesis than single point determinations of accumulated β -galactosidase activity (Schmitz *et al.*, 1996). These data are summarized in Table I. Synthesis of NifL- β -galactosidase or NifA- β -galactosidase hybrid proteins having their natural SD sequences was not inhibited by the presence of specialized ribosomes (rows 1 and 2); therefore, the pool of mutant ribosomes does not seem to interfere with the normal translation of these genes. In fact, their differential rates of production increased slightly in the presence of the specialized 16S rRNA. The increase was similar for both hybrid proteins, suggesting that translational coupling efficiency was not significantly affected. The differential rates of production of NifA- β -galactosidase were higher (~1.5-fold) than those of NifL- β -galactosidase both in the presence and in the absence of specialized ribosomes. The synthesis of a greater amount of the distal gene product has been reported for several translationally coupled operons (Yates and Nomura, 1981; Gerstel and McCarthy, 1989). However, the higher rate of production of NifA- β -galactosidase may be explained in several alternative ways. Quantification of radiolabelled proteins (see below, Figure 5) suggested that this higher rate of β -galactosidase production is due to a slightly higher specific activity of NifA- β -galactosidase.

When the SD of *nifA* was substituted for SDX or SDN (Table I, rows 3 and 4), the differential rates of production of NifA- β -galactosidase by normal ribosomes dropped significantly. The presence of specialized ribosomes did not improve production of the hybrid protein in these constructions, even when *nifA* carried the specialized SDX (row 3, compare 'minus' and 'plus' specialized ribosomes). Thus, translation of *nifL* by wild-type ribosomes does not allow specialized ribosomes to gain access to the SDX, nor can wild-type ribosomes reinitiate efficiently without the proper recognition of the SD sequence at the distal cistron. It is noteworthy that the decrease was more pronounced for the construction bearing SDN than for that bearing SDX (27-fold versus 7-fold). This suggests that, while SDN seems to abolish *nifA* translation almost completely, wild-type ribosomes still can initiate *nifA* translation from SDX, although their efficiency is reduced. Expression of *nifL-lacZ* bearing a specialized SDX sequence was low in the absence of specialized ribosomes (Table I, row 5), confirming the observation above that wild-type ribosomes recognize SDX quite inefficiently. Induction of the specialized rRNA provoked a 9-fold increase in the differential rate of production of NifL- β -galactosidase. Therefore, when the *nifL* SD is substituted by SDX, efficient translation becomes strongly dependent on the presence of specialized ribosomes.

When *nifL* carried the specialized SDX, expression of the *nifA-lacZ* fusion bearing its natural SD was unexpectedly high in the absence of specialized ribosomes (Table I, row 6). Although poor translation of the preceding *nifL* gene by normal ribosomes may be responsible for some coupled translation of *nifA*, the differential rate of production was too high and led to an apparent translational coupling efficiency >100%. One explanation for this observation is that *nifA* is at least partially translated

Table I. Differential rates of production of β -galactosidase activity from *nifL-lacZ* or *nifA-lacZ* fusions bearing different SD sequences, in the absence or presence of specialized ribosomes. Relative translational coupling efficiency is also shown

Row	Plasmid	Type of fusion	Shine-Dalgarno in		Differential rate ^a Specialized ribosomes		% Coupling efficiency ^b Specialized ribosomes	
			<i>nifL</i>	<i>nifA</i>	–	+	–	+
1	pIZ576	<i>nifL-lacZ</i>	WT	–	4528	5309	–	–
2	pIZ574	<i>nifA-lacZ</i>	WT	WT	7219	7769	100.0	100.0
3	pIZ587	<i>nifA-lacZ</i>	WT	SDX	1107	1324	15.3	17.0
4	pIZ589	<i>nifA-lacZ</i>	WT	SDN	268	340	3.7	4.4
5	pIZ575	<i>nifL-lacZ</i>	SDX	–	1203	11348	–	–
6	pIZ573	<i>nifA-lacZ</i>	SDX	WT	3112	7876	162.3	47.4
7	pIZ579	<i>nifA-lacZ</i>	SDX + Δ SacII ^c	WT	738	942	NA	NA
8	pIZ586	<i>nifA-lacZ</i>	SDX	SDX	589	16187	30.7	97.5
9	pIZ588	<i>nifA-lacZ</i>	SDX	SDN	101	508	5.3	3.1

NA, not applicable.

^aEstimated as Δ activity (nmol ONP/min/ml)/ Δ OD₆₀₀.

^bEstimated as the ratio between differential rates of production of NifA- β -galactosidase and its corresponding NifL- β -galactosidase. Values are relative to coupling efficiency when *nifL* and *nifA* have their natural SD.

^c*nifL* bears a 2 bp deletion at the distal *SacII* site, leading to a premature stop codon 68 nucleotides upstream of the *nifA* SD.

in an independent fashion. Row 7 shows the level of independent translation of *nifA* under these conditions. Induction of the specialized 16S rRNA clearly increased the differential rate of production of NifA- β -galactosidase from this construction (2.5-fold). Although this increase can be interpreted as the result of translational coupling, albeit with much lower efficiency than in the wild-type situation, other explanations are equally possible. The simplest one is that translation of *nifL* by specialized ribosomes stimulates independent translation of *nifA* by stabilizing the mRNA. However, a 2 bp deletion at the very distal *SacII* restriction site in *nifL*, which produced a stop codon 68 nucleotides upstream of the *nifA* SD, abolished this increase (compare rows 6 and 7, 'plus' specialized ribosomes). Therefore, it appears that full translation of *nifL* is required for stimulating the expression of *nifA*, suggesting that such increase is indeed due to translational coupling. This is further demonstrated by showing that translation of *nifA-lacZ* under these conditions is essentially carried out by specialized ribosomes (see below and Figure 5).

When both *nifL* and *nifA* bore a specialized SDX (Table I, row 8), the differential rate of production of NifA- β -galactosidase was low in the absence of specialized ribosomes. Comparison of the levels of expression of the *nifL-lacZ* and *nifA-lacZ* fusions in the absence of specialized ribosomes (rows 5 and 8) shows that translational coupling was poor (31%) when translation of *nifL* was accomplished by wild-type ribosomes. Therefore, inefficient recognition of the SDX by wild-type ribosomes effectively reduces coupled translation of *nifA*. Production of the NifA- β -galactosidase hybrid was strongly increased in the presence of specialized ribosomes, achieving an apparent translational coupling efficiency similar to that obtained when both genes carried their natural SD sequences. Thus, coupled translation of *nifA* by either type of ribosome is equally efficient, provided that the SD of *nifA* can be efficiently recognized by the ribosomes translating *nifL*. These data support a reinitiation mechanism for translational coupling in this operon.

When *nifL* carried the specialized SDX and *nifA* carried SDN (Table I, row 9), the differential rate of production

of NifA- β -galactosidase was very low. When specialized ribosomes were present, the expression of this fusion was stimulated 5-fold. Again, this increase could be attributed to mRNA stabilization due to active translation of *nifL*, or to residual translational coupling despite the poor recognition of the SDN by both wild-type and specialized ribosomes. The very low values of translational coupling efficiency for this construct suggest that recognition of the SD sequence at the *nifA* TIR is required for coupled translation in this system.

Translation of both *nifL* and *nifA* is carried out by the same type of ribosome

The fact that maximum translational coupling efficiency was only accomplished when *nifL* and *nifA* carried the same type of SD suggests that translation of *nifA* is at least partially achieved by a reinitiation mechanism. However, when *nifL* was translated from the specialized SDX and *nifA* from its own wild-type SD, a stimulation of the production of NifA- β -galactosidase was observed upon induction of the specialized 16S rRNA. If this increase is due to translational coupling between both genes, then it may be explained by a facilitated binding mechanism allowing ribosomes from solution to gain access to the *nifA* TIR.

If a mechanism of facilitated binding was involved in translational coupling between *nifL* and *nifA*, translation of *nifL* by either wild-type or specialized ribosomes should allow fresh ribosomes to bind the wild-type *nifA* SD from solution. Since the affinity of wild-type ribosomes for a wild-type SD sequence is higher than that of specialized ribosomes, *nifA* would be expected to be translated preferentially by wild-type ribosomes in this situation. However, if translational coupling occurred by a reinitiation mechanism, translation of *nifA* would be necessarily performed by the same ribosomes that translated *nifL*, i.e. translation of *nifL* by specialized ribosomes would also make *nifA* be translated by specialized ribosomes.

In order to determine which type of ribosome translates *nifA* when translation of *nifL* is carried out by specialized ribosomes, we took advantage of the fact that translation by ribosomes harbouring the specialized rRNA is resistant

Fig. 4. Effect of spectinomycin on accumulation of β -galactosidase activity from *nifL-lacZ* (A and C) or *nifA-lacZ* (B and D) fusions, in the presence of specialized ribosomes, when *nifL* bore its natural SD (A and B) or the specialized SDX (C and D); *nifA* always had its natural SD. Values shown are only those after 4 h of induction by IPTG, when spectinomycin was added.

to spectinomycin. Accumulation of β -galactosidase activity in strains bearing *nifL-lacZ* or *nifA-lacZ* fusions was monitored in the presence of specialized ribosomes and the effect of the addition of spectinomycin to the culture medium was determined for each fusion. When *nifL* carried its natural SD, accumulation of NifL- β -galactosidase and NifA- β -galactosidase was blocked shortly after addition of spectinomycin (Figure 4A and B). This suggests that specialized ribosomes could not efficiently recognize the wild-type SD sequences of *nifL* or *nifA* and that, as expected, translation of both cistrons was essentially carried out by wild-type ribosomes.

When *nifL* bore the specialized SDX, accumulation of β -galactosidase activity from the *nifL-lacZ* fusion slowed down significantly after the addition of spectinomycin (Figure 4C). Since most of the translation of this gene fusion is carried out by spectinomycin-resistant specialized ribosomes (Table I, row 5), this reduction may be attributed to a general decline in translation due to blockage of the synthesis of protein components of the translational machinery. Alternatively, specialized ribosomes may exhibit some residual spectinomycin sensitivity. However, NifL- β -galactosidase continued to accumulate for at least 3 h after the addition of spectinomycin, showing that synthesis of the hybrid protein was still taking place in the presence of the drug. Interestingly, the evolution of β -galactosidase activity from the wild-type *nifA-lacZ* fusion located downstream of *nifL* bearing an SDX (Figure 4D) followed the same pattern as that of the activity from the *nifL-lacZ* fusion (i.e. the activity still accumulated several hours after the addition of spectinomycin). Thus, *nifA* was at least partially translated by specialized ribosomes, despite the fact that its TIR carried a wild-type SD. These results strongly suggest that translational coupling at the *nifLA* operon is carried out by a reinitiation mechanism.

One caveat in the latter experiment is that the changes in activity after the addition of spectinomycin are relatively

small when compared with the activity previously accumulated in the cultures. In order to avoid this high background and to obtain a more quantitative view of the data above, the effect of spectinomycin on the synthesis of the NifL- β -galactosidase and NifA- β -galactosidase hybrids was also monitored by *in vivo* protein radiolabelling with [35 S]methionine after the induction of the specialized rRNA. Radioactive bands were subsequently visualized after resolution on SDS-PAGE (Figure 5) and quantitated on an isotope detector. As expected, in the absence of spectinomycin, all strains tested displayed numerous labelled bands corresponding to the normal translational activity of *E.coli* under these conditions. Bands corresponding to each hybrid protein were identified in the corresponding lanes by comparison with lane 1, which lacks any *lacZ* fusion. Their mobilities agree with their predicted sizes.

Addition of 50 mg/l spectinomycin to the culture medium before labelling resulted in a strong reduction in the intensity of most radiolabelled protein bands, indicating that translation by wild-type ribosomes was efficiently repressed at this drug concentration. However, some bands corresponding to cellular proteins were still evident. These bands did not disappear at a 10-fold higher concentration of spectinomycin (not shown). This suggests that, as previously reported (Hui *et al.*, 1987; Brink *et al.*, 1993), specialized ribosomes can direct the synthesis of some *E.coli* proteins. When *nifL* bore its natural SD, labelling of NifL- β -galactosidase was strongly reduced in the presence of spectinomycin (Figure 5, lane 3 versus lane 4). Addition of spectinomycin also prevented the labelling of NifA- β -galactosidase, regardless of the type of SD located at the *nifA* TIR (lanes 5 versus 6 and 7 versus 8). Therefore, specialized ribosomes are unable to initiate independent translation of *nifA*, even when its SD is substituted by the specialized SDX. Although direct quantification of the bands in the absence of spectinomycin was not possible due to the high background, visual inspection shows that *nifA* translation from SDX was much less efficient than that from its own SD.

In close agreement with the results shown in Table I, translation of *nifL-lacZ* from the specialized SDX was highly dependent on the presence of specialized ribosomes (Figure 5, lanes 9 versus 10). As expected, the hybrid NifL- β -galactosidase was efficiently labelled both in the absence and presence of spectinomycin (lanes 10 versus 11). Similar results were obtained for NifA- β -galactosidase when both *nifL* and *nifA* bore the specialized SDX (lanes 15, 16 and 17). When *nifL* bore an SDX and *nifA* bore its own SD, NifA- β -galactosidase was significantly produced in the absence of specialized ribosomes (lane 12). Nevertheless, the presence of specialized ribosomes increased this production (lane 13). Under these conditions, addition of spectinomycin (lane 14) did not prevent labelling of NifA- β -galactosidase, therefore indicating that *nifA* was translated by specialized ribosomes, despite the fact that it bore a normal SD. Since *nifA* cannot be translated by specialized ribosomes unless *nifL* bears a specialized SDX, the specialized spectinomycin-resistant ribosomes responsible for the synthesis of NifA- β -galactosidase observed in lane 14 must have initiated from the SDX in *nifL* and then reinitiated at the wild-type SD of *nifA* in this construction.

Fig. 5. Effect of spectinomycin and specialized ribosomes on synthesis of NifL- β -galactosidase and NifA- β -galactosidase, in relation to their respective SD sequences. Only the upper part of the gel is shown. Bars in the non-numbered lanes represent proteins of 200 and 116 kDa. Arrows show the positions of the two hybrid proteins.

After background subtraction and normalization to the number of methionines, quantitation of the bands indicated that NifL- β -galactosidase and NifA- β -galactosidase bearing the specialized SDX (Figure 5, lanes 11 and 17) were produced in similar amounts. This indicates that the reinitiation frequency is $\sim 100\%$ under these conditions, resulting in a NifL/NifA ratio of 1. Since both *nifL* and *nifA* are translated almost exclusively by specialized ribosomes in this situation (Table I, rows 5 and 8), these data also suggest that the ~ 1.5 -fold higher activity of NifA- β -galactosidase versus NifL- β -galactosidase, consistently observed when translational coupling was optimal (Table I), is probably due to a slightly higher specific activity of NifA- β -galactosidase and not to a higher production of the hybrid protein.

Spectinomycin-resistant production of NifA- β -galactosidase having its own SD (Figure 5, lane 14) was $\sim 40\%$ of that of NifL- β -galactosidase. There is a good correlation between the ratio of NifA- β -galactosidase/NifL- β -galactosidase calculated from differential rates of production (47.4%, see Table I) and that estimated from quantitation of the radioactive bands after the addition of spectinomycin. This suggests that most or all of the translation of *nifA* is also carried out by specialized ribosomes, regardless of the presence of a wild-type or specialized SD. Therefore, the stimulation of NifA- β -galactosidase production from the *nifA* SD in the presence of the specialized 16S rRNA (Table I, row 6; see previous section) is largely due to reinitiation by specialized ribosomes.

Mutational analysis of the putative downstream box in *nifA*

While it is clear that maximum translational coupling is only achieved when *nifL* and *nifA* carry the same type of SD sequence, specialized ribosomes were still able to reinitiate at the *nifA* wild-type SD with $\sim 40\%$ efficiency. The *nifA* TIR shares a number of features with very efficient TIRs, such as extended complementarity to the 3' end of the 16S rRNA and an appropriately spaced start codon (McCarthy and Brimacombe, 1994). Additionally, the 5' end of the coding sequence of *nifA* resembles the bacterial translational enhancer downstream box (DB). This element is complementary to a region of the 16S rRNA (positions 1469–1483) (anti-downstream box or anti-DB) and it has been shown to improve translational

Fig. 6. Complementarity between wild-type or DB mutant coding sequences of the 5' end of *nifA* and the proposed anti-downstream box in the 16S rRNA. Lack of pairing is represented by lower-case letters. Mutated positions are underlined.

efficiency (Sprengart *et al.*, 1990, 1996). Ten out of 12 nucleotides at the 5' end of the *nifA* coding sequence may pair to the anti-DB (Figure 6). The relevance of this putative DB in translational coupling was assessed by analysing the effects on translation of *nifA* of two sets of site-directed mutations which significantly reduced the pairing between the putative DB and the anti-DB (mutations DB-1 and DB-2 in Figure 6). Mutated nucleotides lay on a branch of the secondary structure present at the *nifA* TIR. Changes in DB2 should not greatly affect TIR accessibility and changes in DB1, if they have any effect, should improve access of ribosomes (Figure 3).

DB-1 and DB-2 mutations were transferred to pIZ574 to construct plasmids pIZ584 and pIZ585, and also to pIZ573 to yield plasmids pIZ582 and pIZ583, respectively (see Figure 2). These plasmids were transformed into ET8000 bearing pIZ513 and β -galactosidase production was monitored after the induction of the specialized 16S rRNA. The effects of the mutations on the differential rates of production of NifA- β -galactosidase are shown in Figure 7.

When translation was carried out by normal ribosomes (Figure 7A), the more extensive mutation DB-2 had a slight but reproducible effect on the differential rate of production of NifA- β -galactosidase (77% of that for the wild-type DB). However, the DB-1 mutation had no effect (95%). When *nifL* bore the specialized SDX (Figure 7B), the mutations had a significantly stronger effect (61 and 42% for DB-1 and DB-2 mutations, respectively). These results suggest that the putative DB of *nifA* is functional, although its contribution to translational coupling efficiency is minor when the SD is efficiently recognized by the ribosomes. However, the stronger effect observed

Fig. 7. Effect of DB mutations on coupled translation of *nifA*, when *nifL* bore its natural SD (A) or the specialized SDX (B). All strains produced specialized ribosomes, and bore a plasmid containing *nifA*–*lacZ* fusions with the natural SD in *nifA*, and different DB sequences. As controls, cultures of ET8000 bearing each of the plasmids containing the corresponding *nifL*–*lacZ* fusion were also assayed, although it is not shown for the sake of clarity.

when translation of *nifA* was initiated by specialized ribosomes suggests that the interactions between ribosomes and the DB may be more relevant in systems in which the SD sequence is defective or lacking.

Discussion

In this work we describe a new version of a specialized ribosome system, which allowed highly specific and efficient translation of a gene carrying a specialized SD sequence. The system is based on transcription of a specialized *rrnB* operon from the strong *tac* promoter, which is controlled by the *lacI^q* allele. The specialized 16S rRNA also bears a point mutation that makes it spectinomycin-resistant. The target gene, *K. pneumoniae nifL*, encoding an oxygen and nitrogen sensor protein, was fused in frame to *lacZ* and placed under the control of the *lacUV5* promoter. Addition of IPTG to the culture medium allowed simultaneous induction of the specialized rRNA and the *nifL*–*lacZ* gene fusion and gene expression could then be monitored by assaying β -galactosidase activity. The potential advantages of this system are: (i) the specialized rRNA is transcribed from a strong but regulated promoter, allowing the generation of a large pool of mutant ribosomes in a controlled fashion; (ii) unlike high-temperature induction used in previous versions (Hui and de Boer, 1987; Brink *et al.*, 1993, 1994), IPTG induction does not have a major effect on cell physiology or stability of mRNA secondary structures; (iii) the use of a regulated promoter at the target gene minimizes the background activity problems observed previously (Rex *et al.*, 1994); and (iv) spectinomycin resistance of the specialized rRNA allows direct measurement of the contribution of specialized ribosomes to the translation of the target gene by addition of the drug to the culture medium. When *nifL* bore its natural SD sequence, its expression was unaffected by the presence of specialized ribosomes. However, when the *nifL* SD sequence was substituted by the specialized SDX, its expression was low in the absence of specialized ribosomes and was greatly increased upon induction of the mutant rRNA. Our data clearly show that *nifL* can be efficiently translated by either type of ribosome and that recognition of its SD specifies the type of ribosome which translates the gene.

This system was used to investigate the mechanism of translational coupling between the *nifL* gene and its counterpart *nifA*. The regulatory *nifA* gene is located immediately downstream of *nifL* and we have previously demonstrated translational coupling in this gene pair (Govantes *et al.*, 1996). The use of a specialized ribosome system allowed us to examine *in vivo* both the molecular mechanism underlying the stimulation of the translation of *nifA* by ribosomes translating *nifL* and the role of SD recognition in determining the translational coupling efficiency.

Translation of *nifL* and *nifA* was driven by different combinations of wild-type and specialized SD sequences, in the presence of wild-type and specialized ribosomes. When both genes bore specialized SDX sequences, translation of *nifA* was strongly stimulated by the presence of specialized ribosomes and the translational coupling efficiency in this situation was similar to that achieved when both genes bore their natural SD sequences. Thus, translational coupling between *nifL* and *nifA* by normal or specialized ribosomes is equally efficient provided that the SD sequence of *nifA* can be effectively recognized by the ribosomes translating the preceding *nifL* gene. However, translational coupling efficiency dropped significantly when *nifL* and *nifA* bore different types of SD sequences in any combination (Table I). If a facilitated binding mechanism was operating at the *nifLA* operon, the translational coupling efficiency should not have been greatly affected by the type of ribosome translating *nifL*, since recognition of the downstream SD by ribosomes translating the upstream gene is not required in this model. Therefore, a significant proportion of the coupled translation was carried out by a reinitiation mechanism.

The construction in which *nifL* is translated from the SDX and *nifA* from its own SD displayed an unexpected pattern of expression. The activity from this fusion was fairly high in the absence of specialized ribosomes. Approximately 40% of its expression could not be explained by its translation being coupled to *nifL* translation. In spite of this high background, translation of *nifL* by specialized ribosomes still increased the expression of *nifA*, resulting in an apparent coupling efficiency of ~50% (Table I). This could either be explained by facilitated binding of wild-type ribosomes from solution, or by specialized ribosomes reinitiating translation at the wild-type SD. Both accumulation of β -galactosidase activity and *in vivo* radiolabelling after the addition of spectinomycin to cultures expressing the specialized rRNA, show that virtually all of the *nifA* translation was carried out by specialized ribosomes (Figures 4 and 5). Therefore, reinitiation is also the mechanism operating in this situation, despite the poor recognition of the *nifA* SD by the specialized ribosomes.

Taken together, these data demonstrate that translation of *nifL* cannot facilitate translation of *nifA* by new ribosomes binding from solution and therefore translational coupling in this operon functions by a reinitiation mechanism. Although it is possible for ribosomes terminating translation of *nifL* to melt the secondary structure at the *nifA* TIR, apparently this temporary melting is not sufficient to allow binding of fresh ribosomes to the *nifA* RBS. Also, binding of ribosomal subunits from solution could probably be simultaneously prevented by the terminating

ribosome itself, given the overlapping of the *nifL* translational stop and *nifA* translational start signals. Therefore, reinitiation may be the only possible way to achieve translation of the downstream genes in most of the translationally coupled gene pairs, which have a relatively simple secondary structure and in which the stop and start codons are very close together. However, since reinitiation is limited by the distance between the stop and start codons (Adhin and van Duin, 1990), facilitated binding by ribosomes from solution may still be an operative mechanism for those gene pairs whose coding regions are separated by longer distances.

Quantitation of the labelled bands corresponding to the hybrid NifL- β -galactosidase and NifA- β -galactosidase proteins after the addition of spectinomycin indicates that translation of *nifA* under these conditions is essentially performed by specialized ribosomes. However, independent translation of *nifA* in this construction was significantly high in the absence of specialized ribosomes. These apparently conflicting results may be explained if independent translation initiation of *nifA* is only significant when *nifL* is translated poorly, and it is inhibited when efficient translation of *nifL* promotes frequent reinitiation events at the *nifA* TIR. As stated above, the presence of terminating ribosomes at the *nifA* TIR region may prevent ribosomal subunits in solution from binding the *nifA* SD.

In our system, mispairings in the three central positions between specialized anti-SD and the consensus SD or between normal anti-SD and specialized SDX still allowed significant reinitiation, albeit with reduced efficiency. This is consistent with the view that the requirement of a recognizable RBS, although relevant for reinitiation, is less stringent than it is for independent initiation (Draper, 1996). However, mispairings between either anti-SD and the neutral SDN reduced the translational coupling efficiency to ≤ 5 , suggesting that interaction between the SD and the anti-SD is crucial for efficient reinitiation. Similar results have been reported when the SD of *atpA* was substituted (Rex *et al.*, 1994). Since the mismatched nucleotides in the SDN are in the same three central positions of the SD, this result suggests that reinitiation frequency may be affected not only by the number of mispairings but also by the identity of the mispaired nucleotides.

DB sequences have been described as translational enhancers which may even promote translation in the absence of a SD (Sprengart *et al.*, 1990, 1996), although their function is much less characterized and some conflicting data have been reported (Resch *et al.*, 1996). The identification of a putative DB at the 5' end of the *nifA* coding region prompted us to analyse its possible effect on translational coupling. Mutational analysis showed that the DB markedly increased *nifA* translation by specialized ribosomes, which are expected to bind the *nifA* SD inefficiently. This result suggests that the DB of *nifA* is functional. In contrast, we could only detect a minor effect of the DB on *nifA* translation by normal ribosomes efficiently recognizing the *nifA* SD. However, since the DB is present and is functional, it is possible that it contributes to maximize the expression of *nifA* under different physiological conditions, or is required for the fine tuning that allows the cell to achieve a correct stoichiometry between NifL and NifA.

It has been previously suggested (Resch *et al.*, 1996) that the reported effects of mutations in several DBs may be due to reduced mRNA stability, lower specific activity of the resulting proteins, or the generation of rare codons at the mutated positions, rather than to reduced translation initiation. However, our data show that the magnitude of the effects of the DB mutations largely depend on the ability of the ribosomes translating *nifA* to recognize its SD, thus indicating that these mutations affect the translation initiation process. Therefore, none of the arguments above is likely to apply to our system. Also, it is very unlikely that the observed effects of the DB mutations are due to slight differences in the secondary structure which may reduce ribosome accessibility, since differences in the effects depending on the type of ribosome cannot be explained either. Our results suggest that DB recognition may be most relevant to the reinitiation process when the translating ribosomes are not able to bind efficiently the SD of the downstream gene. This model might also apply to *de novo* translational initiation, since the recognition signals are the same in both processes. In support of this view, a DB has been shown to promote translation initiation even in the absence of a SD (Sprengart *et al.*, 1996). However, a more complete characterization of DB sequences at different cistrons will be required before general conclusions on their relevance and function can be drawn.

In summary, construction of an efficient and specific specialized ribosome system has allowed us to demonstrate that translational coupling between *nifL* and *nifA* operates exclusively by reinitiation, and to assess the relevance of SD recognition and the functionality of a putative DB sequence in *nifA* translation. Similar systems may be very useful for a more detailed characterization of translational coupling in other gene pairs and also to analyse the effect of a DB on reinitiation or *de novo* translation initiation, in relation to the efficiency of the SD recognition.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strains used in this study were ET8000 (*rbs gyrA hutC_{Ka} lacZ::IS1 Mucts62*) (Buck, 1986), DH5 α [*F⁻ ϕ 80d lacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17 (*r_K- m_K-*) supE44 thi-1 gyrA relA1*] (Hanahan, 1983) and CJ236 [*dut ung thi relA*; pCJ105 (Cm^r)] (Bio-Rad Laboratories).

Plasmids used in this work and their relevant features are shown in Figure 2. The complete *rrnB* operon with the specialized ASDX from pP_LASDX-CATX (Brink *et al.*, 1993) was cloned under the control of the *tac* promoter in pMM40 (Kleiner *et al.*, 1988) and then a *Bam*HI-*Eag*I fragment bearing the *rrnB* operon, the *tac* promoter and the *lacIq* repressor was cloned into *Eag*I- and *Clal*-digested pACYC184 to yield pIZ513. pIZ514 is identical to pIZ513, but carries a *Kpn*I-*Sac*I deletion that eliminates the 16S rRNA and rRNA^{23S} genes and the 5' end of the 23S rRNA gene (Brink *et al.*, 1993). pIZ576 is a *nifL-lacZ* protein fusion at the unique *Nco*I site under control of the *lacUV5* promoter, cloned between the *Eco*RI and *Sall* sites in pT7-5 (Tabor, 1994). This fusion encodes a NifL- β -galactosidase hybrid protein bearing 329 amino acids of NifL. pIZ574 is a *nifLA-lacZ* protein fusion at the unique *Nar*I site under control of the *lacUV5* promoter, cloned between the *Eco*RI and *Sall* sites in pT7-5. This fusion encodes a NifA- β -galactosidase hybrid protein bearing 188 amino acids of NifA. pIZ587 and pIZ589 are identical to pIZ574 but the *nifA* SD sequence has been site-directed mutagenized to produce the specialized SDX or the neutral SDN, respectively. pIZ575, pIZ573, pIZ586 and pIZ588 are identical to pIZ576, pIZ574, pIZ587 and pIZ589, respectively, but the *nifL* SD sequence has been substituted by the specialized SDX. This SDX sequence was

introduced as a 20 bp *HindIII*–*EcoRI* fragment (see Hui and de Boer, 1987), which leaves a 10 bp spacer between the specialized SD and the *nifL* start codon. pIZ579 is identical to pIZ573, but carries a 2 bp deletion at the distal *SacII* restriction site in *nifL*. This means that translation of *nifL* stops 68 nucleotides upstream of the *nifA* SD. pIZ582 is identical to pIZ573, but carries the mutant downstream box DB-1 at the 5' end of *nifA*. pIZ583 is based on pIZ573, but carries the mutant downstream box DB-2. pIZ584 and pIZ585 are equivalent to pIZ582 and pIZ583, but are based on pIZ574. Standard cloning procedures were used for these constructions. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase and the Klenow enzyme were purchased from Boehringer and used according to the manufacturer's instructions. All mutations in the final plasmids were confirmed by sequencing.

Site-directed mutagenesis

Oligonucleotide site-directed mutagenesis was used to generate mutant versions of the SD or the putative DB at the 5' end of *nifA*. The *SacII*–*KpnI* fragment containing the 3' end of *nifL* and the 5' end of *nifA* was cloned in pBluescript SK+ (Stratagene) and transformed into CJ236. The procedure was performed following the instructions of the Mutagenesis Phagemid *in vitro* mutagenesis kit (Bio-Rad), and in agreement with the method described by Kunkel (1985). The mutagenic oligonucleotides were: NIFA-SDX (CGTCTGACGTATGTGTCGGAATCGGATTTATGGATCATTTGCTCACACACGGTCAGGG), NIFA-SDN (CGTCTGACGACGTGTCCGAATCGGATTTATGGATCATTTGCTCACGAGCGGTCAGGG), DB-1 (AATCGGATAAAAAGGATCATT) and DB-2 (GAATCGGAAAATAAGATCATT). Underlined nucleotides indicate the mutagenic positions. Mutations were screened by sequencing. All of the mutations preserved the secondary structure occluding the SD of *nifA*, as confirmed with the Zuker's program *mfold* version 2.3, available on the Internet (Zuker's home page: <http://www.ibc.wustl.edu/~zuker/rna/>). To achieve this, oligonucleotides NIFA-SDX and NIFA-SDN carry two sets of mutations to alter the SD sequence of *nifA* and to reconstitute the secondary structure affected by the first set of mutations (Figure 3). Mutations were reintroduced into the appropriate plasmids as *SacII*–*KpnI* fragments.

Growth conditions for β -galactosidase assays

β -galactosidase assays were performed according to Miller (1992). Since ribosomal content of cells is affected by growth conditions (Keener and Nomura, 1996) analysis of expression of *nifL* and *nifA* was performed under nitrogen-limiting conditions, the physiological conditions for expression of these genes. *Escherichia coli* ET8000 cells harbouring the appropriate combinations of plasmids were grown in K medium (MacNeil *et al.*, 1978) with arginine (2 g/l) as the sole nitrogen source. Chloramphenicol (7.5 mg/l) and ampicillin (100 mg/l) were also added to the culture medium to prevent loss of plasmids. Cultures were grown at 30°C to an OD₆₀₀ of 0.2–0.3. IPTG was then added to the cultures at a final concentration of 1 mM, and samples were withdrawn for β -galactosidase assays at timed intervals. Activities per ml were plotted versus OD₆₀₀ and differential rates of production of β -galactosidase were determined from the slopes, as $\Delta\text{activity}/\Delta\text{OD}_{600}$ (Schmitz *et al.*, 1996). Efficiency of translational coupling was estimated as the ratio between differential rates of production of NifA– β -galactosidase and NifL– β -galactosidase hybrid proteins.

To identify the type of ribosomes translating *nifL* and *nifA*, cultures were split in two flasks at 4 h after the addition of IPTG. Spectinomycin was added to one of the samples to a final concentration of 50 mg/l and accumulation of β -galactosidase was monitored.

Radiolabelling of proteins

As above, cultures of ET8000 with the appropriate combinations of plasmids were split in two flasks 3.5 h after the addition of IPTG. Spectinomycin was added to one of the samples at a final concentration of 50 mg/l. 45 min later, 2.2 $\mu\text{Ci}/\text{ml}$ [³⁵S]methionine was added to each culture. Samples were taken 30 min after addition of [³⁵S]methionine and run on a 7.5% SDS–PAGE gel (Laemmli, 1970). After drying the gel, radioactive bands were quantitated with an isotope detector Fuji BAS1000 Image Plate scanner.

Acknowledgements

We are grateful to Dr de Boer for providing pPLASDX-CATX. This work was funded by the Comisión Interministerial de Ciencia y Tecnología (grant BIO93-0407), a fellowship from El Monte-Junta de Andalucía

to F.G. and a fellowship from the Spanish Ministerio de Educación y Ciencia to E.A.

References

- Adhin,M.R. and van Duin,J. (1989) Translational regulation of the lysis gene in RNA bacteriophage ϕ requires a UUG initiation codon. *Mol. Gen. Genet.*, **218**, 137–142.
- Adhin,M.R. and van Duin,J. (1990) Scanning model for translational reinitiation in eubacteria. *J. Mol. Biol.*, **213**, 811–818.
- Brink,M., Verbeet,M. and de Boer,H. (1993) Formation of the central pseudoknot in 16S rRNA is essential for initiation of translation. *EMBO J.*, **12**, 3987–3996.
- Brink,M., Nels,R., Verbeet,M. and de Boer,H. (1994) Specialized ribosomes allow for the study of mutations in functionally important regions in 16S rRNA, without affecting cell growth. The identification of functional regions in the central domain of 16S rRNA. *J. Mol. Biol.*, **237**, 368–377.
- Buck,M. (1986) Deletion analysis of the *Klebsiella pneumoniae* nitrogenase promoter: importance of spacing between conserved sequences around positions -12 and -24 for activation by the *nifA* and *ntnC* (*glnG*) gene products. *J. Bacteriol.*, **166**, 545–551.
- Dallmann,H. and Dunn,S. (1994) Translation through an *uncDC* mRNA secondary structure governs the level of *uncC* expression in *Escherichia coli*. *J. Bacteriol.*, **175**, 1242–1250.
- Draper,D. (1996) Translational initiation. In Neidhart,F.C., Curtis,R.,III, Ingraham,J.L., Lin,E.C.C., Low,K.B., Magasanik,B., Reznikoff,W.S., Riley,M., Schaechter,M. and Humbarger,H.E. (eds), *Escherichia coli and Salmonella. Cellular and Molecular Biology*. ASM Press, Washington DC, pp. 902–908.
- Gerstel,B. and McCarthy,J.E.G. (1989) Independent and coupled translation initiation of *atp* genes in *Escherichia coli*: experiments using chromosomal and plasmid-borne *lacZ* fusions. *Mol. Microbiol.*, **3**, 851–859.
- Govantes,F. and Santero,E. (1996) Transcription termination within the regulatory *nifLA* operon of *Klebsiella pneumoniae*. *Mol. Gen. Genet.*, **250**, 447–454.
- Govantes,F., Molina-López,J.A. and Santero,E. (1996) Mechanism of coordinated synthesis of the antagonistic regulatory proteins NifL and NifA of *Klebsiella pneumoniae*. *J. Bacteriol.*, **178**, 6817–6823.
- Grodzicker,T. and Zipser,D. (1968) A mutation which creates a new site for the re-initiation of polypeptide synthesis in the Z gene of the *lac* operon of *Escherichia coli*. *J. Mol. Biol.*, **38**, 305–314.
- Hanahan,D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, **166**, 557–580.
- Henderson,N., Austin,S. and Dixon,R. (1989) Role of metal ions in negative regulation of nitrogen fixation by the *nifL* gene product from *Klebsiella pneumoniae*. *Mol. Gen. Genet.*, **216**, 484–491.
- Hill,S., Kennedy,C. and Kavanagh,E. (1981) Nitrogen fixation gene (*nifL*) involved in oxygen regulation of nitrogenase synthesis in *K. pneumoniae*. *Nature*, **290**, 424–426.
- Hui,A. and de Boer,H.A. (1987) Specialized ribosome system: preferential translation of a single mRNA species by a subpopulation of mutated ribosomes in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **84**, 4762–4766.
- Hui,A., Jhurani,P. and de Boer,H.A. (1987) Directing ribosomes to a single mRNA species: a method to study ribosomal RNA mutations and their effects on translation of a single messenger in *Escherichia coli*. *Methods Enzymol.*, **153**, 432–452.
- Ivey-Hoyle,M. and Steege,D.A. (1992) Mutational analysis of an inherently defective translation initiation site. *J. Mol. Biol.*, **224**, 1039–1054.
- Keener,J. and Nomura,M. (1996) Regulation of ribosome synthesis. In Neidhart,F.C., Curtis,R.,III, Ingraham,J.L., Lin,E.C.C., Low,K.B., Magasanik,B., Reznikoff,W.S., Riley,M., Schaechter,M. and Humbarger,H.E. (eds), *Escherichia coli and Salmonella. Cellular and Molecular Biology*. ASM Press, Washington DC, pp. 1417–1431.
- Kleiner,D., Paul,W. and Merrick,M. (1988) Construction of multicopy expression vectors for regulated over-production of proteins in *Klebsiella pneumoniae* and other enteric bacteria. *J. Gen. Microbiol.*, **134**, 1779–1784.
- Kunkel,T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA*, **82**, 488–492.
- Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–683.

- Lesage,P., Chiaruttini,C., Graffe,M., Dondon,J., Milet,M. and Springer,M. (1992) Messenger RNA secondary structure and translational coupling in the *Escherichia coli* operon encoding translational initiation factor IF3 and the ribosomal proteins L35 and L20. *J. Mol. Biol.*, **228**, 366–383.
- MacNeil,T., MacNeil,D., Roberts,G.P., Supiano,M.A. and Brill,W. (1978) Fine-structure mapping and complementation analysis of *nif* (nitrogen fixation) genes in *Klebsiella pneumoniae*. *J. Bacteriol.*, **136**, 252–266.
- McCarthy,J.E.G. and Brimacombe,R. (1994) Prokaryotic translation: the interactive pathway leading to initiation. *Trends Genet.*, **10**, 402–407.
- McCarthy,J.E.G. and Gualerzi,C. (1990) Translational control of prokaryotic gene expression. *Trends Genet.*, **6**, 78–85.
- Merrick,M., Hill,S., Hennecke,H., Hanh,M., Dixon,R. and Kennedy,C. (1982) Repressor properties of the *nifL* gene product in *Klebsiella pneumoniae*. *Mol. Gen. Genet.*, **185**, 75–81.
- Miller,M. (1992) *A Short Course in Bacterial Genetics. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 72–74.
- Resch,A., Tedin,K., Gründling,A., Mündlein,A. and Bläsi,U. (1996) Downstream box-anti-downstream box interactions are dispensable for translation initiation of leaderless mRNAs. *EMBO J.*, **15**, 4740–4748.
- Rex,G., Surin,B., Besse,G., Schneppe,B. and McCarthy,J.E.G. (1994) The mechanism of translational coupling in *Escherichia coli*. *J. Biol. Chem.*, **269**, 18118–18127.
- Sarabhai,A. and Brenner,S. (1967) A mutant which reinitiates the polypeptide chain after chain termination. *J. Mol. Biol.*, **27**, 145–162.
- Schmitz,R.A., He,L. and Kustu,S. (1996) Iron is required to relieve inhibitory effects of NifL on transcriptional activation by NifA. *J. Bacteriol.*, **178**, 4679–4687.
- Sprengart,M., Fatscher,H. and Fuchs,E. (1990) The initiation of translation in *E. coli*: apparent base-pairing between the 16S rRNA and downstream sequence of the mRNA. *Nucleic Acids Res.*, **18**, 1719–1723.
- Sprengart,M.L., Fuchs,E. and Porter,A. (1996) The downstream box: an efficient and independent translation initiation signal in *Escherichia coli*. *EMBO J.*, **15**, 665–674.
- Tabor,S. (1994) Gene expression using the T7 TNA polymerase/promoter system. In Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds), *Current Protocols in Molecular Biology*. John Wiley, New York.
- Yates,J.L. and Nomura,M. (1981) Feedback regulation of ribosomal protein synthesis in *E. coli*: localization of the mRNA target sites for repressor action of the ribosomal protein L1. *Nature*, **294**, 190–192.

Received December 30, 1997; revised February 16, 1998;
accepted February 17, 1998