A Method for Isolation of *Escherichia coli* Mutants with Aberrant RNA Methylation using Translocatable Drug Resistance Elements*

GLENN R. BJÖRK and ARNE OLSÉN

Department of Microbiology, Umeå University, S-90187 Umeå, Sweden

Both ribosomal RNA(rRNA) and transfer RNA(tRNA) in *Escherichia coli* contain a large number of modified nucleosides of which the methylated nucleosides constitute the major group. The transfer of methyl groups from *S*-adenosyl-L-methionine to a preformed polynucleotide forming methylated nucleosides is catalyzed by a set of highly specific RNA methyltransferases. There is at least one enzyme involved in the biosynthesis of each methylated nucleoside in rRNA and tRNA, respectively. We have earlier isolated mutants following chemical mutagenesis which lost the capacity to methylate certain nucleosides in either rRNA or tRNA.[1,2] The phenotype of these mutants was difficult to score and there was a high probability that the mutant harboured additional mutations. A careful and laborious genetical work had therefore to be done before bacterial strains suitable for physiological studies could be constructed. Thus, it would be of great advantage if a method was devised which could avoid chemical mutagens and would render the mutated cell an easy scorable phenotype.

Translocatable drug resistance elements (transposons) have recently been described and their use in bacterial genetics was reviewed.[3] Such elements translocate at low frequency (10^{-4}, per viable cell) from one replicon to another and carry genes conferring resistance to various antibiotics. Furthermore, since one selects for a rare event it is unlikely that the antibiotic resistant clone has arisen from a double event. One such transposon, Tn5, appears to insert itself randomly into the chromosome and simultaneously renders the cell kanamycin resistant. When structural genes are split by such transposon the gene function is completely destroyed. At the same time the transposon acts as a positive selective marker by virtue of its kanamycin resistance and thus permits selection of any mutation caused by it. If Tn5 was inserted into a structural gene for an rRNA- or tRNA methyltransferase, such an event should cause a complete lack of the corresponding methylated nucleoside in the RNA species. This paper describes an isolation procedure of Tn5 induced mutations in genes responsible for the methylation of rRNA or tRNA and the successful isolation of a mutant which has aberrant methylation of tRNA.

Results and discussion. *E. coli* strain GB7 (HfrP4X, *argH1*, *trmA5*, *thiA1*) was grown overnight in TB medium (1 % Difco tryptone[5] 0.5 % NaCl and 10 mM $MgCl_2$. To 1.5×10^9 cells λkan ($\lambda cI_{857}b221$ rex::Tn5, kindly given to us by Dr Hennecke, Regensburg, Germany) was added at a multiplicity of five. This phage is unable to integrate into the bacterial chromosome due to the b221 deletion. Adsorption of the phage was for 30 min at 32 °C after which the mixture was diluted 10-fold in TB medium lacking Mg^{2+}. This avoids reinfection of the phage. The culture was grown for 30 min at 32 °C when the cells were concentrated by centrifugation and resuspended in 0.9 % NaCl. A proper amount of the culture was plated on fully supplemented agar plates containing kanamycin (50 μg/ml) and 2.5 mM sodium pyrophosphate. The latter compound kills vegetative phages containing more than the minimum amount of DNA in their head. The agar plates were incubated for 2 h at 32 °C, shifted to 43.5 °C for 4 – 5 h to lower the probability of appearance of lysogens and incubated overnight at 32 °C. The frequency of kanamycin resistant clones was between 10^{-4} to 10^{-5} per viable cell. No lambda lysogens were detected among 6 000 clones following such treatment. Kanamycin resistant clones were purified once on the above-mentioned plates and single cell colonies were grown overnight in 1.5 ml Rich-MOPS medium containing (*methyl*-^{14}C)-L-methionine (11 μg/ml, 20 μC/μmol). This medium contained 20 amino acids, two purines, two pyrimidines and five vitamines[4]. Each clone was saved by transfer of a sample to agar plates containing kanamycin. To the rest of the culture carrier cells (10^9 cells), DNAse (50 μg) and lysozyme (400 μg) were added. The suspension (2 ml) was frozen and thawed three times and incubated for 10 min at 37 °C. Tris-HCl, pH 8.0 (10 mM) saturated phenol (2 ml) together with 0.2 ml of 2 % sodium dodecyl sulfate was added and the mixture was shaken for 10 min at 30 C. Following low speed centrifugation in the cold the aqueous phase was withdrawn and total RNA was precipitated with 5 ml of ethanol containing 1 % potassium acetate. Transfer RNA was extracted from the precipitate by 0.4 ml 2 M LiCl in 25 mM Tris-HCl pH 8.0[5] and transferred to an Eppendorf centrifuge tube. Following precipitation with ethanol as above the tRNA was dissolved in 0.4 ml water and precipitated again with ethanol. This latter precipitation is necessary for efficient removal of LiCl which will otherwise interfere with the following chromatographic separation. Transfer RNA was washed once with 0.4 ml 67 % ethanol, lyophilized and dissolved in 50 μl of a mixture containing snake venom phosphodiesterase, pancreatic RNase A and alkaline phosphatase as described earlier.[2] The digestion to nucle-

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Fig. 1. Chromatographic pattern of methylated nucleoside from tRNA of *E. coli* labelled *in vivo* with (*methyl*-^{14}C)-L-methionine. Transfer RNA was from parent strain (left slot) and mutant (right slot). The chromatographic development was upwards. The arrows denote the two missing radioactive methylated nucleosides in the tRNA from the mutant.

osides was performed for 38 – 40 h at 37 °C. A portion (20 µl) was applied on a 55 cm long thin layer cellulose-coated aluminium foil (E. Merck, Darmstadt, Germany). The chromatogram was developed descending in butanol – isobutyric acid – concentrated ammonium hydroxide – water (75:27,5:2,5:25 v/v [6]) for 36 to 38 h.

Material which did not dissolve in 2 M LiCl consisted of rRNA. Such material was dissolved in 0.4 ml water containing 25 µg rRNA and 50 µg tRNA as carrier. Following precipitation by ethanol the contaminating tRNA was removed by 2 M LiCl extraction. The rRNA was dissolved in water and precipitated by ethanol and treated as described above for the tRNA. A portion (10 µl) of the nucleoside mixture was applied on a similar chromatogram developed in a solvent as above but with the isobutyric acid exchanged with butyric acid. On each chromatogram 12 nucleoside mixtures were spotted simultaneously using a multisyringe dispenser. (Desaga, Heidelberg, Germany) fitted into an apparatus designed by ourselves to facilitate the application. The position of the (*methyl*-^{14}C) – labelled nucleosides was visualized by autoradiography. The X-ray film was exposed for about one month before being developed. The separation of methylated nucleosides from tRNA is shown in Fig. 1 and the separation of the methylated constituents from rRNA was similar (data not shown). This procedure separates 17 and 13 different methylated nucleosides from tRNA and rRNA, respectively. Since 5-methyluridine in tRNA is normally the major methylated nucleoside and since it migrates in a region where some other methylated nucleosides migrate the absence of this nucleoside should improve the sensitivity of the method. Therefore we use as parent strain a *trmA5* mutant, which completely lacks 5-methyluridine.[1]

One expects transposon induced mutations in a particular gene at a frequency of one in 5 000.[3] Since we monitored the activity of at least 25 different genes we should find transposon induced mutants in RNA methylation genes in about one in 200 clones tested assuming the translocation to be random. Although this assumption seems to be true as a first approximation there exist preferred sites within some genes. Furthermore, since the procedure requires that the cells are able to grow no mutation in essential RNA methylation will be isolated. At present we have analyzed about 150 clones and one mutant with aberrant methylation in the tRNA was found (Fig. 1). This mutant lacks two radioactive methylated nucleosides and the detailed analysis of it will be published elsewhere. However, preliminary genetical analysis revealed that the Tn5 is located between *trp* and *his* genes on the *E. coli* chromosome, a region where no gene involved in tRNA methylation so far has been identified. The successful isolation of the mutant shows

that our method is working and we believe that the transposon induced mutants in the RNA methylation will facilitate the study of the biosynthesis and function of this group of modifying enzymes and contribute to a more thorough understanding of the translational apparatus in *E. coli*.

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Modification of the Binding Site for Pyridine Nucleotides of Glutathione Reductase by 2,3-Butanedione*

VIJAYAKUMAR BOGGARAM and BENGT MANNERVIK

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

Glutathione reductase has two different binding sites per subunit: one, N-site, for the pyridine nucleotide substrate (NADPH**) and one, G-site, for the disulfide substrate (GSSG).[1,2] The two sites, whose openings are located on opposite sides of the same subunit, communicate *via* an isoalloxazine ring of FAD at the bottom of the N-site and a juxtaposed cystine disulfide at the bottom of the G-site. Thus, reducing equivalents are transferred from NADPH in the N-site to GSSG in the G-site *via* two redox-active groups in the interior of the enzyme. Site-specific inhibitors could help to unravel the catalytic events and locate them topographically in the enzyme molecule. Two such inhibitors have recently been found, which affect the G-site: ethoxyformic anhydride which modifies an essential histidine[3] and 2,4,6-trinitrobenzenesulfonate which appears to interact with the reduced form (dithiol) of the redox-active disulfide.[4] However, no inhibitor modifying the N-site has been reported. The widespread occurrence of arginine in anion-binding sites[5] prompted the present study, which shows that the arginine-modifying reagents 2,3-butanedione and phenylglyoxal inhibit the enzyme by reacting with the N-site.

Glutathione reductase from human erythrocytes (*cf.* Refs. 3 and 6 for experimental procedures) was irreversibly inhibited by 2,3-butanedione in 50 mM sodium borate (pH 8.3). The inhibition increased with time and with the concentration of 2,3-butanedione, and was strongly dependent on the presence of borate, as expected for arginine modification.[5] The absorption spectrum of the enzyme was not affected in the region 350 – 600 nm by the modification. The enzyme was similarly inactivated by phenylglyoxal. Fig. 1 shows the progress curves of the inactivation with 2,3-butanedione in the absence and presence of NADPH. It was found that 2 mM NADPH

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** Abbreviations: FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetate; GSSG, glutathione disulfide; NADP+ and NADPH, oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate.

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