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Translationally coupled initiation of protein synthesis in *Bacillus subtilis*

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ABSTRACT

The neomycin phosphotransferase gene (neo) from Transposon Tn5 is active in Gram-negative bacteria but silent in B.subtilis since it lacks an appropriate ribosome binding site for Gram-positive bacteria. Neo translation could be reactivated by coupling its initiation to the translational termination of the highly expressed β-lactamase gene (penP) from B.licheniformis. This initiation occured at the authentic neo start codon. Its efficiency was independent of the nucleotide sequence 5' to the neo gene, but strongly affected by the distance between the termination and initiation codon. It was the highest if both codons overlapped in the sequence ATGA. In B.licheniformis, a translationally coupled neo gene was inducible expressed as the penP gene demonstrating the potential of the technique to monitor the activity of expression units for which no direct assays exists.

INTRODUCTION

In procaryotic systems translational initiation is strongly influenced by sequences 5' to the initiation codon which usually contains a polypurine stretch complementary to the ribosomal 16S RNA, the Shine-Dalgarno (SD)-sequence (1), and which may also be involved in the formation of RNA secondary structures making the initiation codon more or less accessible for the ribosomes (2,3). In addition, translational initiation can be affected by tranlational termination events close to the initation codon. Originally only suggested by polar effects on the translation of the polycistronic trp operon in Escherichia coli (4) this phenomenon, termed "translationally coupling", has been verified in a variety of experimental situations in E.coli demonstrating that translation termination enhances translation initiation of closely associated genes (5,6).

In most cases studied, translational coupling is superimposed on de novo initiation but there is also experimental evidence indicating that "restart events" may be induced within a gene in the absence of a SD-sequence (7,8,9,10). To test directly that translationally coupled initiation was completely independent and different from de novo initiation, we aimed to study this reaction in the absence of functionally active SD-sequences. Therefore, B.subtilis was used as a host, since this Gram-positive bacterium requires a much more extensive SD-complementarity for de novo initiation than the E.coli system (11,12) used in the studies of translational coupling mentioned above. As model system for translationally coupled genes two antibiotic resistance marker genes encoding easily measurable enzymes were coupled. The leading reading unit used was the penP gene from B.licheniformis encoding the highly expressed β-lactamase (13,14) and, as the following indicator for translationally coupled initiation, the "Gram-negative" neo gene from Transposon Tn5 encoding the neomycin phosphotransferase II (NPT II; 15,16). Using this experimental system, we examined the influence of sequences 5' to the initiation codon and of the distance between the termination and initiation codons on the efficiency of translational coupling. By introducing a translationally coupled penP/neo construct into B.licheniformis, we also demonstrated the usefulness of translational coupling in monitoring the gene expression of an inducible chromosomal gene.

MATERIALS AND METHODS

Bacterial strains

E.coli K-12 strain C600 r^+c^- (17) and B.subtilis 168 strain BD170 trpC2, thr-5 (18) were used in all experiments. B.licheniformis RHO311 strain 9945A arg13$^-$, pep7, penI (19) and the penP constitutive mutant IHO4917 obtained after NNG-treatment of RHO311 were gifts from R.Palva, B.licheniformis 749/C (20) was a gift from K.Simons.

Transformation

Bacterial transformation by plasmid DNAs was carried out using standard procedures described for E.coli (21) and

B.subtilis (22). B.licheniformis cells were transformed essentially as described by Sherratt and Collins (23). In cases where the penP gene should be induced, Cephalosporin C (1µg/ml) was added 30 min after the addition of the DNA and after an additional incubation for 2 hrs, the transformed cells were spread on indicator plates containing Cephalosporin C (1µg/ml) and Kananmycin (Km; 5µg/ml).

Media and growth conditions

E.coli cells were grown in Standard 1 bouillon (Merck, Darmstadt), B.subtilis cells in VY broth (Difco) supplemented with yeast extract (5mg/ml)(Difco). Antibiotic resistant cells were selected for in the presence of tetracycline (Tet; E.coli 10 µg/ml and B.subtilis 20 µg/ml), ampicillin (Amp; 50 µg/ml), and Km (E.coli, 25 µg/ml, B.subtilis, 5 µg/ml). For the quantification of the kanamycin resistance (KmR), transformed cells, selected by Tet, were transfered with toothpicks onto agar plates containing different concentrations of Km (1, 2.5, 5, 7.5, 10, 15, 30, 50, 70, and 100 µg Km per ml). After 16 hrs incubation at 37 °C, relative growth was monitored by colony size.

Preparation and analysis of cell extracts

Cells from 1ml of an overnight culture were collected by centrifugation, resuspended in 1 ml 50 mM Tris HCl pH8, 10% sucrose, 2mg/ml lysozyme, 100mM EDTA, incubated for 15 min at 37°C, sonicated and the cellular debris were removed by centrifugation. For the analysis, 20 µl of these crude extracts were separated by 10% SDS-polyacrylamide gelelectrophoresis (SDS-PAGE) using the gel system of Lämmli (24).

NPT II-ndPAGE assay

Crude cell extracts (10 µl) were seperated by nondenaturing PAGE (10% acrylamide) and the position of enzymatically active NPT II-like protein was determined in the gel by in situ phosphorylation of Km using [γ-^{32}P] as substrate (25).

β-lactamase assay

For the detection and quantitative evaluation of the β-lactamase production, the chromogenic cephalosporin Nitrocefin [Glaxo research Ltd.] was used which undergoes a

color change from yellow to red upon hydrolysis by β-lactamase. For colony screening, 2µl Nitrocefin (500µg/ml) was spotted on each colony.

Plasmid purification

Preparative plasmid purification from *E.coli* cells was carried out as described by Ish-Horowicz and Birke (26), small scale preparations from *E.coli* and from different *Bacillus* strains as described by Birnboim and Doly (27).

Plasmid constructions

All molecular cloning procedures were performed essentially as described by Maniatis et al. (17). Plasmid pJKK3-1 (28) was used as standard recipient for the *penP* or/and *neo* gene fragments.

pPEN plasmids: Plasmid pPEN1500-2 was constructed by inserting a 1500bp EcoRI* fragment from *B.licheniformis*, previously cloned in fdpen1540-1 (13), into vector pJKK3-1. For the construction of pPEN1240 the EcoRI/BclI *penP* gene fragment (pos.1-1240; 13) was isolated and ligated to a 34 bp BamHI adaptor fragment encoding two internal HindIII sites (AD16, H.Schaller unpublished). The ligated DNA fragments were cut with EcoRI and HindIII and the resulting EcoRI/HindIII *penP* restriction fragment were inserted into the EcoRI/HindIII digested plasmid pJKK3-1 creating pPEN1240 (see Figure 1). Starting from the HindIII site at the 3'end of the *penP* gene in pPEN1240 the *penP* gene was shortened further by Bal31 exonuclease digestion (Beck et al., 1982) followed by the addition of BamHI linkers. One plasmid obtained with this procedure, pPEN1180, contains an EcoRI/BamHI *penP* gene fragment (pos.1-1180) inserted into the plasmid pJKK3-1 cleaved by EcoRI and BamHI (29).

pRSB and pPN plasmids: Plasmid pRS2 was constructed by the insertion of the *neo* gene as a 1600pb BglII/BamHI fragment of pKm1 (16) into the BglII linearized plasmid pPEN1500-2. For the construction of pPRSB2 the same *neo* gene fragment was inserted into the plasmid pPEN1500-2 cleaved by BglII and BamHI. In pRSB-9 the BglII restriction site of pRSB2 was destroyed by treating the BglII linearized plasmid pRSB2 with nuclease S1 and religation. For the construction of pRSB+4,

the sticky ends of the restriction site BglII of pRSB2 were filled in with DNA-polymerase I (30). Plasmid pRSB+43 was obtained by the insertion of the 43 bp BamHI adaptor fragment containing a synthetic *lac* operator sequence and two EcoRI sites (AD3; 31) into the BglII site of pRSB2. The junction sequences of plasmids pRSB-9, pRSB+4, pRSB+43 were confirmed by sequence analysis using the BclI site, next to the BglII site, for 5'end labelling (32). For the construction of pPN2, the *neo* gene was inserted as a 1170bp BglII/SalI fragment from pKm2 (16) into plasmid pPEN1180 cleaved by BamHI and SalI. Plasmids pRSB3 and pPN3 were obtained by replacing the BglII/SalI and BamHI/SalI vector fragments containing the *neo* gene of pPEN1240 and pPEN1180, respectively, with the *neo* gene fragment from pKm3, containing a modified *neo* gene variant (33). To construct pPN34, the EcoRI site at the 5' end of the *penP* insert in pPN3 was destroyed by a filling-in reaction using DNA-polymerase I and, in a second step, the adaptor fragment AD3 (see above) was inserted into the BamHI site localized in the *penP*/*neo* junction sequence. Subsequently the internal *EcoRI* adaptor fragment was deleted creating pPN34. Plasmid pPN4 was obtained by the substitution of the EcoRI/SalI fragment of pPN34 containing the *neo* gene variant with the EcoRI/SalI fragment of pKm2 carrying the authentic *neo* gene.

pPKm plasmids: For the construction of pPKm2, a 270bp EcoRI/HindIII fragment containing the *penP* promoter and the *penP* ribosome binding site (pos.1-264) was inserted together with a HindIII/SalI fragment encoding the authentic *neo* gene into plasmid pJKK3-1 cleaved by EcoRI and SalI (see Figure 2). The EcoRI/HindIII *penP* fragment was provided by a plasmid, pPKm22, which was obtained in the same experiment as pPKm16, the construction of which is described in Reiss et al. (34). The HindIII/SalI *neo* fragment was provided by a plasmid in which the BglII site of pPKm2 was converted to a HindIII site. This was achieved by the insertion of a synthetic oligonucleotide HindIII linker into the BglII linearized plasmid pKm2 after filling in the sticky ends of the BglII restriction site with DNA-Polymerase I (29).

RESULTS

Plasmid constructions

Two series of plasmids (pRSB and pPN) encoding penP/neo bicistronic transcription units, were constructed starting from plasmids that carry penP genes inserted into the E.coli/B.subtilis shuttle vector pJKK3-1 (Figure 1).

In the first series of plasmids, the pRSB family, the coding sequence of the neo gene was linked downstream to the amino-terminal part of the penP gene containing the first 104 amino acid codons of the penP gene. The reading units of both genes were combined such that the translation of the penP reading unit terminates close to, or overlapping with, the start-codon of the neo gene. These constructs were obtained using the BglII restriction site in the penP gene as junction for the inserted neo gene sequences (Figure 1).

In the second series of analogous penP/neo constructs, the pPN family, a functionally intact penP gene was used. These

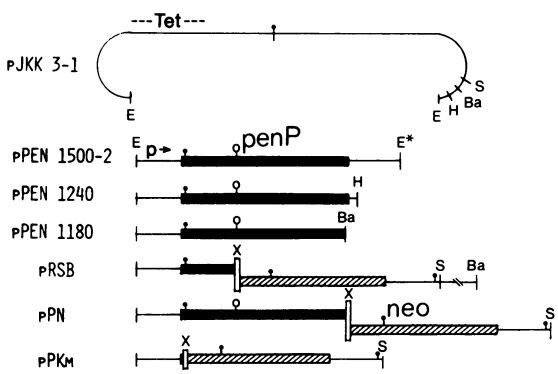

Figure 1: Schematic outline of penP/neo gene fusions and the penP segments used for their construction. pJKK3-1 is a B.subtilis/E.coli shuttle vector used as cloning vehicle and pRSB, pPN and pPKm represent plasmid families differing by the penP DNA fragments inserted. The open boxes (X) indicate sequences varying within a plasmid family at the penP/neo junction (see Figure 2). The structural sequences of the penP gene are represented as a black bar and that of the neo gene as a hatched bar. The promoter region of the penP gene is indicated by P->. Important restriction sites are marked by E=EcoRI, E*=EcoRI*, H=HindIII, Ba=BamHI and S=SalI. The restriction sites BglII and PstI are indicated by an open and closed circle, respectively.

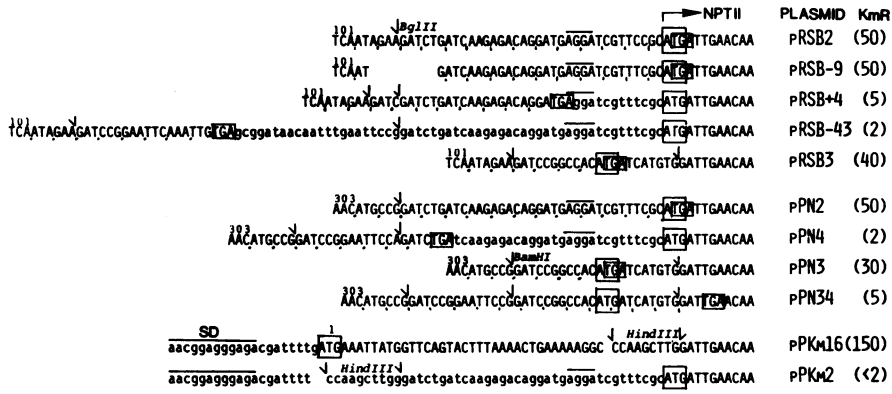

Figure 2: penP/neo junction sequences in plasmids from series pRSB, pPN and pPKm. The first start codon ATG in phase within the neo coding region is framed in a large box and the stop codon of the preceding penP reding unit is framed in a small box. Amino acid codons 101 and 303 of the penP gene are indicated as reference points and synthetic oligonucleotides sequences separating authentic penP and neo sequences are placed between arrows. The SD-region of the neo and the penP genes are overlined and marked by SD if functionally active in B.subtilis. The names of the plasmids and the KmR (μg/ml) induced in B.subtilis BD170 host cells are shown on the right hand side.

bicistrons contain all but the last three amino acid codons of the penP gene placed in front of the structural part of the neo gene. Compared to the pRSB series these constructs have the advantage that the translation of the leading reading unit could also be monitored, since the penP part still encodes functionally active β-lactamase (29). In the pPN plasmids the BamHI restriction site, introduced into the penP gene with a synthetic oligonucleotide linker 9 nucleotides upstream of its translational stop codon, is used as junction to the inserted neo gene sequences (Figure 1).

In addition, a third group of constructs, plasmids pPKm, were used as controls. These contain penP/neo gene fusions with drastically shortened penP reading units such as plasmid pPKm16 (Figure 2) where all but the 13 amino-terminal penP codons were deleted. The remaining amino acid codons of the penP gene were fused in phase to the structural part of the neo gene to express an amino-terminal NPT II fusion protein

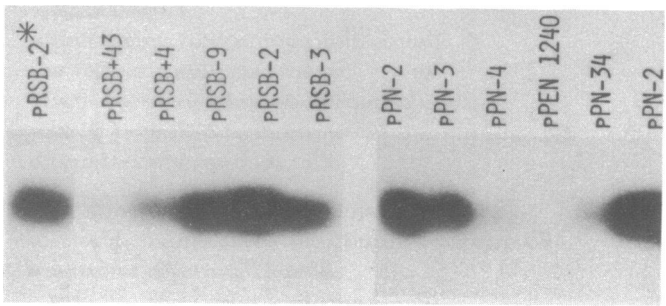

Figure 3: Qualitative and quantitative analysis of NPT II produced by B.subtilis cells transformed with the plasmids indicated. Crude extracts of $3x10^7$ cells from overnight cultures were separated by non-denaturing PAGE and the position and the amount of NPT II activity were determined as described (25). Lanes 1-6 and lanes 7-12 show the results of two independent experiments. The slot marked by an asterix shows a reference for the NPT II enzyme synthesised in E.coli ($1x10^7$ cells) harboring plasmid pRSB2.

under the control of the penP expression signals. In another plasmid, pPKm2, all codons of the penP gene were removed such that the penP promoter fragment lacking the translational start signal (and all of the penP structural gene) was directly linked to a neo gene fragment containing the neo coding region together with its ribosome binding site (Figure 2). Both plasmids were constructed using Bal 31 deletions in the penP gene starting from the PstI site 16 codons downstream of the penP initiation codon (34).

The expression of the neo gene can be coupled to the preceding penP translation

Ten bicistronic penP/neo constructs, six of series pRSB and four of series pPN (Figure 2) were analysed for NPT II synthesis to test whether the expression of the neo gene could be coupled to the translation of the preceding penP reading unit. Fur this purpose we quantitated the neo gene expression by measuring the KmR of B.subtilis cells harboring the corresponding plasmids, and by the NPT II-ndPAGE enzyme assay (25) which allowed a qualitative and quantitative evaluation of the NPT II protein produced.

The results obtained in these assays (Figures 2 and 3) indicate that all plasmids of the pRSB and pPN family express

the *neo* gene. The NPT II enzyme synthesised had the identical electrophoretic mobility as the authentic NPT II protein from *E.coli* cells (Figure 3) which suggests that the native *neo* start codon was used to initiate the NPT II protein synthesis. Different amounts of NPT II enzyme were produced by *penP*/*neo* constructs that differ in the distance between the *penP* termination and *neo* initiation codon indicating that the NPT II protein synthesis is activated by the translation of the preceding *penP* reading unit. Activation of *neo* gene expression was highest if the *neo* start codon overlapped with the termination codon of the *penP* reading unit in the sequence ATGA.

Sequences immediately in front of the this stop/start sequence do not seem to influence this coupled initiation reaction since the NPT II synthesis was not significantly affected by an exchange of sequences 5' to the ATGA sequence. This is exemplified by constructs in which the *penP* reading unit was linked, via the ATGA stop/start overlap, either to the authentic *neo* gene (plasmids pRSB2 and pPN2) or to a *neo* gene variant with altered sequences 5'to the *neo* coding region (plasmids pRSB3 and pPN3). Compared to the constructs carrying the authentic *neo* gene, the constructs with the *neo* gene variant mediate a slightly reduced KmR to their host cells (Figure 2) which is most likely due to a lower specific activity of the amino-terminally modified NPT II protein (34) and not to the exchange of sequences at the *penP*/*neo* junction. Therefore we conclude that sequences immediately in front of the initiation codon of the translationally coupled gene are not involved in the initiation reaction following translational termination, even if these sequences contain a ribosome binding site active in *E.coli*, as present in the constructs carrying the authentic *neo* gene.

This notion is supported further by the results obtained with plasmid pPKm2 (Figure 2) in which the *neo* gene expression is controlled directly by the *penP* promoter and the *neo* ribosome binding site and not by translational coupling. This *neo* gene chimera, pPKm2, was silent in *B.subtilis* (Figure 2) and active in *E.coli* (29). Thus, a contribution of the native

neo ribosome binding site in translational initiation could be ruled out for *B.subtilis* and therefore, the initiation of *neo* translation in our *penP*/*neo* constructs must be mediated exclusively by the chain termination of preceding translation.

Translational coupling depends on the distance between the termination and initiation codon

In the plasmids discussed so far the *penP* and the *neo* reading frames overlap at their termination and initiation codons to form the sequence ATGA. To investigate whether this particular situation was responsible for the translational coupling observed, we compared the *neo* gene expression of pRSB2 to that of constructs differing at the *penP*/*neo* junction sequence by the deletion of 9 nucleotides (pRSB-9), or by the insertion of 4 (pRSB+4) and 43 nucleotides (pRSB+43), respectively. As shown in Figures 2 and 3, an at least ten-fold reduced NPT II synthesis, compared to the parental plasmid pRSB2, was determined by the NPT II-ndPAGE assay or the KmR of the *B.subtilis* host cells for pRSB+4 and pRSB+43. In these constructs, the nucleotides inserted lead to a frameshift and an earlier termination of the *penP* translation either 12 (pRSB+4) or 57 nucleotides (pRSB+43) upstream of the translational start codon of the *neo* gene. In contrast, in pRSB-9, where the *penP* reading frame remained in phase, no change in *neo* gene expression was observed demonstrating that the reduced level of *neo* gene expression in pRSB+4 and pRSB+43 was not caused by the insertion of nucleotides into the junction sequence, but rather was the result of the earlier termination of *penP* translation.

This conclusion was confirmed by the results obtained with plasmids pPN4 and pPN34 which differ from their progenitors pPN2 and pPN3 by the insertion of the same 14 nucleotides at the *penP*/*neo* junction sequence (Figure 2). This insert moves translation termination 29 nucleotides upstream (in pPN4) or 11 nucleotides downstream (in pPN34) of the *neo* start site which leads to a NPT II synthesis drastically decreased compared to that of the parental constructs with overlapping termination and initiation codons. The expression of the

leading penP reading unit was not affected by the manipulation at the penP/neo junction since all pPN constructs induced equal β-lactamase synthesis as determined by the amount of β-lactamase detected in SDS-PAGE analysis of cell extracts harboring these plasmids (data not shown). Thus the reduced NPT II synthesis in penP/neo bicistronic constructs with different stop-start overlaps seems to reflect different reinitiation efficiencies of previously terminating ribosomes.

Efficiency of ribosomal reinitiation

The relative efficiency of the ribosomal reinitiation was determined by comparing the expression of translationally coupled neo genes to the translational activity of the preceding penP reading unit whose control elements remained unchanged in all constructs. The activity of this penP unit was determined by measuring the NPT II production of plasmid pPKm16 (Figure 2). In this plasmid the penP promoter and penP ribosome binding site including a few nucleotides downstream of the penP initiation codon were used to express a fusion protein consisting of the 13 amino-terminal amino acids from the penP reading unit and the whole NPT II protein. The amount of NPT II fusion protein expressed by this plasmid was as high as the amount of β-lactamase expressed by pPEN plasmids (app. 10^5 molecules per cell) as estimated from the intensity of the protein bands on a SDS-PAGE (29). Thus, the KmR mediated by pPKm16 seems to represent fairly well the expression rate induced by the penP expression signals in B.subtilis.

For the determination of the ribosomal reinitiation efficiency we therefore could compare the KmR induced in B.subtilis by pPKm16 (350μg/ml) to that induced by various constructs containing translationally coupled neo genes. Taking into account that the amino-terminally modified NPT II enzyme expressed by pPKm16 is 2-3 times less active than the authentic NPT II protein (34) we estimate from the KmR levels of 50μg/ml that about 5-10% of the ribosomes reinitiate at the neo initiation codon overlapping with the termination codon in the sequence ATGA as present in the constructs pRS82 and pPN2.

Inducible expression of translationally coupled neo gene in B.licheniformis

The above results suggest that a translationally coupled neo gene could be used as a marker to follow changes in the expression of the leading gene. To test this prediction experimentally we used this coupling effect to investigate the induction phenomenon of the chromosomal penP gene in B.licheniformis wildtype strains.

As a first step in these experiments we wanted to replace the chromosomal penP gene of B.licheniformis with a penP/neo construct expressing the neo gene coupled to the translation of preceding penP sequences as described for pRSB2. It had been shown before for B.subtilis that chromosomal genes can exchange genetic information by homologous recombination with fragments carried on plasmids (35,36). Assuming that this was also true for B.licheniformis, the inducible strain RHO311 (Materials and Methods) was transformed with the plasmid pRS2. This plasmid contains the penP/neo bicistron from pRSB2 plus 1kb of distal penP sequences 3' to the neo sequences in order to facilitate a double crossover leading to the replacement of the chromosomal penP gene by the penP/neo indicator gene. After transformation and selection for KmR in the presence of the β-lactamase inducer Cephalosporin C, 36 KmR transformants were isolated. 24 out of these were negative in β-lactamase production and Tet sensitive indicating that in these cells the integration of the penP/neo construct had occured at the penP locus of the chromosome destroying the penP gene with concomitant loss of the plasmid encoded Tet resistance marker. The remaining 12 transformants were also resistant to Tet and positive for β-lactamase production as assayed by Cephalosporin hydrolysis (see Material and Methods) indicating that these cells carried plasmid pRS2 as well as the intact endogenous penP gene. This interpretation was supported by the isolation and identification of plasmid pRS2 from the second, but not from the first class of transformants.

Two types of KmR transformants were also obtained using the penP constitutive strain B.licheniformis IHO4917 as recipient for pRS2. One class was β-lactamase negative and Tet sensitive

Table 1

Kanamycin resistance (µg/ml) of B. licheniformis strains [penP$^+$] transformed with pRS2 [Tet$^+$Km$^+$]

B. licheniformis strain	RH0311		IH04917	
phenotype of transformants [1]	type A	type B	type A	type B
KmR uninduced	< 10	50	350	350
KmR induced [2]	150	150	350	350

[1]) type A penP$^-$Tet$^-$Km$^+$ (chromosomal penP/neo integrate)
type B penP$^+$Tet$^+$Km$^+$ (chromosomal penP and plasmid pRS2)

[2]) induced by Cephalosporin C (1 µg/ml)

indicaitng that it contained the penP/neo gene integrated into the chromosome. The second class seems to carry the neo gene still on the transforming plasmid pRS2 since it exhibited KmR, Tet resistance and β-lactamase production.

We investigated next whether the neo gene was inducible by Cephalosporin C in these isolates. As shown in Table 1 this was the case in the inducible strain RH0311 no matter whether the penP/neo marker was integrated or present on the replicating plasmid pRS2. The two types of transformants of the constitutive strain were both resistant to high levels of Km (see Table 1) even in the absence of inducer.

From these results several conclusions can be drawn about the penP locus and its regulation. First, the penP locus is regulated by a diffusable component differing from the β-lactamase itself. Second, the penP sequences used in our constructs, isolated from a constitutive mutant 749/C (20) do not encode the information for a constitutive phenotype since these cloned penP sequences are nevertheless regulated by the pen sytem when introduced into the inducible strain RH0311. Thus, the mutation leading to a constitutive β-lactamase production in strain 749/C must be located outside the penP promoter region suggesting that the constitutive expression may be due to a defect in a diffusable factor, a repressor protein, acting in trans on the penP regulatory signals. These findings are consistent with the results obtained in gene

transfer experiments using less well defined chromosomal (23) or plasmid encoded *pen* genes (37).

DISCUSSION

The results presented in this study demonstrate, at least for procariotic systems, that translationally coupled initiation provides an alternative way to induce protein synthesis, which in contrast to the de novo initiation, does not depend on the host specific sequences in the ribosome binding site. Recent data suggest that this mode of initiation may function also in animal cells (38,39) and plant viruses (40). Thus, although of low efficiency this mechanism of translational initiation seems to be used in verry different biological systems.

Our data indicate that translationally coupled initiation is primarily affected by the distance between the start codon and the codon terminating the translation of the preceding gene. The initiation was as high as 10% in cases where the stop and the start codon overlapped in the sequence ATGA. Initiation was much lower (0,5%) if the translation terminated 12 or 28 nucleotides upstream of the start codon and also low (1%) if translation stopped 11 nucleotides downstream after traversing the initiation site. Thus, translational activation cannot be related to changes in the potential mRNA secondary structure at the ribosome binding site by the readthrough of ribosomes (2) but rather appears to depend on the enhanced local concentration of ribosomes or ribosomal subunits that are not released immediately from the mRNA after translational termination. A contribution of the nucleotide sequence in front of the *neo* gene is rendered very unlikely by the extensive sequence variation in our constructs which involve quite different oligonucleotide inserts and which use chain termination of *penP* translation either in the middle or at the very end of the *penP* gene.

Taken together, these data strongly argue that translationally coupled initiation is the consequence of ribosomes or ribosomal subunits scanning a mRNA chain for some distance after translational termination and leading to

restarts at nearby initiation codons independent of the sequence context and possibly also of ribosomal initiation factors. A similar conclusion has recently been drawn from an experiment in E.coli using a construct coupling the trpB and trpA genes in an overlapping stop/start sequence (TGATG) lacking the naturally occuring SD-sequence (6). Again, a restart efficiency of about 10% could be estimated supporting the notion that the close overlap of stop and start codon is required for efficient restart initiation.

The independence of host specific initiation signals opens the opportunity to use translational coupling to an indicator gene as a new universal method to indirectly monitor the expression of a preceding gene whose gene product cannot be assayed directly. As the stop/start initiation allows the authentic gene product of the indicator gene to be produced, this technique seems to be of advantage compared to the currently used alternative of measuring the strength of expression units by determining the synthesis of fused gene products which are of variable stability and biological activity (34,41,42). As an example of this use of a translationally coupled neo gene, we have presented an analysis of the regulation of the penP gene in its natural host B.licheniformis.

Another potential application of our finding relates to the fact that the translationally coupled neo gene can be used not only to monitor, but also to select for the expression of a preceding non-selectable gene by KmR. This is of special importance for B.subtilis expression systems (such as used in this study) which usually suffer from the instability of heterologous DNA inserts. Thus, we have recently used this technique to stabilize highly instable penP gene derivatives in B.subtilis which allowed us to study the synthesis and secretion of genetically altered β-lactamases. (Sprengel and Schaller submitted).

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