REPORT

Methylation of 23S rRNA nucleotide G745 is a secondary function of the RlmA^I methyltransferase

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ABSTRACT

Several groups of Gram-negative bacteria possess an RlmA^I methyltransferase that methylates 23S rRNA nucleotide G745 at the N1 position. Inactivation of *rlmA^I* in *Acinetobacter calcoaceticus* and *Escherichia coli* reduces growth rates by at least 30%, supposedly due to ribosome malfunction. Wild-type phenotypes are restored by introduction of plasmid-encoded *rlmA^I*, but not by the orthologous Gram-positive gene *rlmA^{II}* that methylates the neighboring nucleotide G748. Nucleotide G745 interacts with A752 in a manner that does not involve the guanine N1 position. When a cytosine is substituted at A752, a Watson-Crick G745-C752 pair is formed occluding the guanine N1 and greatly reducing RlmA^I methylation. Methylation is completely abolished by substitution of the G745 base. Intriguingly, the absence of methylation in *E. coli* rRNA mutant strains causes no reduction in growth rate. Furthermore, the slow-growing *rlmA^I* knockout strains of *Acinetobacter* and *E. coli* revert to the wild-type growth phenotype after serial passages on agar plates. All the cells tested were pseudorevertants, and none of them had recovered G745 methylation. Analyses of the pseudorevertants failed to reveal second-site mutations in the ribosomal components close to nucleotide G745. The results indicate that cell growth is not dependent on G745 methylation, and that the RlmA^I methyltransferase therefore has another (as yet unidentified) primary function.

Keywords: rRNA methylation; RNA methyltransferase; ribosome tunnel

INTRODUCTION

All living organisms modify their stable RNAs post-transcriptionally, and to date almost one hundred different types of nucleotide modification having been reported (Rozenski et al. 1999). In bacteria, each rRNA modification is generally the product of a specific enzyme, contrasting with the mechanisms in eukaryal cells where numerous modifications are guided by a variety of snoRNAs that function together with a limited set of enzymes (Kiss 2002; Ofengand 2002; Decatur and Fournier 2003). Whatever the mechanism involved, any nucleotide modification will have a finite energetic cost, and thus each rRNA modification might be expected to prove its worth by performing a distinct function. Several nucleotide modifications in rRNA have indeed been shown to be important for translation (Green and Noller 1996), and a number of additional modifications provide advantageous phenotypic traits under particular

conditions, such as conferring resistance against ribosome-targeting antibiotics (Cundliffe 1989; Weisblum 1995; Mankin 2001). However, the functions of most rRNA modifications are far from fully understood. Moreover, there is a small but growing number of reports of rRNA modifying enzymes that are necessary for efficient cell growth while the products of these enzymes, the nucleotide modifications, appear to be superfluous (Persson et al. 1992; Lafontaine et al. 1998; Gutsell et al. 2001).

The rRNA modifications in *Escherichia coli* are the best characterized of any species (Rozenski et al. 1999). The *E. coli* 16S and 23S rRNAs contain 11 and 23 modifications respectively, and in many cases the enzymes responsible for these modifications are known. Several classes of Gram-negative bacteria, including *E. coli*, are methylated at the conserved nucleotide G745 in domain II of 23S rRNA (Fig. 1) by the methyltransferase enzyme RlmA^I (formerly RrmA) (Gustafsson and Persson 1998; Liu and Douthwaite 2002a). Some Gram-positive species possess an orthologous methyltransferase, RlmA^{II} (formerly TlrB), which methylates the neighboring and similarly conserved nucleotide G748 (Liu and Douthwaite 2002a), and confers resistance to the macrolide antibiotic tylosin (Fouces et al. 1999; Wilson and Cundliffe 1999; Liu and Douthwaite 2002b). Methylation at G745 does not confer antibiotic resistance, and

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FIGURE 1. (Left panel) Secondary structure of the *E. coli* 23S rRNA stem-loop containing the target nucleotides for the RlmA^I and RlmA^{II} methyltransferases at G745 and G748, respectively. Base substitutions were made at G745 and A752 by site-directed mutagenesis. The *Acinetobacter* rRNA has essentially the same sequence. (Right panel) Tertiary fold of same stem-loop determined by NMR (Lebars et al. 2003); the structure is an average of the 17 coordinate sets in the database file. The sheared base pair between G745 and A752 is evident. The N-1 of G745 is oriented toward the RNA major groove; the N-1 of G748 is more readily accessible at the tip of the hairpin loop. The structure of the *E. coli* RlmA^I has recently been solved, and a mechanism of substrate recognition has been proposed (Das et al. 2004).

although the exact function of RlmA^I is less clear, it has been shown that inactivation of the *E. coli* *rlmA*^I gene reduces the efficiency of protein synthesis and concomitantly lowers growth rates (Gustafsson and Persson 1998).

In the course of using several different *E. coli* strains in which the *rlmA*^I genes had been inactivated (Björk and Isaksson 1970; Gustafsson and Persson 1998; Liu and Douthwaite 2002c), we observed that the slow-growth phenotype tended to be spontaneously lost on continual replating and regrowth of cultures. The resultant cells were all pseudorevertants that had regained the wild-type growth rate without restoring methylation at G745. It was not immediately clear to us whether we were observing a phenomenon peculiar to *E. coli*, or whether these results indicated that the RlmA^I class of methyltransferases had a more important function than the modification of G745.

Here, we investigate the importance of G745 methylation for cell growth by blocking RlmA^I activity in two independent ways. First, the *rlmA*^I gene was inactivated in another bacterial model; and second, methylation was blocked by altering the structure of the rRNA substrate at nucleotide G745. For the first study, the Gram-negative bacterium *Acinetobacter* was chosen as methylation of its rRNA at G745 (Liu and Douthwaite 2002a) indicated that it possesses an *rlmA*^I gene, and because this bacterium is suitably phylogenetically distant from *E. coli* to be able to make a comparison of the RlmA^I methyltransferases worthwhile. We identified the *Acinetobacter* *rlmA*^I prior to inactivating the gene within the original strain and evaluating how this affected cell growth. We then attempted to rescue the original *Acinetobacter* growth characteristics by introducing homologous *rlmA*^I and orthologous *rlmA*^{II} genes on plasmids, and also by selecting for spontaneous revertant strains. In parallel experiments, we mutagenized the rRNA target to pre-

vent methylation of nucleotide 745 in *E. coli* strains that possess an authentic and active copy of *rlmA*^I. These studies conclusively show that methylation at G745 is not required to maintain growth rates, and is thus not the primary function of the RlmA^I methyltransferase.

RESULTS AND DISCUSSION

Cloning of *Acinetobacter* *rlmA*^I

In this study, we investigate the function of the RlmA^I methyltransferase that modifies 23S rRNA nucleotide G745 in several groups of Gram-negative bacteria. There is a high degree of similarity between RlmA^I and the orthologous methyltransferase RlmA^{II}, which modifies the neighboring nucleotide G748 in Gram-positive species (Liu and Douthwaite 2002a). Both classes of enzyme methylate at the N1 position of their respective guanine targets (Douthwaite et al. 2004).

Alignment of previously characterized RlmA^I and RlmA^{II} sequences (Bujnicki et al. 2002; Liu and Douthwaite 2002a) revealed two suitably spaced blocks of conserved amino acids: CGEGYY in the N-terminal region, corresponding to domain I of the methyltransferase S-adenosylmethionine binding site (Kagan and Clarke 1994); and MTPF/yA/gW within the methyltransferase C-terminal sequence. The primers mf51 and mf52, respectively corresponding to these amino acid sequences, gave a PCR product of approximately 450 bp from the *Acinetobacter* chromosome template, fitting with the expected sequence and size of an *rlmA*^I gene fragment. This fragment (cloned in plasmid pSD50) was used to probe Southern blots of *Acinetobacter* genomic DNA, and the intact *rlmA*^I gene was found in a *Hind*III fragment (~2.6 kb), which was subsequently cloned into plasmid pSD82 (Table 1).

The coding region *Acinetobacter* *rlmA*^I is 828 bp long, corresponding to a protein of 275 amino acids. As was expected, the putative protein is more closely related to RlmA^I proteins from Gram-negative bacteria than to RlmA^{II} from Gram-positives, and shows 41% identity (51% similarity) with *E. coli* RlmA^I, compared to 31% identity (39% similarity) with *Streptomyces fradiae* RlmA^{II}. The 1002 bp sequence of the *Acinetobacter* *rlmA*^I gene with its promoter and terminator has been deposited in the GenBank (accession number AY029296).

The *Acinetobacter* RlmA^I methylates 23S rRNA nucleotide G745

The *Acinetobacter* RlmA^I protein was expressed in *E. coli* and purified to over 95% homogeneity, as estimated by SDS-PAGE mini-gel electrophoresis. This protein was then incubated with 23S rRNAs from *Acinetobacter* ASD89 and *E. coli* IB10 both of which lack methylation at nucleotide G745. Primer extension analysis showed that the *Acineto*-

TABLE 1. Bacterial strains, plasmids and oligodeoxynucleotide primers

	Description	Reference
Strains		
<i>Acinetobacter calcoaceticus</i> ADP1	Wild type	Juni 1972
<i>A. calcoaceticus</i> ASD89	Derived from ADP1: <i>rlmA^I</i> , Kan ^R	This study
<i>Escherichia coli</i> DH10B	F [−] <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80d <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR recA1 endA1 araD139</i> Δ(<i>ara, leu</i>)7697 <i>galU galK λrpsL nupG</i>	Life Technologies, Inc.
<i>E. coli</i> IB10	<i>thr leu his argA relA rrmA10</i>	Björk and Isaksson 1970
<i>E. coli</i> TA531	Δ(<i>rrnE rrnB rrnH rrnA</i>) <i>rrnC::LacZ⁺ rrnD::cat⁺ rrmC::cat⁺ ΔrecA</i> /pTRNA66, pHK- <i>rrnC⁺</i>	Asai et al. 1999
Plasmids		
pHK- <i>rrnC⁺</i>	Contains the entire, wild-type <i>E. coli rrnC</i> ; used to maintain <i>E. coli</i> strain TA531: Kan ^R	Asai et al. 1999
pNK	Contains the entire, wild-type <i>E. coli rrnB</i> : Amp ^R	Xiong et al. 2000
pSD50	<i>Acinetobacter rlmA</i> fragment cloned in pUC19 at <i>HincII</i> : Amp ^R	This study
pSD82	<i>Acinetobacter HindIII</i> genome fragment with complete <i>rlmA^I</i> sequence; cloned in pUC19: Amp ^R	This study
pSD88	<i>Acinetobacter rlmA^I</i> gene cloned in pUHE24-2 at <i>NedI</i> / <i>BglII</i> : Amp ^R	This study
pSD88-KanR	Disruption of pSD88 <i>rlmA^I</i> gene at <i>Eco47III</i> : Amp ^R , Kan ^R	This study
pSD114	<i>Streptomyces fradiae rlmA^{II}</i> (<i>tlrB</i>) gene cloned in pWH845: Amp ^R , <i>lacI^q</i>	This study
pSD118	<i>Corynebacterium glutamicum</i> pAG1 <i>rlmA^{II}</i> gene cloned in pWH845: Amp ^R , <i>lacI^q</i>	This study
pSD189	<i>Acinetobacter rlmA^I</i> gene cloned in pWH845: Amp ^R , <i>lacI^q</i>	This study
pUC19	Cloning/sequencing vector: Amp ^R	Yanisch-Perron et al. 1985
pUHE24-2	Cloning/expression vector: Amp ^R	Bujard et al. 1987
pWH845	<i>Acinetobacter/E.coli</i> shuttle vector: Amp ^R , <i>lacI^q</i>	Schirmer et al. 1997

Amp^R and Kan^R indicate ampicillin and kanamycin resistance.

bacter RlmA^I methylated G745 in both 23S rRNAs in vitro by a mechanism that is dependent on the methyl donor S-adenosylmethionine (data not shown).

The knockout experiment confirmed that *Acinetobacter* RlmA^I carries out the same G745 methylation reaction in vivo. The chromosomal DNA of six randomly chosen *Acinetobacter* Kan^R recombinants gave rise to a 2-kb PCR product (0.8 kb *rlmA^I* + 1.2-kb Kan^R cassette). Reverse transcriptase primer extension showed that G745 was no longer methylated in any of these strains.

Slow growth of the *Acinetobacter rlmA^I* knockout

Inactivation of the *rlmA^I* gene caused a significant reduction in the growth rate of *Acinetobacter* on agar plates. The doubling time of one of the knockout clones, ASD89, was measured to be 39 min in liquid medium, versus 30 min for the wild-type strain grown under the same conditions. Comparable growth retardation has been observed after inactivation of *rlmA^I* in *E. coli* (Gustafsson and Persson 1998).

Plasmid-encoded *rlmA^I* genes from *E. coli* and *Acinetobacter* and *rlmA^{II}* genes from *Streptomyces fradiae* and *Corynebacterium glutamicum* (Table 1) were expressed in *Acinetobacter* ASD89, and methylated > 95% of the 23S rRNA molecules at the respective G745 and G748 targets (not shown). The growth defect of *Acinetobacter* ASD89 was cor-

rected by both the *rlmA^I* genes, but not by either of the orthologous *rlmA^{II}* genes. The successful rescue by plasmid-encoded *rlmA^I* matches an earlier observation for *E. coli* (Gustafsson and Persson 1998), and shows that the slow growth phenotype of the knockout strains was not caused by co-disruption of some other chromosomal gene. We note in this context that *rlmA^I* is tightly flanked on the *Acinetobacter* chromosome by the penicillin-binding transpeptidase gene. It is unclear whether this arrangement is significant, although it is similar to that in *Salmonella enterica* and *Streptomyces fradiae*, where the *rlmA* gene is within 13 to 360 bp of the gene for cell wall synthesis.

Spontaneous reversion from the slow grow phenotype

The slower growth of *E. coli rlmA^I* knockouts was previously thought to reflect the requirement for methylation at nucleotide G745. While this idea is consistent with many observations, including the inability of G748 methylation to rescue the *rlmA^I* defect, it does not fit with a closer analysis of revertant strains. The growth defect of the *Acinetobacter rlmA^I* knockout was lost during serial passaging of the strain on agar plates, consistent with our observations for *E. coli rlmA^I* knockouts. The reversion to wild-type growth rate is a spontaneous event, and no slow growing *Acinetobacter* colonies were observed at the fourteenth passage. None of

the *Acinetobacter* or *E. coli* cells tested had regained their ability to methylate G745, and all were thus pseudorevertants.

The pseudorevertants offer a potential means of elucidating the function of RlmA^I and G745 methylation. From the ribosome crystal structures (Ban et al. 2000; Harms et al. 2001; Yusupov et al. 2001), the N1 group of G745 can be seen to intrude into a narrow region of the subunit tunnel that is involved in the gating of peptides (Nakatogawa and Ito 2002; Tenson and Ehrenberg 2002), and is adjacent to the macrolide antibiotic binding site (Schlünzen et al. 2001; Hansen et al. 2002). Thus, it was tempting to speculate that the methylation state of G745 could play a role in one or more of these processes. We therefore took two of the *E. coli* pseudorevertants (in preference to *Acinetobacter*, for which the genome is not yet known) and sequenced the genes for the ribosomal components that are situated close to G745. However, we found no second-site mutations in any of these components, including the rRNA sequences surrounding nucleotides G745 and A2058, and the r-proteins L4 and L22, which have been shown to influence the subunit structure close to G745 (Gregory and Dahlberg 1999; Gabashvili et al. 2001).

Mutagenizing the rRNA target for RlmA^I

In a final series of experiments, we prevented RlmA^I methylation by mutagenizing its rRNA target site. The RlmA^I methyltransferase modifies its substrate before the rRNA associates with the r-proteins to assemble into functional 50S subunits (Hansen et al. 2001). The structure of this RNA region, in the conformation that is recognized by the methyltransferase, has been solved by NMR (Lebars et al. 2003) and shows that G745 and A752 stack onto helix 35 to form an irregular, noncanonical base pair, which does not involve the N1 position of G745 (Fig. 1). Single base substitutions were introduced in the *E. coli* 23S rRNA gene and were expressed in *E. coli* strain TA531 (Table 1), which enabled us to observe the phenotypes conferred by homogeneous populations of mutant ribosomes. TA531 cells with the mutant rRNAs were all kanamycin sensitive, indicating that the original plasmid with the wild-type *rrnC* operon had been lost. Furthermore, direct sequencing of the rRNA verified that ribosomes contained only mutant 23S rRNA encoded by the pNK-encoded *rrnB* operon.

From the NMR structure (Fig. 1), substitution of guanine or uracil at nucleotide 752 would not be expected to decrease the accessibility of G745. Accordingly, methylation in vivo by RlmA^I remains complete in the 752G and 752U mutants (Fig. 2). Similarly, mild alteration of the RNA loop conformation by deletion of one of the adenosines between 749 and 753 had no detectable effect on in vivo methylation, nor was any effect on RlmA^I methylation observed after substitution of nucleotide 748. However, methylation by RlmA^I was greatly reduced by introduction of a cytosine at position 752 (Fig. 2). This latter effect is presumably due to

FIGURE 2. Gel autoradiographs showing the effects of rRNA mutations on methylation at nucleotide 745 by RlmA^I. Mutant and wild-type rRNAs were extracted from *E. coli* strain TA531 that contains an active RlmA^I methyltransferase, and were sequenced by reverse transcriptase extension from the mfl1 primer. Methylation at the G745 N1 aborts reverse transcription. (A) All four sequencing reactions for the wild-type strain (98% of the rRNA molecules were methylated); for the 752C mutant (14% methylation); and for the 745C mutant (zero methylation). (B) Selected sequencing reactions (horizontal letters above the lanes) from the other mutant rRNA strains discussed in the text (vertical labeling). ΔA, deletion of one of the adenosines between 749 and 753.

the formation of a Watson–Crick G–C pair, which engages the N1 proton of G745 in a hydrogen bond. Methylation was completely abolished after removal of the RlmA^I target base by substitutions at G745.

Despite the loss of methylation (and the loss of phylogenetically conserved bases), none of the substitutions at positions 745 and 752 altered the growth rate of *E. coli*, which was consistently around 57 min (with a standard deviation of ± 3.5 min) under the growth conditions used. The phenotypes of these rRNA mutants assuage our lack of success at identifying second-site mutations in the ribosomal components of the pseudorevertants. The putative second-site mutations are possibly not rescuing the lack of G745 methylation, but are perhaps compensating for some other function of RlmA^I.

Alternative function for RlmA^I

There are other reports of enzymes that modify stable RNAs while presumably having another, more important, func-

tion. Two such examples of methyltransferases are TrmA in *E. coli* that is specific for 5-methylation of U54 in tRNA (Persson et al. 1992), and yeast Dim1p that dimethylates the N6 of both A1779 and A1780 in 18S rRNA (Lafontaine et al. 1998). Both methyltransferases are essential for cell viability, whereas the modifications they carry out are dispensable. This type of phenomenon is not limited to methyltransferases, and has also been observed for RluD, an enzyme that converts three uridines in *E. coli* 23S rRNA to pseudouridines. Knocking out *rluD* expression adversely affects growth, whereas point mutations in *rluD* that allow RluD expression while abolishing its pseudouridine synthetase activity do not appear to have a detrimental effect (Gutgsell et al. 2001).

Our rRNA mutagenesis study closely parallels that of RluD: In both studies, cells express a full length and (presumably) correctly folded modification enzyme which fails to modify its substrate, either because of a substitution in the active site of the enzyme (RluD) or a substitution in the rRNA substrate (this study). As in the RluD study, we conclude that rRNA modification is a secondary function of the enzyme under investigation. The primary functions of these enzymes are most likely ribosome-related, and the involvement of RlmA^I in a chaperone-like role aiding ribosome assembly, as has been suggested for RluD (Gutgsell et al. 2001), remains a strong possibility.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

All the strains and plasmids employed in this study are listed in Table 1. *Acinetobacter* and *E. coli* strains were grown at 37°C in LB medium (Sambrook et al. 1989). For transformation of *Acinetobacter*, cells were cultured in minimal medium (Juni 1972) supplemented with 10 mM succinate. *E. coli* strain DH10B was used for recombinant work including protein expression. Plasmid pUC19

was used for cloning of PCR products to identify the *Acinetobacter rlmA^I* gene, and as the vector for site-directed mutagenesis of *E. coli* 23S rRNA gene fragments. The complete *Acinetobacter rlmA^I* gene was cloned and expressed in plasmid pUHE24–2; mutant 23S rRNAs were expressed within their original *rrnB* context in plasmid pNK. Plasmid pWH845 was used for expressing various *rlmA^I* and *rlmA^{II}* genes in the *Acinetobacter rlmA^I* knock-out strain, and as a shuttle vector between *Acinetobacter* and *E. coli*. Where appropriate, the antibiotic ampicillin was used at a concentration of 100 µg/ml for *E. coli*, and at 300 µg/ml for *Acinetobacter*; kanamycin was used at 25 µg/ml for both bacteria.

Phenotypes conferred by the different mutant rRNAs were evaluated in *E. coli* strain TA531. All the seven chromosome-coded rRNA (*rrn*) operons have been disrupted in TA531 (Asai et al. 1999); rRNA production in this strain is dependent on plasmid-encode *rrn* that is initially provided on pHK-*rrnC⁺* and subsequently on pNK-derivatives with mutant *rrnB*. For cell growth rate determinations, one thousand-fold dilutions of overnight cultures were grown in Luria broth with the appropriate antibiotics, and cell densities were followed at OD₆₀₀.

DNA manipulations

Acinetobacter chromosomal DNA was isolated essentially as described for *E. coli* (Sambrook et al. 1989). Standard DNA techniques were employed for plasmid DNA isolation, DNA manipulations and *E. coli* transformation (Sambrook et al. 1989). *Acinetobacter* was transformed by exploiting the natural competency of the bacterium (Palmen et al. 1993). Plasmid DNA was sequenced using the CEQ 2000 DNA Analysis System (Beckman), primed from the vector or within the gene. Restriction enzymes, ligase and *Pwo* polymerase (Roche Molecular Biochemicals) and AMV reverse transcriptase (Finnzymes) were used according to the suppliers' recommendations.

Identification and cloning of the *Acinetobacter rlmA^I* gene

Two degenerate PCR primers, mf51 and mf52 (Table 2), were designed based on the sequences of previously characterized

TABLE 2. Oligodeoxynucleotide primers used in this study

Primer name	Sequence	Application
–21	5'-AACAGCTATGACCATG	Binds after the 3' end of the pUC19 polycloning site
–40	5'-GTTTTCCAGTCACGAC	Binds in front of the 5' end of the pUC19 polycloning site
gwn1 to gwn6	5'-CGACTAATGTTGAAAATTAGCGG	Mutagenesis ^a of G745 and A752
gwn11 to gwn16	5'-CCGCTAATTTTCAACATTAGTCG	Mutagenesis ^a of G745 and A752
mf11	5'-TTTCGGGGAGAACCAGCTA	Primer extension from 23S rRNA nucleotides 800 to 817
mf51	5'-TGYGGNGARGGNTAYTAY	Degenerate PCR primer ^b <i>rlmA^I</i>
mf52	5'-CCANGCRWANGGNGTCAT	Degenerate PCR primer ^b <i>rlmA^I</i>
mf71	5'-TAACACTCATATGcatcaccatcaccatcac AATGTGTTGATGTGCCCTGTG	5'-end <i>rlmA^I</i> with NdeI site (underlined) plus 6xHis tag (small-case)
mf72	5'-GCAGAAAGCTTCACTGGAAGGATTGATTAATCAAGTGA	3'-end <i>rlmA^I</i> with HindIII site (underlined)
sd33	5'-CATCCGCTAATTTTC	Complementary to 23S sequence 748–763

^aThe positions underlined in bold correspond to nucleotides 745 and 752 where base substitutions were introduced in the *rrnB* 23S rRNA gene. A single change was made in each of the 5'-mutagenesis primers and was used with the complementary change in the 3'-primer.

^bN = G/A/T/C; Y = C/T; R = A/G; W = A/T.

RlmA^I and RlmA^{II} proteins. PCR reactions on *Acinetobacter* chromosomal DNA using mf51 and mf52 (annealing temperature 50°C) gave rise to a main product of approximately 450 bp, which was then ligated into the HincII site of pUC19. The first three plasmid clones that were sequenced contained the same fragment, and this was homologous to known *rlmA* sequences (Bujnicki et al. 2002; Liu and Douthwaite 2002a); one plasmid clone (dubbed pSD50) was used for further study.

Acinetobacter chromosomal DNA was digested with BamHI, EcoRI, HindIII, XbaI, and XhoI, and was run in duplicate sets on a 0.7% agarose gel. The one set of DNA digestion products was denatured, blotted to Zeta-Probe Membrane (Bio-Rad) and probed under stringent conditions (Sambrook et al. 1989) with the ³²P-labeled insert from pSD50. Signal bands in the XbaI and HindIII lanes were used to locate the corresponding gel bands in the second set of digestion products, which were then extracted using a gel purification kit (Qiagen) and cloned into the respective restriction sites in pUC19. Recombinants were screened by colony hybridization using the same pSD50 probe, and positive clones were sequenced.

The complete *Acinetobacter rlmA^I* gene was identified in plasmid pSD82 that contained a HindIII fragment. The *Acinetobacter rlmA^I* gene was lifted out of pSD82 by PCR using primers mf71 and mf72 (annealing temperature 55°C), which correspond to the 5'- and 3'-ends of the gene and encode NdeI and HindIII sites, respectively; mf71 additionally includes the code for a histidine tag. The PCR product of slightly less than 900 bp was inserted into the NdeI/HindIII sites of the expression vector pUHE24–2. The first three plasmids analyzed contained the correct *Acinetobacter rlmA^I* sequence, and one plasmid (pSD88) was taken for further study.

Insertional inactivation of the *Acinetobacter* chromosomal *rlmA^I* gene

A kanamycin resistance gene cassette was inserted into the Eco47III site of the pSD88 *rlmA^I* gene, creating plasmid pSD88-Kan^R. The latter plasmid was linearized and used to transform *Acinetobacter* ADP1. Cells were selected on plates containing kanamycin, and Kan^R *rlmA^I* knock-out candidates were screened by PCR using the mf71 and mf72 primers. The loss of methylation at nucleotide G745 in knock-out clones was verified by primer extension from the 23S rRNA primer mf11, and one *Acinetobacter* clone that passed all the screening criteria was called ASD89.

Expression of *rlmA^I* and *rlmA^{II}* genes in *Acinetobacter* ASD89

The *rlmA^I* genes from *E. coli* (Hansen et al. 2001) and *Acinetobacter*, and the *rlmA^{II}* genes from *Streptomyces fradiae* and *Corynebacterium glutamicum* (Liu and Douthwaite 2002a) were cloned in plasmid pWH845 (Table 1). The pWH845-encoded methyltransferases were expressed in *Acinetobacter* ASD89, and the degree of methylation at nucleotides G745 or G748 was assessed by reverse transcriptase analysis of the 23S rRNA (method description below).

RlmA^I protein purification and activity

The N-terminal histidine-tagged *Acinetobacter* RlmA^I protein was expressed from plasmid pSD88 in *E. coli* and was purified by nickel

ion affinity chromatography (Qiagen). Total RNA from *Acinetobacter* ASD89 and *E. coli* IB10 was isolated using RNeasy mini kit (Qiagen). The action of RlmA^I on unmethylated 23S rRNA substrates from these strains was investigated in vitro as previously described (Liu et al. 2000).

Site-directed mutagenesis of *E. coli* rRNA

Nucleotides 745 and 752 in the *E. coli rrnB* 23S rRNA gene were mutagenized by PCR using two sets of oligonucleotides (Higuchi et al. 1988; Ho et al. 1989). Briefly, the 900 bp SacI/SphI fragment from domain II of the *rrnB* 23S rRNA gene was inserted into the same sites of plasmid pUC19. Complementary oligonucleotide primers containing the desired rRNA mutations were designed (Table 1), and each was used in separate PCR reactions with the -21 and -40 primers that bind outside the SacI and the SphI sites in pUC19. The products from the first two reactions were mixed in a second PCR reaction to regenerate the 900 fragment, which was cloned back into pUC19. The SacI/SphI fragment was sequenced in plasmid clones, and those containing the desired mutations were inserted into the original *rrnB* context in plasmid pNK.

Expression of mutant 23S rRNAs

The pNK plasmid-derivatives were moved into *E. coli* strain TA531. Cells were grown for at least twenty generations in LB medium containing ampicillin, before being diluted 10⁵-fold and plating onto agar with ampicillin. Individual colonies were screened for loss of kanamycin resistance, which indicated displacement of the resident plasmid pHK-rrnC⁺ by a pNK-derivative. As the only source of rRNA genes in these strains is the pNK-encoded *rrnB* operon, each strain will possess a homogeneous population of ribosomes containing mutant 23S rRNA. The structures of the pNK-derivatives were verified by restriction digestion and resequencing the SacI/SphI region for each rRNA mutant strain of TA531.

Purification of ribosomes and sequencing of rRNA

Ribosomes were prepared from the *Acinetobacter* and *E. coli* strains by sonication and centrifugation, followed by rRNA extraction as previously described (Stern et al. 1988). The 23S rRNA region around G745 was sequenced by extension with reverse transcriptase (Stern et al. 1988) from the primer mf11.

Methylation at G745 was measured using a modified version of the primer extension procedure devised by Ed Morgan's laboratory (Sigmund et al. 1988; Hansen et al. 2001). Briefly, 5'-³²P-labeled sd33 primer (Table 2) was annealed to the G748-G763 region of the RNA and was extended with reverse transcriptase using a mixture of 100 μM each of dATP, dCTP, and ddTTP. Extension terminates directly before G745 on methylated rRNA, or at A743 on unmethylated rRNA due to incorporation of the dideoxynucleotide. The degree of methylation was calculated from the relative intensities of the A743 and G745 bands on denaturing gels.

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