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Title (English)

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Title (Swedish)

Abstract

In order to identify proteins associated to the ribosome, a fast and simple purification method for the native ribosome was developed. Attempts were made to insert a tag in the chromosomal gene *L30*, which codes for a ribosomal protein, using the ?-RED system for homologous recombination. However, this system did not work during the time span of this study. Instead, a vector-construct with a His-tag on the *L30* gene was made and it could be seen that the ribosome, or at least parts of it, does bind to metal affinity resins.

Keywords

Ribosome, associated proteins, ?-RED, FLAG-tag, His-tag

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A one-step purification method of the *E. coli* ribosome with associated proteins

Christina Alm

Sammanfattning

Ribosomen är ett gigantiskt RNA- och protein-komplex, som översätter den genetiska koden till proteiner. Översättningen är en del i det komplicerade maskineri som utgör cellens genuttryck. Flera stora komplex och även mindre faktorer är inblandade i den, till synes, väl synkroniserade processen. En ingående studie av de proteiner som är associerade med ribosomen skulle därför kunna ge en bild av hur de olika molekylära systemen i cellens genuttryck är kopplade till varandra. Ett första steg i en sådan studie är att utveckla en metod som möjliggör rening av ribosomen i dess nativa tillstånd, med associerade proteiner.

Strategin var att sätta samman det ribosomala proteinet L30 med en peptid som har affinitet för en immobiliserbar matris. För att optimera reningen var tanken att DNA sekvensen för peptiden skulle kopplas samman med den kromosomala *L30* genen. För detta ändamål användes rekombinationssystemet ?-RED. Transformationsmetoden fungerade dock inte under tiden för denna studie. Istället utvecklades en vektorkonstruktion med *L30* genen kopplad till en His-tag, en peptid med sex histidin aminosyror. Då rening utfördes på en matris med affinitet för His-taggar, kunde ett UV-spektra typiskt för ribosomer urskiljas.

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1. INTRODUCTION

1.1 Aim and background

In spite of the remarkable advancement in ribosome research in the recent years there is a lack of extensive proteomic and genomic analysis of the ribosome-associated proteins and factors. This is mainly due to the lack of efficient and simple purification methods of the ribosome and its associated complexes. Last year the yeast ribosome was purified using epitope tagging followed by immuno-affinity purification (Inada *et al.* 2002). This study aims at designing a similar system for the bacterial ribosome in order to facilitate investigation of complexes associated to the ribosome.

The bacterial ribosome is a huge molecular machine that translates the messenger RNA as it emerges from the RNA polymerase. The process of translation is but a part of the complex machinery of gene expression, which includes inducers of gene transcription, the assembly of the RNA polymerase, splicing, translation, post translational editing and folding of the peptide chain, degradation of mRNA and finally, degradation of the synthesized protein. Each of these systems includes several factors, which can play important roles in coupling different activities to each other (Alberts, 1998). In recent years it has also become clear that prokaryotic molecular processes are spatially organised. As in eukaryotes there are molecular structures that build up a cytoskeleton (Jones *et al.* 2001, van den Ent *et al.* 2001). How all these structures interact and what makes the cellular processes so well synchronized and accurate are not clear at all.

Several non-ribosomal proteins that bind to the ribosome have already been identified; for example, factors and chaperons involved in ribosome biogenesis (Harnpicharnchai *et al.* 2001, Maki *et al.* 2002) and protein translocation (Prinz *et al.* 2000). The latter interaction has also been suggested to promote chromosome segregation due to movements of the translocons towards the membranes when membrane proteins are to be co-translationally inserted into the membrane (Woldringh 2002). This is one example of ideas that may be the outcome when looking into the proteins associated to the ribosome. Identifying cellular components that interact with the ribosome will certainly give further insight into the details of the gene expression machinery and the other molecular processes in the cell. Apart from the fundamental research interest that surrounds the ribosome, the ribosome is also of a medical interest, since it is the target of many antibiotics.

Interactions between proteins are usually studied using yeast two-hybrid assays or protein-chips. However, when investigating complex networks of molecules, the strategy is to purify one of the proteins in the complex under such mild conditions that all other molecules associated with it come along. Thereafter two-hybrid assays may be useful to determine the nature of each protein-ribosome interaction. Such a one-step purification method involves epitope tagging followed by affinity chromatography. Once the protein complex is purified, the components can be resolved in SDS-PAGE or in a 2D-gel followed by mass spectrometry analysis. The main long-term goal of this study is to take the ribosome through this procedure, but in the short time span of this project, the aim has been to develop a fast and simple purification technique for the bacterial ribosome, in its native condition.

Until now, ribosomes have been purified using centrifugation techniques. However, affinity purification on resins represents a more direct method over cosedimentation for assessing the associated proteins. Moreover, affinity purification is faster and simpler than the centrifugation techniques (Inada *et al.* 2002).

1.2 Ribosome structure and function

The bacterial ribosome is a giant complex of RNA and proteins. The total molecular weight is about 2.6 x 10^6 Da. Two thirds of the mass consists of RNA and one third consists of proteins. The bacterial ribosome is usually denoted 70S, a measure of its sedimentation rate in sucrose density gradients.

The 30S and the 50S subunits come together during initiation of translation to build up the 70S ribosome. The 30S subunit consists of a 16S ribosomal RNA (rRNA) and about 20 proteins. The 50S subunit weighs twice as much as the 30S subunit and is composed of a 23S rRNA, a 5S rRNA and over 30 proteins. The proteins are situated on the surface of the ribosome, stabilizing the RNA structure (Ban *et al.* 2000) and enhancing conformational changes in the functional regions (Gao *et al.* 2003). The small subunit mediates the contact with the messenger RNA (mRNA) to be translated whereas the large subunit contains the active site where the peptidyltransferase reaction occurs.

The elongation cycle of the amino acid incorporation to the polypeptide-chain is a complex process, involving several factors, where many details remain to be explained. Three binding sites accommodate amino acyl-tRNAs and free tRNA. These are the A (amino acyl), P (peptidyl) and E (exit) sites. Every codon-anticodon pair passes these three sites during translation. The synthesis starts with initiation where the Shine-Dalgarno sequence of the mRNA binds to the 16S rRNA in the 30S subunit. Initiation factors IF2 and IF3, help selecting the initiator aminoacyl-tRNA, fMet-$tRNA^{fMet}$ and promote 70S ribosome formation (for a review see Ramakrishnan, 2002). Initiation factor IF1 is also necessary for initiation of protein synthesis, but its precise role remains obscure (Dahlquist and Puglisi, 2000).

The elongation cycles involve the elongation factor EF-Tu, which in complex with the aminoacyl-tRNA and GTP is called a ternary complex, which presents the aminoacyl-tRNA to the A-site. When the GTP is hydrolyzed, the EF-Tu and GDP are released from the ribosome and the peptidyl transferase reaction takes place. It is still not known exactly how this reaction occurs. To translocate the peptide chain from the A-site to the P-site, EF-G along with GTP is believed to bind near the A-site. When the GTP is hydrolyzed, translocation occurs and EF-G with GDP is released. The E-site tRNA is released when the next ternary complex binds in the A-site.

Termination of synthesis happens when a stop codon (UAA, UAG or UGA) enters the A-site. One of the two peptide release factors RF1 or RF2 binds to the codon along with the release factor RF3. This binding triggers the hydrolysis and release of the peptide chain from tRNA in the P-site.

During the elongation cycles, there are at least two proofreading steps, discriminating the non-cognate codon-anti codon bindings from the cognate ones (for a review see Ramakrishnan, 2002).

1.3 The cloning strategy

1.3.1 The fusion

When tagging the ribosome, one of the 50 proteins can be chosen for the fusion. However, it is important to select a protein with suitable characteristics such that it does not severely interfere with ribosome

function; it must not have any catalytical function, it must not be situated so that a fused tag prevents the mRNA, the aminoacyl-tRNAs or the nascent peptide from entering or exiting the ribosome. However, the problem of placing the tag in a way that it does not prevent docking proteins or is restricted by them, being prevented from binding the column, remains unsolved.

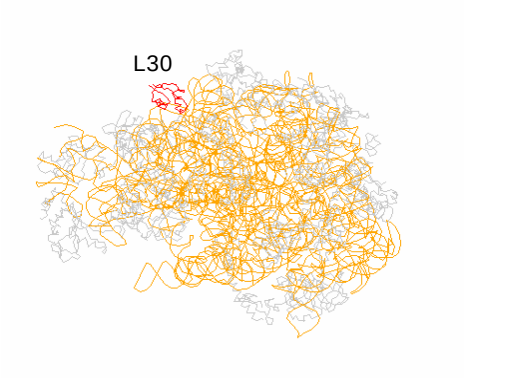

Fig 1. *The ribosomal 50S subunit in EF-G·GTP bound state. The structure is solved by Gao et al. (2003) using cryo-electron microscopy (PDB accession number 1P85). From this view, the 30S subunit should be placed below the 50S subunit to obtain the 70S ribosome. RNA is shown in yellow and ribosomal proteins are shown in grey. The L30 protein is indicated in red.*

Fig 2: *The E. coli ribosomal L30 protein backbone. The protein consists of 58 amino acids, weighs 6411 Da and is located on the surface of the 50S subunit, having both the N-terminal (upper) and the C-terminal (lower) directed outwards from the ribosome. The structure is a part of the 50S structure shown in figure 1.*

The small ribosomal protein L30 (also known as rpmD), located on the surface of the large subunit, was found to be a suitable candidate. It has both C- and N-terminals pointing outwards from the ribosome, it has no known catalytic function and it is far away from both entrance and exit channels.

To obtain cells homogenous for the epitope tagged protein, and to avoid tagged L30 protein in excess, the modified gene should preferably be inserted in the chromosome, replacing the original gene. The alternative is to insert the gene in a vector and to erase the wildtype gene from the chromosome, which involves double screening. Moreover, expression of the tagged protein from a vector may lead to a large amount of free tagged L30 protein, which makes an additional purification step necessary in order to separate the tagged ribosomes from the tagged L30 proteins.

Since the screening of the chromosome insertion was very time consuming, a transformation with a vector construct was made in parallel to examine the binding efficiency of the fusion tag on the ribosome.

1.3.2 The transformation

Two transformation methods were used; one traditional vector cloning and one newer method, called λ-RED, where linear single stranded oligonucleotides are added to the bacterial chromosome via electroporation with *E. coli* strains containing λ prophage genes. This latter method was developed by Yu *et al.* (2002) and has quickly become widely used. The method for chromosomal engineering is based on homologous recombination and therefore the methodology is often referred to as recombineering (for review see Court *et al.* 2003).

The prophage genes *exo*, *bet* and *gam* give the gene products Exo, Beta and Gam. These proteins take active part in recombination. The prophage genes are

under transcriptional control of the temperature sensitive ?cI857 repressor. Transcription is therefore induced by a temperature shift to 42°C for 15 minutes, which gives a tight regulation and coordinate expression, leading to high recombination frequencies. Gam inhibits the RecBCD nuclease from digesting linear DNA and Exo and Beta generate recombination activity (Yu *et al.* 2000). Exo creates sticky ends on dsDNA, enabling recombination. However, in this study ssDNA was used, making the exo gene product superfluous. Deletion of *gam* only decreases the number of transformants five-fold when recombining with single-stranded oligonucleotides (Ellis *et al.* 2001). This is probably due to the fact that RecBCD degrades single-stranded oligonucleotides much more slowly than double-stranded ones (Sriprakash *et al* 1975). Hence, Beta is the only absolutely needed recombinase since it binds to the oligonucleotide and protects it from single-strand nuclease attack. The Beta protein also helps the linear fragment to anneal to the complementary sequence on the chromosome.

This method works for fragments with DNA homology sequences as short as 30-50 bp on the ends of linear DNA (Yu *et al.* 2000). The linear DNA therefore contains the tag surrounded by 30 bp on each end, which will be incorporated in the chromosomal gene via homologous recombination.

Insertion of ssDNA oligos most probably happens when the chromosomal DNA is single stranded, as it is during replication. At the replication fork, the lagging strand is exposing a larger stretch of single stranded region than the leading strand does, and therefore the oligonucleotide identical to the Okazaki fragment would be expected to give a tenfold higher frequency of transformants (Ellis *et al.* 2001). The efficiency of this system when using a single-stranded oligo complementary to the lagging strand is in the order of one transformant per thousand surviving cells after electroporation (Yu *et al.* 2003). However, the oligo sequence itself can affect the recombination efficiency ten times down or a hundredfold up (Constantino and Court 2003). Using double-stranded oligos seems to always give fewer recombinants (Yu *et al.* 2003).

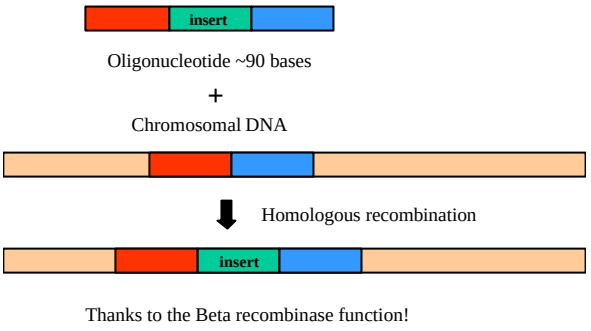

Fig 3: *Transformation of linear DNA into the chromosome using the ?-RED system.*

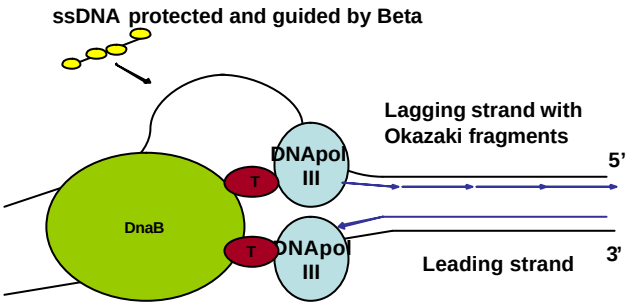

Fig 4: *The situation at the replication fork. The lagging strand exposes more ssDNA than the leading strand does, resulting in larger recombination frequencies for ssDNA oligos identical to the Okazaki fragments.*

1.3.3 Tags

There are a number of available fusion tag systems, ranging from one amino acid to whole enzymes with a size of more than 100 kDa (Einhauer and Jungbauer 2001). The means of interaction with the solid phase during chromatography vary as well as the elution approach, which can involve changing the pH, salt concentration or the concentration of a competitor to the binding. Even more advanced systems, which involve restriction enzymes, can be found.

In this study a very mild elution method is required; changing the pH or salt concentration can decrease the stability of the complex with associated proteins. Moreover, the tag should be quite small to avoid interaction with the complex itself. In order to make the system useful for other scientists, the components of the system should be commonly available and not too costly. Two such tags are the His and the FLAG™ tag. In this case, the His-tag consists of six or seven histidines, fused into one of the terminals of the L30 protein. Purification is accomplished by the histidines chelating to divalent ions in a solid phase and elution is made by competition with imidazole, an aromatic molecule mimicking the side chain of histidine. The drawback of this method is that imidazole degrades RNA, demanding a dialysis step immediately after elution. Using the immunogenic FLAG™ tag AspTyrLysAspAspAspAspLys instead requires a very expensive antibody. The great advantage of the FLAG™ tag, though, is the large specificity due to the immunogenic properties. Since these two tags have different characteristics, advantages and drawbacks, it is interesting to try both systems and to find out which one meets our demands the best.

For the vector construct, the FLAG™ tag was not used since the large amount of tagged proteins would demand large scale affinity chromatography, leading to the use of large amounts of costly antibody. Instead the histidine tag was used for the vector construct.

2. METHODS

2.1 Transformation

2.1.1 ?-RED cloning

Oligonucleotides for the recombination are described in Table 2 and fig 5. Since the *L30* gene is located on the left replichore of the chromosome and its position is counter clockwise (Colibri World-Wide Web server: www.genolist.pasteur.fr/colibri/ (14 Nov. 2003)), oligos complementary to the gene sequence itself should be used in order to make them anneal as Okazaki fragments during replication.

Competent cells for induction of the λ-RED system and for electroporation were prepared as described by Yu *et al* (2000): Overnight cultures of the strains described in Table 1, grown at 30°C, were diluted 50 fold in 50 ml LB and grown at 30° C until OD_{600} was 0.5-0.6. The recombination system was induced in a 42°C water bath with shaking for 15 min. All subsequent steps were performed at 0°C-4°C.

Table 1: Strains used in transformation experiments

Strain	Genotype
DY329	W3110 ?*lacU169 nadA*::Tn10 *gal490 λcI857* ?(*cro-bioA*)
DY380	DH10B {λ*cI857* (*cro-bioA*) <> *tet*}
NC397	Tet^S Kan^R *gal490* {λ*cI857*?(*cro-bioA*)}
HME5	W3110 ?(*argF-lac*) *U169*{λ*cI857*?(*cro-bioA*)}
HME6	W3110 ?(*argF-lac*) *U169*{λ*cI857*?(*cro-bioA*)}*galK* *tyr145UAG*
TOP10	F- *mcrA* ?(*mrr-hsdRMS-mcrBC*) *f80lacZDM15* ?*lacX74* deo^R *recA1 araD139* ? (*ara-leu*)7697 *galU galK rpsL* (Str^R) *endA1 nupG*

All strains except TOP10 were kindly provided by D. Court, National Cancer Institute-Frederick. TOP 10 cells were purchased from Invitrogen (cat nrC404006).

The samples were transferred to pre-cooled centrifuge tubes and were spinned down in 5 000x g for 10 min at 4° C. Each pellet was dissolved in 2 ml water and thereafter diluted in 30 ml water, followed by a second spin down and a repetition of the washing. The pellets were dissolved in 1 ml water each and spinned down in eppendorf tubes at maximum speed in a micro centrifuge for 10 min at 4°C. The pellets were resuspended in 400 µl water.

50 µl ice-cold cell suspension was mixed with oligonucleotides (1 pmol in the weo first transformations and 5 pmoles in the third) and was electroporated using pre-cooled 0.1 cm cuvettes in a Bio-Rad gene pulser set at 1.8 V and 25 µF with a pulse controller set at 200 Ω.

Electroporated cells were added to 5 ml LB and were grown for two hours and were thereafter diluted 10^6 times and spread on LB-plates. Cells electroporated with the control oligo were spread on MacConkey agar containing 1% galactose. The plates were incubated at 30.

```
                                          5'ccggt
      atcatcgccggtggtgcaatgcgcgccgttctggaagtc
      gctggggttcataacgttctggctaaagcctatggttcc
      accaacccgatcaacgtggttcgtgcaactattgatggc
      ctggaaaatatgaattctccagaaatggtcgctgccaag
      cgtggtaaatcc gttgaagaaattctggggaaataaacc

  1 - atg gca aag act att aaa att act caa acc
 31 - cgc agt gca atc ggt cgt ctg ccg aaa cac
 61 - aag gca acg ctg ctt ggc ctg ggt ctg cgt
 91 - cgt att ggt cac acc gta gag cgc gag gat
121 - act cct gct att cgc ggt atg atc aac gcg
151 - gtt tcc ttc atg gtt aaa gtt gag gag taa

      gagatgcgtttaaatactctgtctccggccgaaggctcc
      aaaaaggcgggtaaacgcctgggtcgtggtatcggttct
      ggcctcggtaaaaccggtggtcgtggtcacaaaggtcag
      aagtctcgttctggcggtggcgtacgtcgcggtttcgag
      ggtggtcagatgcctctgtaccgtcgtctgccgaaattc
      ggctt3'
```

***Fig 5**: The L30 gene with surrounding sequences. The flanking regions of the oligos used for the ?RED transformation will anneal to the underlined sequences.*

Table 2: oligonucleotides used for ?-RED transformation, and the final sequences

Oligonucleotide	5'-3' Sequence	Producer
FLAG	GCGGGTTTGAGTAATTTTAATAGTCTTTGC**TTTGTCATCGTCGTC(** **TGTAGTC**CATGGTTTATTTCCCCAGAATTTCTTCAAC	Invitrogen
Final gene sequence	GTTGAAGAAATTCTGGGGAAATAAACCATG**GACTACAAGGACGA** **GATGACAAA**GCAAAGACTATTAAAATTACTCAAACCCGC	
HIS	GCGGGTTTGAGTAATTTTAATAGTCTTTGC**ATGATGATGATGATG**/ **GATG**CATGGTTTATTTCCCCAGAATTTCTTCAAC	Sigma-Genosys
Final gene sequence	GTTGAAGAAATTCTGGGGAAATAAACCATG**CATCATCATCATCAT(** **ATCAT**GCAAAGACTATTAAAATTACTCAAACCCGC	
Control oligo	AAGTCGCGGTCGGAACCGTATTGCAGCAGCTT**TAT**CATCTGCCGCTG GACGGCGCACAAATCGCGCTTAA	Sigma-Genosys

The final gene sequence will arise after a second replication. The sequences in bold represent the actual tag, which will be N-terminal fused to the L30 gene. The bold TAT in the control oligo is the codon that will be back-mutated from the stop codon TAG.

2.1.2 USER friendly cloning

The L30 gene was amplified with PCR using primers designed for cloning with the USER kit (New England Biolabs), see Table 3. The PCR reaction ingredients were as described for the PCR method, with 55°C annealing.

10 µl PCR products were mixed with 20 ng of the 2.7 kb linear vector pNEB205A (containing *lacZa*, multiple cloning site, origin of replication and *bla*) and one unit USER (uracil-specific excision reagent) enzyme, which will produce sticky ends on the PCR product, by removing the ggagacau sequence (see fig 7). The sticky ends on the DNA fragment can then anneal to the complementary sticky ends (ggagacat) in the linearized vector pNEB205A, which there after will be circular.

The mixture was incubated for 15 min at 37°C and for additional 15 min at room temperature, where after 100 µl TOP10 Chemically Competent E. coli cells (Invitrogen) were transformed with 10 µl of the mixture by incubating on ice for 30 minutes followed by 30 seconds heat-shocking at 42°C and chilling on ice for 1 min.

Table 3: Primers used for USER friendly cloning and sequencing of the insert.

Primers	5'-3' Sequence	Producer
Start	GGAGACAUAAGGAGGT**AACCATGGCAAAGACTATTAAAATT**	Invitrogen
End	GGGAAAGUTTAGTGGTGGTGGTGGTGGTG**CTCCTCAACTTTAACCATGAA**	Invitrogen
M13 forward	TGTAAAACGACGGCCAG	
M13 reverse	CAGGAAACAGCTATGACC	

Sequences in bold will anneal to the L30 *gene during the PCR. In the end-primer six GTG are included. These will give six CAC codons in the 3' end of the L30 gene, just before the end codon, yielding a C-terminal His-tag. The sequencing primers will anneal to the* lacZ *gene, outside the multiple cloning site.*

```
                                        5′ccggt
      atcatcgccggtggtgcaatgcgcgccgttctggaagtc
      gctggggttcataacgttctggctaaagcctatggttcc
      accaacccgatcaacgtggttcgtgcaactattgatggc
      ctggaaaatatgaattctccagaaatggtcgctgccaag
      cgtggtaaatccgttgaagaaattctggggaaataaacc

  1 - atg gca aag act att aaa att act caa acc
 31 - cgc agt gca atc ggt cgt ctg ccg aaa cac
 61 - aag gca acg ctg ctt ggc ctg ggt ctg cgt
 91 - cgt att ggt cac acc gta gag cgc gag gat
121 - act cct gct att cgc ggt atg atc aac gcg
151 - gtt tcc ttc atg gtt aaa gtt gag gag taa

      gagatgcgtttaaatactctgtctccggccgaaggctcc
      aaaaaggcgggtaaacgcctgggtcgtggtatcggttct
      ggcctcggtaaaaccggtggtcgtggtcacaaaggtcag
      aagtctcgttctggcggtggcgtacgtcgcggtttcgag
      ggtggtcagatgcctctgtaccgtcgtctgccgaaattc
      ggctt3′
```

Fig 6: *The L30 gene with flanking chromosomal regions. The primers start and end will anneal to the underlined sequences.*

```
                      5′g gag aca uaa gga ggt
      gag gta acc atg gca aag act att aaa att
  1 - atg gca aag act att aaa att act caa acc
 31 - cgc agt gca atc ggt cgt ctg ccg aaa cac
 61 - aag gca acg ctg ctt ggc ctg ggt ctg cgt
 91 - cgt att ggt cac acc gta gag cgc gag gat
121 - act cct gct att cgc ggt atg atc aac gcg
151 - gtt tcc ttc atg gtt aaa gtt gag gag taa
      act ttc cc 3′
```

Fig 7: *The final PCR product for the USER™ friendly cloning. The upper underlined sequence will be removed, whereas the lower underlined sequence will constitute the sticky end. The double stranded DNA molecule may there after anneal to complementary sticky ends in the linear pNEB205a vector, making it circular.*

2.2 Screening

2.2.1 PCR method

To detect ?-RED transformants, the forward primers were designed to anneal to the tags and the reverse primer was designed to anneal 200 bases downstream, just at the end of the L30 gene. Control PCRs were performed using a forward primer, complementary to a sequence 200 bases upstream of the L30 gene.

Conditions were varied to achieve an optimized PCR system for each of the two inserts using non-homogenous colonies and cultures (2 µl culture to 20 µl reaction mix) as template. Homogenous single colonies were screened by adding a small amount of cells to 20 µl reaction mixes containing 10 µl 10x PCR buffer (Amersham), 8 µl dNTP (Amersham), 1 µl of each primer and 1 µl Taq DNA polymerase (Amersham) per 100 µl reaction mix. For primers annealing to the His tag 0.2 mM Mg^{2+} was added to the mixture and the PCR was run with 52°C annealing temperature. PCRs with the start and end primers and the primer annealing to the FLAG™ tag were run with 55°C annealing and the control primer was run with either 52°C or 55°C. The thermal

cycling program was 95°C for 5 min, 95°C for 40 seconds, the actual annealing temperature for 45 seconds, back to step two 30 times and finally 72°C for 7 min.

The PCR products were resolved on a 1.5% agarose gel containing EtBr (0.5 ng/l). Bands were visualized under a UV-lamp.

PCR with the primers start and end was run on plasmids purified from the transformed TOP10 cells (1 µl template to a 25 µl reaction mix).

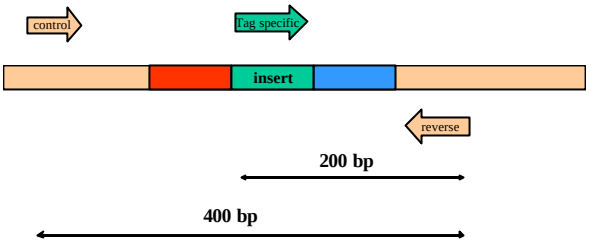

Fig 8: *Scheme of the chromosomal DNA, primers and PCR/products. Primers from the left are: Control, tag specific and reverse. Purified PCR products from a PCR with control and reverse primer can be used as template for a PCR with the tag specific primer. See also fig 12.*

```
                                          5'ccggt
      atcatcgccggtggtgcaatgcgcgccgttctggaagtc
      gctggggttcataacgttctggctaaagcctatggttcc
      accaacccgatcaacgtggttcgtgcaactattgatggc
      ctggaaaatatgaattctccagaaatggtcgctgccaag
      cgtggtaaatccgttgaagaaattctggggaaataaacc

  1 - atg gca aag act att aaa att act caa acc
 31 - cgc agt gca atc ggt cgt ctg ccg aaa cac
 61 - aag gca acg ctg ctt ggc ctg ggt ctg cgt
 91 - cgt att ggt cac acc gta gag cgc gag gat
121 - act cct gct att cgc ggt atg atc aac gcg
151 - gtt tcc ttc atg gtt aaa gtt gag gag taa

      gagatgcgtttaaatactctgtctccggccgaaggctcc
      aaaaaggcgggtaaacgcctgggtcgtggtatcggttct
      ggcctcggtaaaaccggtggtcgtggtcacaaaggtcag
      aagtctcgttctggcggtggcgtacgtcgcggtttcgag
      ggtggtcagatgcctctgtaccgtcgtctgccgaaattc
      ggctt3'
```

Fig 9: *The 180 bases gene L30 with 200 bases surrounding sequence. Underlined sequences correspond to the control primer and to the reverse primer.*

Table 4: Primers used in screening and sequencing of DNA recombined using the ?-RED system.

Primer	Sequence	Producer
Control	CCGCTATCATCGCCGGTGG	Sigma-Genosys
His	ATGCATCATCATCATCATCATCATGCA	Invitrogen
Flag	GACTACAAGGACGACGATGACAAA	Invitrogen
Reverse	TTACTCCTCAACTTTAACCATGAA	Invitrogen

The control primer will anneal to a sequence 200 bp upstream of the L30 gene. The His and Flag primers will anneal to the inserts and the reverse primer will anneal to a sequence in the end of the L30 gene.

2.2.2 Colony hybridization

270 ng of the His and Flag oligos were labeled with ^{32}P-?ATP (Amersham) using 30 units of Polynucleoteide Kinase T4 (USB) in PNK buffer (USB). The mixtures were incubated at 37°C for 3 h and the oligos were purified from free ATPs using a G50 gel column (Amersham) in a micro-centrifuge.

Colonies were transferred to a Hybond™-N nylon membrane (version LRPNN/95/10, Amersham). The membranes were thereafter put with the cell side up on 3MM papers, which were soaked with the following solutions: 10% SDS, denaturation buffer (1.5 M NaCl, 500 mM NaOH) and twice in neutralization buffer (150 mM NaCl, 500 mM trizma base), for three minutes each. The membranes were washed vigorously in 2x SSC (30 mM tri-sodium citrate, 300 mM NaCl, pH 7-8).

After air drying the membranes, DNA was fixed to them using a Bio-Rad UV-crosslinker (150 mJ). The membranes were put into hybridization tubes and were treated with hybridization buffer (0.5% SDS, 75 mM tri-sodium citrate, 750 mM NaCl, 5% ssDNA gel, 5% 100x Denhardt's solution) for 30 minutes at 65°C.

The labeled oligonucleotides were denatured and added to the hybridization buffer (10 µl oligo per 20 ml buffer) for incubation over night at 65°C with gentle agitation. The membranes were washed with 1x SSC, 0.1% SDS at 65° for 15 min followed by a wash with 0.1x SSC, 0.1% SDS at 65° for 15 min. The membranes were exposed to a phosphorimage cassette for three days.

The cassettes were scanned with a 400 series PhosphorImager (Molecular Dynamics) and the images were analyzed using ImageQuaNT Version 4.2a (Molecular Dynamics).

2.3 Confirmation of insert

2.3.1 DNA purification and PCRs on PCRs

PCR products were purified using the Promega kit "Wizard PCR preps DNA purification system" using a vacuum manifold.

Plasmids were purified using the QIA Spin Miniprep Kit Protocol with a microcentrifuge.

Purified PCR products from a control PCR (see PCR screening) were used as template for tag specific PCRs (1 µl purified DNA in 50 µl reaction mix).

2.3.2 Western Blot

30 µl culture (diluted 10x from OD_{600} =0.2) was loaded onto an SDS-PAGE gel (5% acryl amide stacking gel and 18% acryl amide resolving gel) which was run at 200 V. 200 ng of a positive control (N-terminal FLAG™ fusion protein) was run in the same gel.

The gel was blotted onto a nitrocellulose membrane over night at 30 V, 4°C.

Thereafter the membrane was blocked with BLOTTO (5% nonfat Semper dry milk in PBS buffer) for 90 min with shaking.

Primary antibodies (Sigma poly-clonal antiFLAG), diluted 1:10 000 in BLOTTO, immersed the membrane for 2 h. Thereafter the membrane was washed three times with BLOTTO. Secondary antibody (Anti-rabbit Ig, Amersham), diluted 1:5 000 in BLOTTO, immersed the membrane for 30 min, where after the membrane was washed with PBS-Tween (0.5 % Tween 20 in PBS buffer). 2 ml of each ECL reagent (Pharmacia) were mixed and incubated the membrane for one minute. After exposing the membrane to an ECL-film for 30 min, the film was developed by hand.

2.3.3 Sequencing

Sequencing of purified control PCRs was performed with a BECKMAN COULTER kit, using 10 pmoles of primers and 100-200 fmoles of template DNA. The thermal cycling program was 96°C for 20 seconds, 50°C for 20 seconds, 60°C for 4 min for 30 cycles.

The sequencing products were precipitated according to the BECKMAN COULTER protocol: 2 µl 3M NaOAc pH 5.2, 2 µl 100 mM EDTA and 1 µl glycogen (20 mg/ml) was added to every sequencing reaction mixture. After mixing, 60 µl 95% ethanol from -20°C freezer was added and the solution was centrifuged at maximum speed in a microcentrifuge for 15 minutes. The supernatant was discarded and the pellet was washed with 200 µl 70% ethanol from -20°C freezer and spinned down. The procedure was repeated once. The pellet was air dried under a lamp and resuspended in 35 µl Sample Loading Solution (BECKMAN COULTER). The gel running and analysis were performed by Siv Strömberg, Department of Immunology, Uppsala University.

Sequencing of purified nested PCRs and of the insert in the pNEB205a vector, was performed with a Mega BASE 1000 kit (Amersham Biosciences) by Ulla Gustafson, Dept. of Animal Breeding and Genetics, Uppsala University.

2.3.4 Stability of insert

The stability of the insert was investigated both with the PCR method and with Western Blot on colonies that had been grown in cultures and been respread once and twice. Glycerol stocks were also checked for the insert with the PCR method.

2.4 Expression and purification

Overnight cultures of transformed TOP10 cells were grown at 37°C in LB medium containing 1mM IPTG and 50µg/ml ampicillin. Cells were spinned down at the late log-phase and cell pellets were frozen in -20°C.

Before lysis, cells were thawed on ice and resuspended in lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole) with 20µg DNaseI per gram cells. The cellsuspension was frenchpressed twice and cell debris was spinned down at 34 500x g for 20 min at 4°C. 1 ml cobalt resins (TALON™ Superflow™ Metal Affinity Resin, BD Biosciences) were added to the supernatant and incubated for one hour with agitation at 4°C. The resins were collected in a minicolumn and were washed twice with 8ml wash buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole). Bound proteins were eluted with 0.5 ml elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole) and the fraction was immediately dialyzed into polymix buffer (95mM KCl, 5mM NH_4Cl, 5mM MgOAc, 0.5 mM $CaCl_2$, 8mM Putrecine, 1mM spermidine) with 5mM KPO_4 (pH 7.5) and 1mM DTT at 4°C over night.

2.5 Detection

The UV-spectra (220-320 nm) of the dialyzed samples were investigated in a spectrophotometer (HITACHI U-2001).

3. RESULTS AND DISCUSSION

3.1 Transformation

3.1.1 ?-RED

The electroporation time constants were all in the range of 4.4 to 4.9 msec, which is a bit too low to be optimal; it should be at least 5.0 msec.

From the first transformation experiment, 85 colonies were screened for the FLAG™ tag insert and 275 for the His tag insert. One FLAG™ clone was found; the 200-bp band in fig 10A is very sharp and the corresponding lane for non-transformed cells shows no such band (fig 10B). Since FLAG™ specific PCR products on purified control PCR products from transformed cells gave a band as sharp as that from the tag specific PCR directly on the transformed cells, the insert and its position seemed correct (fig 11).

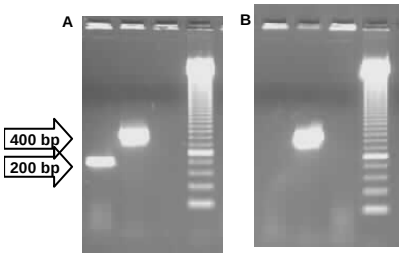

Fig 10: FLAG™ *tag specific (lane 1) and control PCR (lane 2) products resolved on a 1.5% agarose gel with a 50 bp marker (Amersham Biosciences). Line 3 shows a control PCR with no template.* ***A:*** *transformed colony.* ***B:*** *non-transformed colony.*

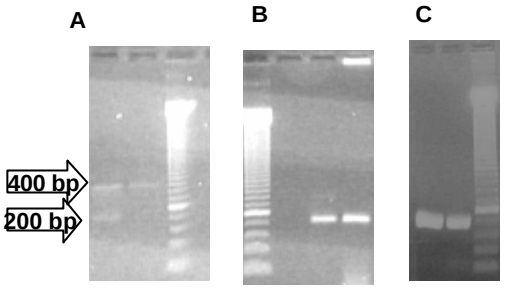

Fig 11: ***A:*** *FLAG™ tag specific PCR on purified control PCRs from non-transformed cells (lane 1), the purified control PCRs, diluted as when used as template (lane 2), 50 bp marker from Amersham Biosciences (lane 3).* ***B:*** *50 bp marker(lane 1), purified control PCR on transformed cells diluted as when used as template (lane 2), FLAG™ tag specific PCR on the purified control PCR products from transformed cells (lane 3), FLAG™ tag specific PCR directly on transformed cells.* ***C:*** *FLAG™ specific PCRs on purified PCR products from FLAG™ specific PCRs on purified control PCR products from transformed cells (lane1). FLAG™ specific PCRs on purified PCR products from FLAG specific PCRs on purified control PCR products from non- transformed cells (Lane 2), 50 bp ladder from Amersham (lane3).*

The 200-bp band in the first lane in fig 11A is puzzling, though. FLAG™ specific colony PCR on non-transformed cells never gave a PCR product, but when the PCR was run on purified control PCR fragments from non-transformed cells, they showed products from the FLAG™ specific PCR. Since the corresponding band from the PCR on induced cells was much sharper, the weak band could at first be considered as not significant, but when a FLAG™ specific PCR was run on the purified products from the FLAG™ specific PCR on the purified control PCRs, the bands from the PCR on

DNA from transformed and non transformed cells were equally intense (Fig 11C). A systematic explanation of the PCRs on different PCR products is given in fig 12.

To explain this observation, sequencing of the FLAG™ specific PCR products on the control PCR products was performed. Despite several attempts and help from experts, no sequence could be read out from these fragments. However, sequencing of purified control PCR products from transformed and non-transformed cells worked and they showed the same sequence, with no tag present in the transformed cells.

Tag specific PCRs on cells streaked from the positive colony showed no insert (fig 13), indicating that the insert was not stable. This idea was further supported by the Western blot, which showed that there was no expression of the FLAG™ tag in the cell culture from the positive colony (fig 14).

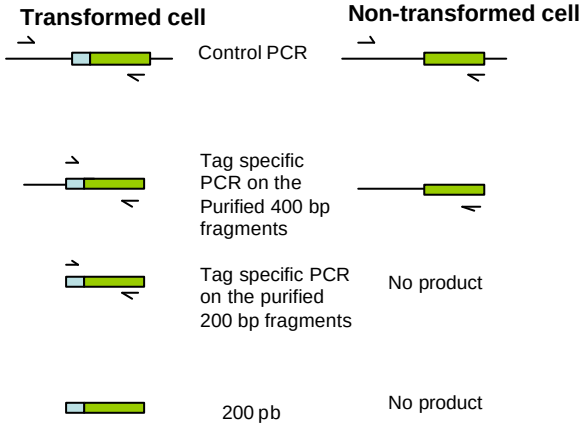

Fig 12: *The expected results from PCRs on different PCR products. However, in this study there are PCR products where they are not expected, as shown in fig 11. Between each PCR the fragments are purified.*

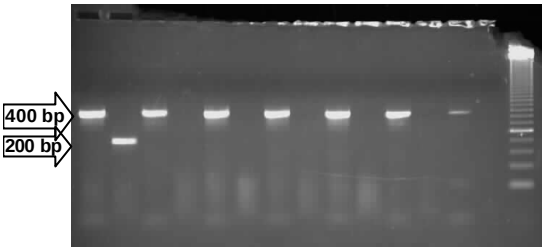

Fig 13: *Lane 1 contains control PCR products from the positive FLAG™ tag clone and lane 2 contains FLAG™ tag specific PCR products from the same colony. Lanes 3, 5, 7, 9 and 11 show PCR products from control PCRs on cells grown from the positive clones. Lanes 4, 6, 8, 10 and 12 contain the FLAG™ tag specific PCR products from the same colonies as in the lanes 1, 3, 5, 7, 9 and 11 respectively. The cells used for the PCRs in lanes 3 and 4 were streaked from the positive clone onto a LB plate. The cells used for the PCRs in lanes 5 and 6 were grown in a liquid culture from the positive clones and were thereafter spread on a LB plate. The cells used for the PCRs in lanes 7 and 8 were streaked from the cells used in lanes 5 and 6. The cells used for the PCRs in lanes 9 and 10 were taken from a glycerol stock from the positive clones. Lanes 13 and 14 are positive controls with no template.*

Fig 14: *The developed film from the Western blot. The positive control is the only present band.*

Several explanations for these observations are possible. One is that the FLAG™ specific PCR products did not contain the insert, but some features in the *L30* gene of the particular cell caused the

FLAG™ primer to anneal. This could be the same feature that makes the primer annealing to the purified control PCR fragments from non-transformed cells. A second possible explanation for these results is that the insert somehow was excluded from the genome as the cells grew. Another explanation is that some secondary structure, pre-terminating transcription, arose due to the insertion. Since *L30* is situated in the spc-operon with genes coding for other ribosomal proteins and the protein export protein *secY* (Cerretti *et al.* 1983), a stopped transcription will be lethal to the cell. This explanation is contradicted by the fact that the transformed cell has grown both in liquid culture and on a plate after the electroporation and still showed the insert in the colony-PCR.

Despite extensive research in the field of genetics, some properties remain more or less elusive, for instance duplication. Yu *et al.* (2000) have reported about diploid transformants arising when using the ?-RED recombination system. This could partly explain the observed instability.

The surprising feature of the transformants in this study is that they all seem to lose the insert at exactly the same stage.

The technique of recombineering is highly sensitive to mistakes; one may have to perform several transformations to obtain any recombinants (*E. coli* strain provider, personal communication). Therefore, the experiment was repeated.

After the second transformation, 170 colonies expected to have the FLAG™-insert and 100 colonies expected to have the His-insert, were screened using colony hybridization. 13 colonies hybridized with the His-oligo and 19 colonies hybridized with the FLAG™-oligo, showed signals that were believed to be positive (fig 15). However, when checking these colonies with the PCR-method, they all turned out to be negative.

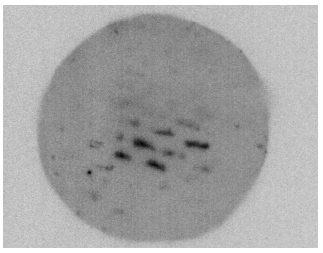

Fig 15: *The colony hybridization membrane, developed on a phosphorimage plate, from 50 NC397* E. coli *colonies hybridized with the FLAG™ oligo. The seven strongest signals were investigated with PCR. However, they all turned out to be negative.*

At this point it became clear that the screening strategy was inefficient. Outgrowth of the cells directly after transformation leads to situations where several cells with the same origin will be investigated and this is only reasonable to do when screening for antibiotic resistance. Therefore, a third transformation experiment was performed, without growing the cells in liquid culture before spreading on plates. 350 colonies were hybridized with the His-oligo and 12 of them seemed to be positive, but the tag-specific PCRs once again showed that they were all negative.

During the third transformation, a control-oligo was used to monitor the transformation. Since the sequence itself can affect the efficiency (Constantino *et al.* 2003), the tagging may not result in the same number of transformants, but the control tells if the transformation succeeded at all. In this case the transformation turned out not to work. The control oligo gives on average one transformant per thousand surviving cells, but in this study no transformants were seen, though more than 10^5 cells were screened.

The experiments made so far can not clearly state that the transformation will not work. However, it is clear that a different approach should be used in order to facilitate the screening process. Such an approach would be to insert an antibiotic resistance gene next to the tag. If, however, the problem is that insertion of extra DNA in the operon is lethal to the cell, a vector construct should be made and the chromosomal *L30* should be made non-functional, for instance by an amber mutation.

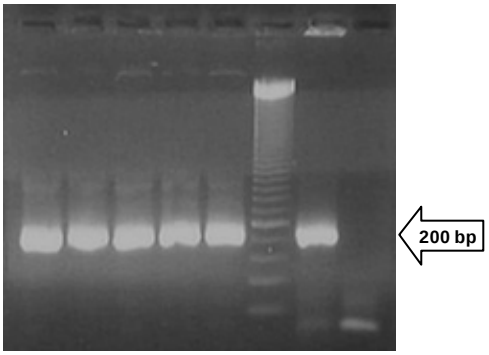

Fig 16: *Products from PCR with start and end primers. The first five lanes: PCR products from purified plasmids containing a C-terminal His tagged L30 gene. 6th lane: 50 bp ladder (Amersham). 7th lane: Positive control,* E. coli *cells (the primers will anneal to the L30 sequence on the chromosome). 8th lane: Negative control, no template.*

3.1.2 USER friendly cloning

Tag-specific PCR showed that five out of five colonies contained the plasmid with the insert (fig 16) and sequencing confirmed the transformation.

3.1.3 Expression and purification

His-tag purification using the TOP10 cells transformed with the vector construct showed that the ribosome, or at least parts of it, does bind to the metal affinity resins. UV-absorption spectra of the elution from the metal affinity resins showed that the quote A_{260}/A_{280} (absorption for RNA divided by absorption for proteins) was near 1.8 (fig 17 and table 5), which is the expected value of this quote for ribosomes.

The concentration of the purified ribosomes was very small, 0.9 µM or 4.4 pmol in 500 µl, (1 A_{260} unit is equivalent to 23 pmol ribosomes) and needs to be increased tenfold to see the ribosomal proteins on an SDS-PAGE gel.

Table 5: UV-absorptions.

Nm	Absorption
320	0.052
300	0.079
280	0.237
260	0.379
240	0.325
220	1.630

UV-absorptions of the first 500 µl elution from cleared lysate from TOP10 pNEB205a L30::HIS cells collected at late log-phase.

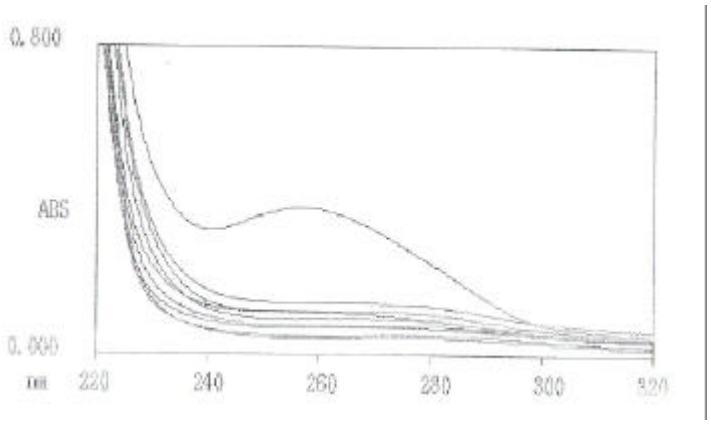

Fig 17: *UV absorption spectra of eluted fractions from a minicolumn with Talon™ Superflow™ Metal Affinity Resin (BD Biosciences). The cleared lysate was prepared from 1.3 gram pellet of transformed (top10 pNEB205a L30::HIS) and non-transformed (TOP10) cells, collected at late log phase. The top spectrum shows the first 500 µl elution of lysate from transformed cells. The lower spectra show the second, third and fourth 500µl elutions and all four elutions of lysate from non-transformed cells.*

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