Cloning, in vitro transcription, and biological activity of Escherichia coli 23S ribosomal RNA

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ABSTRACT

The 23S rRNA gene was excised from the rrnB operon of pKK3535 and ligated into pUC19 behind the strong class III T7 promoter so that the correct 5' end of mature 23S RNA was produced upon transcription by T7 RNA polymerase. At the 3' end, generation of a restriction site for linearization required the addition of 2 adenosine residues to the mature 23S sequence. In vitro runoff transcripts were indistinguishable from natural 23S RNA in size on denaturing gels and in 5'-terminal sequence. The length and sequence of the 3' terminal T1 fragment was also as expected from the DNA sequence, except that an additional C, A, or U residue was added to 21%, 18%, or 5% of the molecules, respectively. Typical transcription reactions yielded 500 - 700 moles RNA per mole template. This transcript was used as a substrate for methyl transfer from S-adenosyl methionine catalyzed by Escherichia coli cell extracts. The majority (50 - 65%) of activity observed in a crude (S30) extract appeared in the post-ribosomal supernatant (S100). Activities catalyzing formation of m^5C, m^5U, m^2G, and m^6A residues in the synthetic transcript were observed.

INTRODUCTION

Transcription in vitro by T7 RNA polymerase of genes for a number of stable cellular RNAs has been used to produce RNA for the study of both structure and function. Examples include Escherichia coli 16S rRNA and reconstituted 30S subunits (1 - 4), domains of 16S (5) and 23S(6) RNA, tRNA species (7 - 11), tRNA precursors (12 - 14), the M1 RNA of ribonuclease P (15), 5S RNA (16), self-splicing RNAs (17), and viroid ribozyme RNAs (18, 19). Most of these authors also described straightforward techniques for site-directed mutagenesis of these RNAs.

Here we describe methodology for the in vitro production of full length transcripts of E. coli 23S ribosomal RNA using T7 RNA polymerase. The transcript has a sequence at the 5'-end identical to that of mature 23S RNA isolated from E. coli ribosomes (20) and only two to three additional nucleotides at the 3'-end. The synthetic transcript also lacks the post-transcriptional modifications found in mature ribosomal RNA. The availability of full-length 23S ribosomal RNA is expected to be of considerable utility in studies on 50S ribosomal subunit assembly, structure, and function in ways analogous to that described for 16S RNA (3, 21). Such transcripts are also ideal substrates for the identification, isolation, and characterization of post-transcriptional modification enzymes such as the methyltransferases (22, 23). In this work, we show that the unmodified 23S RNA transcript is a substrate for methyltransferases, at least one of which has not been characterized previously.

MATERIALS AND METHODS

General

Restriction enzymes and T7 RNA polymerase were from New England Biolabs except for Cfr10I which was from Amersham. T4 DNA ligase, competent E. coli DH5 and DH5α cells, and electrophoresis size markers for DNA (1Kb ladder) or RNA (0.24 - 9.5 Kb ladder) were from BRL, pKK3535, pUC19, and natural 23S RNA were obtained as described (24). pKK3535 was renumbered as described in the legend to Figure 1. Numbering of both pKK3535 and pCW1 refers to the non-template strand. One A260 unit was assumed equal to 40 μg of RNA or 50 μg of DNA. Powdered glass for DNA purification (GeneClean kit) was from Bio 101 (La Jolla, CA.). Ultrapure agarose (SeaKem GTG) was from FMC. [32P]pCp and [35S]α-thioATP were from Amersham. Unlabeled pCp was obtained from Pharmacia. Buffers used were TE (24); TAE (25); E21 (for Cfr10I, Amersham); React 2, 3 and 4 (BRL); R4-2 (80 mM Tris pH 8.0, 15 mM MgCl₂, 50 mM KCl); HEN (50 mM Hepes pH 7.3, 100 mM NaCl, 10 mM EDTA); RNA Buffer (5 mM KOAc pH 7.0, 1 mM Mg(OAc)₂), and R Buffer (20 mM Hepes pH 7.5, 10 mM Mg(OAc)₂, 100 mM NH₄Cl and 6 mM β-mercaptoethanol). Electrophoresis in 0.7 - 1.0% agarose gels was performed in TAE buffer containing 0.5 μg/ml ethidium bromide. Electrophoresis of RNA after glyoxal-Me^2O denaturation was as described by Denman, et al. (3) except using 1% agarose. DNA bands were purified from agarose by dissolving the agarose slice in 3 vols of saturated NaCl, adsorbing the DNA to powdered glass (GeneClean), washing, and elution in water or TE buffer.

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according to the manufacturer's protocol. Plasmid DNA was sequenced by the dideoxy method. DNA (13.4 µg, 3.6 pmoles), was denatured in 0.2 N NaOH and 0.2 mM EDTA for 5 min at room temperature, and precipitated at -20°C for 30 min after addition of 0.1 vol 3M NaOAc and 2 volts EtOH. The pellet was washed with 70% EtOH, dried briefly in vacuo, and stored at -20°C. Three 21-nucleotide primers were made, complementary to residues 43-63, 869-889, and 2538-2558 (32-52 bases past the 3' end of the transcript) of plasmid pCW1 (Figure 1). Each primer (1.9 pmol) was annealed to the denatured plasmid by incubation for 15 min at 37°C in 20 mM Tris HCl pH 7.5, 10 mM MgCl₂, 25 mM NaCl, and cooling to room temperature. Sequencing was performed with [³²P]α-thioATP, modified T7 DNA polymerase (Sequenase), and the reagents and protocols from U. S. Biochemicals, using a 1:10 dilution of the labeling mixture to obtain sequences close to the primer.

**rrlB gene fragments (Fig. 1A)**

pKK3535 at 167 µg/ml was digested for 1 h with 67 units/ml EcoRI followed by addition of a second aliquot of enzyme and
an additional 1h incubation. The digest was separated on 0.7% agarose gels. The 3538 and 2151 bp bands were excised, and electroeluted. DNA was concentrated and freed from ethidium bromide and gel fragments by n-butanol and phenol/chloroform extraction, and precipitation with EtOH. 5 µg of the 3538 bp fragment was further digested in 100 µl of E21 buffer with 7.5 units of Cfr10I for 2 h at 37°C, an additional 7.5 units of enzyme was added and incubation continued for another 2 h. Preparative agarose gels showed bands of ca. 2000 bp and ca. 1500 bp, as predicted (Fig.1A). The 2030 bp band was excised and purified by the powdered glass procedure. 4.5 µg of the 2151 bp band from the EcoRI digest was digested with 4.5 units SfAlNI in 100 µl of 10 mM Tris pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 100 µg/ml BSA for 2 h at 37°C. An additional 4.5 units of enzyme were added, and incubation continued for 2 h longer. Preparative agarose gel electrophoresis yielded the expected fragments. The 826 bp fragment was excised and purified as above.

**Synthetic linkers (Fig. 1B)**

The 5'-linker oligodeoxynucleotides were purified by detritylation, desalting, and purification by ion-exchange HPLC on a Nucleogen DEAE 60-7 column (Machery-Nagel, Diihren, FRG) using 20 mM NaOAc pH 6.0 in 20% acetonitrile and a 30 ml gradient from 0 to 1 M LiCl. The 3'-linker oligodeoxynucleotides were synthesized in the trityl-on mode, deprotected, and purified on OPC columns (Applied Biosystems) using the manufacturer's protocol. Duplexes were formed by annealing as described (1) except at 2 µM oligomers and 90°C.

**Plasmid pCW1 (Fig. 1C)**

pUC19 at 500ng/ml was digested with 50 units/ml Kpnl in React 4 buffer at 37°C for 1 h, an additional 50 units/ml enzyme added and incubation continued for 1 h longer. An equal volume of R4-2 buffer was added and the plasmid digested with 56 units/ml enzyme at 37°C, followed by addition of a second aliquot of enzyme and another 1 h incubation. Doubly-cut plasmid was purified by electrophoresis (1% agarose), excised, and isolated of enzyme and another 1 h incubation. The resulting material was used to divide the HSW into supernatant [HSW(A/50s)] and precipitate [HSW(A/50p)]. Methylation conditions were modified (22), except that isolated 23S RNA was used as carrier, the digests were brought to 35 mM HOAc before analysis, the HPLC buffer was replaced with 10 mM NH₄OAc pH 7.0, 200 mM NH₄Cl, 0.01 mM EDTA, using the manufacturer's instructions except that the ladder was obtained by heating at 100°C instead of 90°C, and 25% gels were used. CL3 nuclease (BRL) digestion was in 5 µl of 10 mM Tris pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 10 mM β-mercaptoethanol, and 100 µg/ml BSA for 2 h at 37°C, a second 50 units of enzyme were added and incubation continued for an additional 2 h. Seven or 14 µg of linearized plasmid (2 or 4 pmol) were transcribed in a 1 ml reaction containing 40 mM Tris·HCl pH 8.0, 8 mM Mg(OAc)₂, 25 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 1000 units of RNAsin (Promega), 2.5 mM each of ATP, CTP, UTP, and GTP (sodium salts, Sigma), 5 units yeast inorganic pyrophosphatase (Sigma), and 1000 units T7 RNA polymerase at 37°C for 5 h. At the end of the incubation, DNAse (12 or 25 units from Cooper Biomedical or Promega Biotec, respectively) was added and incubation continued for 15 min. The mixture was phenol extracted and low molecular weight components removed by Sephacryl S200 chromatography (Pharmacia) in HEN buffer. Transcripts were precipitated with EtOH, dialyzed against RNA Buffer, and stored at -70°C. Dideoxy sequencing of RNA was performed using AMV reverse transcriptase and reagents from Promega according to the manufacturer's instructions with [³²P]α-thioATP, and primers complementary to residues 43-63 or 869-889 of 23S RNA. Sequencing of the 3' end of the transcript and of control natural 23S RNA was accomplished by labeling with [³²P]pCp as described (1) except as follows. Ligation mixtures (100 µl) containing 0.4 µM RNA, 16 µM [³²P]pCp (123,000 dpm/µmole), 120 units/ml of T4 RNA ligase, and 0.2 mM ATP were incubated for 12-16 h. The extents of labeling of natural 23S RNA and synthetic transcript were 58% and 66%, respectively. Digestion to completion was with 0.5 or 2.0 Sankyo units/µg RNA in 17 mM Na citrate pH 5.0, 0.9 mM EDTA, 3.5 M urea, 0.5 mg/ml tRNA for 30 min at 55°C. Enzymatic sequencing of the isolated fragments with Phy M, U2, and B. cereus ribonucleases (Pharmacia RNA sequencing kit) was according to the supplier's instructions except that the ladder was obtained by heating at 100°C instead of 90°C, and 25% gels were used. CL3 nuclease (BRL) digestion was in 5 µl of 10 mM sodium phosphate pH 7.0, 20 mM KCl, 0.01 mM EDTA, using 4 units /µg RNA for 30 min at 55°C. Analysis of the 3'-terminal residue was by RNase T2 digestion and thin layer chromatography essentially as described (1).

**Methylation of transcript**

All procedures were as described (22) except as follows. Extracts of E. coli were prepared from cells broken in R buffer, ribosomal high salt wash (HSW) was prepared by sedimenting the 100,000×g pellet (resuspended in 1 ml R buffer/g cells) through 0.67 volumes of the same buffer made 500 mM in NH₄Cl, and only one concentration of ammonium sulfate (50%) was used to divide the HSW into supernatant [HSW(AS50s)] and precipitate [HSW(AS50p)] fractions. Methylation conditions were modified to 100 mM Hepes pH 7.5, 200 mM NH₄Cl, and 200 mM RNA. Digestion to nucleosides and HPLC analysis were as described previously (22), except that isolated 23S RNA was used as carrier, the digests were brought to 35 mM HOAc before analysis, the HPLC buffer was replaced with 10 mM NH₄OAc,
pH 4.1, and 55% methanol replaced 50% acetonitrile as the organic solvent.

RESULTS AND DISCUSSION

Plasmid Construction

The generation of a plasmid containing the strong class III T7 promoter fused to the 5'-end of the rrlB gene [the 23S DNA sequence of the rrlB operon (28)], and a unique cleavage site with the required 5' overhang (29) at the 3'-end of the gene to allow runoff transcription, closely parallels the methods described previously for 16S RNA (24). This construction (Figure 1) differs from that for the 16S rDNA-containing plasmid in two respects. First, the paucity of unique cleavage sites within 100 bases of the ends of the rrlB gene necessitated its isolation as two fragments. Second, the resulting 5-way ligation into pUC19 was performed in 2 steps.

pKK3535 (30) was digested with EcoRI, and the 3 fragments, 6176, 2151, and 3538 bp in length (all numbers referring to the non-template strand), were separated on agarose gels. The fragment containing the 5' end of the rrlB gene (2151 bp fragment) was further processed with SfaNI. The resulting 275 bp fragment, 1050 bp fragment, and 826 bp fragment were readily separable. The 826 bp fragment contained the 5'-segment of the rrlB gene except for the 5'-terminal 17 nucleotides. The fragment containing the 3' end was processed with Cfr10I to yield a 2030 bp fragment and a 1508 bp fragment. The 2030 bp fragment contained the remainder of the rrlB gene minus the 3'-terminal 31 residues.

Synthetic deoxyoligonucleotides were synthesized and annealed to produce the two duplex segments illustrated in Fig. 1B. At the 5'-end, the synthetic duplex contained a Kpnl site for ligation into pUC19, and the strong class III T7 promoter fused to the first 17 bases of the mature 23S RNA sequence. As indicated in Fig. 1B, transcription of this construct is predicted to produce a precise replica of the 5'-end of mature E. coli 23S RNA. At the 3'-end, a synthetic duplex linker was constructed containing a Cfr10I site, the 3' 31 bases of the mature 23S RNA sequence, an AfiI restriction site for linearization, and a PstI site for ligation into pUC19. The 3' end sequence of mature 23S RNA is such that a unique restriction site could not be engineered which would produce a 5' overhang (29) as well as the correct RNA sequence upon digestion and transcription. Therefore, we added 2 A-T base pairs to the 3' end of the construct to allow linearization with AfiI (Fig. 1B).

Attempts at a 5-way ligation of the 826 bp fragment, the 2030 bp fragment, and the 2 linkers shown in Fig. 1B into Kpnl-PstI double-digested pUC19 did not produce lac Z transformants. We reasoned that intermolecular ligation of Kpnl-Kpnl sites and PstI-PstI sites was responsible. Therefore, both 5'-phosphorylated termini of the Kpnl-PstI double-digested pUC19 were first blocked by incubation with ligase in the presence of a 14-fold excess of each duplex linker (Fig. 1B). Since the synthetic duplexes have neither 3'- nor 5'-phosphates, intermolecular ligation cannot occur. To verify that blockage had occurred, the reaction mixture was electrophoresed on a 0.7% agarose gel along with a control incubation which did not contain any linkers. The mixture which contained linkers gave a band at the location of marker linear pUC19 (obtained by cleavage at the unique Kpnl site), whereas the control lacking synthetic duplexes produced only a smear of high molecular weight material (data not shown). The defined band, presumed to contain the double-digested vector ligated to both linkers, was extracted from the gel.

Table 1. Yield of 23S RNA Transcript

<table>
<thead>
<tr>
<th>DNA</th>
<th>Pase</th>
<th>Transcript</th>
<th>mole RNA/ mol DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.14</td>
<td>+</td>
<td>35.4 ± 1.4</td>
<td>786 ± 30</td>
</tr>
<tr>
<td>0.29</td>
<td>+</td>
<td>45.5 ± 3.9</td>
<td>504 ± 43</td>
</tr>
<tr>
<td>0.29</td>
<td>-</td>
<td>18.6</td>
<td>206</td>
</tr>
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</table>

The two 23S fragments, SfaNI-EcoRI (826 bp) and EcoRI-Cfr10I (2030 bp) were added to the isolated vector at stoichiometries of 1:3 and 1:1 and ligated. Transformation efficiency was 20 to 30 colonies per ng (11 - 17 per fmol) based on the pUC19-linker input, whereas a control transformation using pUC19 yielded 1300 per ng (733 per fmol). Thus, we estimate a correct ligation frequency of approximately 2%.

Characterization of the Plasmid

Seventeen colonies out of 107 lac Z transformants were screened by digestion with PstI (expected: 1 band of 5589 bp), Kpnl (expected: 1 band of 3589 bp), and EcoRI (expected: 2 bands of 878 and 4711 bp). Sixteen of the 17 showed the predicted

Fig. 2. Electrophoresis of 23S RNA transcript on a denaturing agarose gel. Lanes from left to right contained, 4 μg each of 23S RNA from E. coli ribosomes (N), RNA from transcription 1 (T1), RNA from transcription 2 (T2), 23S RNA from E. coli ribosomes, (N) and RNA size standards (L; 0.24, 1.4, 2.4, 4.4, 7.5, and 9.5 x 10^3 bases, as indicated). Transcriptions 1 and 2 were independent transcription reactions.

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patterns. Four plasmids were sequenced by the dideoxy sequencing method using primers complementary to residues 43–63, residues 869–889 (26 residues 3′ to the internal EcoRI site), and 32–52 bases past the transcription stop in pCW1 (Fig. 1). All showed the expected sequences through all the ligation junctions (data not shown). Since the remainder of the gene was cut directly from pKK3535, it was not sequenced.

Transcription of 23S RNA
Plasmid pCW1 was linearized with Afl II and transcribed as described in Materials and Methods. Transcriptions were performed at two concentrations of DNA with or without inorganic pyrophosphatase (PPase). The amount of RNA obtained is shown in Table 1. These results show a clear effect of PPase on transcription yield, as was also observed for 16S RNA transcriptions (P. R. Cunningham and J. Ofengand, unpublished results). Doubling of the DNA input had only a small effect on yield (1.3-fold), but since we were able to obtain 2–4 mg of plasmid DNA from 100 ml of culture, most transcriptions were performed under the conditions specified in the second row of Table 1 to maximize the yield of RNA.

Characterization of the 23S RNA transcripts
The synthetic transcript and isolated 23S RNA as a control were subjected to denaturing gel electrophoresis (Fig. 2). Both the synthetic and natural 23S RNA migrated to the same position, and comparison with standards indicated a size of approximately 3000 bases. Segments of the RNA were sequenced using reverse transcriptase. Both the 5′ end including the SfaM site, and the EcoRI joint at position 843 of the transcript gave the expected sequence, although the nature of the 5′ terminal nucleotide could not be determined by reverse transcription (for example, see (1)). The 3′ terminus was sequenced after ligation of [32P]pCp and electrophoretic isolation of the 3′ terminal RNase T1 fragment. Although labeled natural 23S RNA produced a single T1 fragment of the expected size, the labeled transcripts produced 2 fragments. One (56% of the total) was the expected length whereas the other (44% of the total) was one residue longer. The ratio of the two oligomers remained constant despite a 4-fold increase in the amount of RNase T1 indicating that the two oligomers resulted from heterogeneity at the 3′ end rather than from incomplete cleavage after G2895. Direct enzymic sequencing (Fig. 3) verified that the shorter oligomer (Panel B) had the expected sequence while the longer oligomer (Panel A) had an additional residue at the 3′-end. The oligomer from the control natural 23S RNA (Panel C) also yielded the expected sequence. The longer oligomer (A) was heterogeneous at the 3′-end, since the NpCp region of the gel contained two distinct oligomers, labelled N and M. To verify the nature and amount of each 3′-terminal nucleotide, the labelled RNase T1 fragments were digested to completion with RNase T2 and analyzed by thin layer chromatography. 98% of the [32P] in Up in the oligomer from the control natural RNA, and 95% was in Ap in the shorter RNase T1 oligomer from the transcript.

Table 2. MethylaOon Activity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
<th>m5C</th>
<th>m5U</th>
<th>m5G</th>
<th>m6A</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100</td>
<td>10.3</td>
<td>0.29</td>
<td>0.53</td>
<td>0.24</td>
<td>0.04</td>
</tr>
<tr>
<td>HSW(A50s)</td>
<td>3.2</td>
<td>0.21</td>
<td>0.24</td>
<td>0.43</td>
<td>0.06</td>
</tr>
<tr>
<td>HSW(A50p)</td>
<td>1.3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*Enzyme fractions are described in Materials and Methods.

pmole [3H]CH3 incorporated per hr per mg of cells used as starting material.

Analysis was by enzymatic digestion to nucleosides and HPLC as described in Materials and Methods. Values have been corrected by subtraction from each value of the amount of total incorporation in the absence of added RNA; 0.027 for S100 and 0.013 for HSW. Thus the values given are minimum amounts, n.d., not determined.
RNase T1 oligomer contained 47% C, 41% A, and 12% U, but no G. The addition of an extra nucleotide during transcription by T7 RNA polymerase is not uncommon (1, 29). In this case, 56% of the transcripts were the correct length and sequence, 21% had an extra 3'-C, 18% an extra 3'-A, and 5% an extra 3'-U.

**Methylation of the 23S RNA transcript**

We have previously shown that 16S rRNA methyltransferases display strong sequence and structural specificity for their substrate (22, 23). Therefore, we tested the ability of the 23S RNA transcript to act as a substrate for the methyltransferase activity in *E. coli* extracts (Table 2). Most of the methylation activity for 23S RNA was found in the S100 (100,000×g supernatant) fraction in contrast to 16S RNA methyltransferases, which are enriched in the HSW fraction (23). The fact that fully-methylated 23S RNA isolated from *E. coli* ribosomes was not methylated (≤ 4% of the synthetic transcript (23)) indicates that the sites methylated in *vitro* were the same as those methylated in *vivo*. Although Table 2 shows that 30% of the methyltransferase activity was found in the ribosomal high salt wash (HSW) fractions, vigorous washing of the surface of the 100,000×g pellet removed an additional 5–15% of the total methyltransferase activity (ca. 1–3 units per mg cells) into the S100 fraction. This distribution contrasts with 16S RNA methyltransferases responsible for formation of m^5^C967 (22, 23) and m^5^A1518m^5^A1519 (31) which are enriched in the HSW fraction.

Activities for the synthesis of m^3^C, m^2^G, m^2^U and small amounts of m^6^A were found in both the HSW and S100 fractions. This may be due to a single methyltransferase for each modification which distributes between both fractions. However, we cannot exclude the possibility that the activities in the HSW and S100 fractions producing the same methylated nucleotide are different enzymes which make the same methylated nucleotide at different sites, since all of the methylated bases we detected occur two or more times in mature 23S RNA from *E. coli* (32, 33). Less than stoichiometric amounts of the methylated nucleotides were obtained even though the methylation reactions had reached a kinetic plateau. This is probably due to the crude nature of the enzyme fractions used. Even so, from 0.2 to 0.5 moles of each specific methylated base were produced per mole of 23S RNA showing that a major methylation site was involved in each case.

The m^2^U activity detected with this newly available substrate has not been reported previously, although it is known that at least two m^2^U residues occur in 23S RNA (34, 20). The m^3^C activity described here could be the one reported previously (35) or it could be a new activity since there are two known m^3^C residues in 23S RNA (32, 33). Similarly, the m^2^G activity detected in our experiments could be the one described by Isaksson (36) or it could be a new one since 2–3 m^2^G residues have been reported in 23S RNA (32, 33), although none have been located in the 23S primary sequence to date. Location of the observed methylations in the primary sequence is an obvious target for further research.

**CONCLUSIONS**

Milligram amounts of 23S RNA are readily produced from the subclone of the *rrlB* gene described in this work. The transcript is recognized by cognate methyltransferases, demonstrating the utility of the synthetic transcript in the identification and isolation of methyltransferase activities. Two avenues of research are thus opened up by the availability of this transcript. First, the application of the techniques of site-directed mutagenesis already developed for the analogous 16S RNA transcript (1, 24) and the existence of procedures for complete 50S subunit reconstitution (37) should allow structure-function mapping of the 23S RNA in the 50S subunit. Second, both the characteristics of individual methyltransferases and the roles of individual methylations in assembly and function of the 50S subunits can be studied with the availability of methyl-free substrates.

**REFERENCES**