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Structural basis for selectivity and toxicity of ribosomal antibiotics

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Ribosomal antibiotics must discriminate between bacterial and eukaryotic ribosomes to various extents. Despite major differences in bacterial and eukaryotic ribosome structure, a single nucleotide or amino acid determines the selectivity of drugs affecting protein synthesis. Analysis of resistance mutations in bacteria allows the prediction of whether cytoplasmic or mitochondrial ribosomes in eukaryotic cells will be sensitive to the drug. This has important implications for drug specificity and toxicity. Together with recent data on the structure of ribosomal subunits these data provide the basis for development of new ribosomal antibiotics by rationale drug design.

INTRODUCTION

The ribosome is the target for many important antibacterial agents; these compounds interfere with essential steps of protein synthesis (Gale *et al.*, 1981; Noller, 1991): 2-deoxystreptamine aminoglycosides cause codon misreading and inhibit translocation (Davies and Davies, 1968); compounds of the streptomycin family are related to 2-deoxystreptamines and have a similar mode of action, but a different binding site (Moazed and Noller, 1987); macrolides, lincosamides and streptogramin B antibiotics are chemically distinct drugs which interact with the peptidyl transferase center of the large subunit (Gale *et al.*, 1981).

In spite of decades of use of ribosomal drugs we still do not understand the principles governing selectivity and toxicity of these agents. Most antibiotics that bind to the ribosome have been shown to interact directly with ribosomal RNA (Gale *et al.*, 1981; Moazed and Noller, 1987; Fourmy *et al.*, 1996). The high cross-species conservation of functional sites within ribosomal RNA, targeted by ribosomal drugs, implies limitations with respect to selectivity and toxicity.

Eukaryotic cytoplasmic ribosomes are insensitive to macrolides, lincosamides, streptomycin and 2-deoxysteptamines (Kurtz, 1974; Palmer and Wilhelm, 1987; Wilhelm *et al.*, 1987a,b); bacterial ribosomes are sensitive to the action of these drugs (Gale *et al.*, 1981). *In vitro* mitochondrial ribosomes show a mixed susceptibility pattern: lower eukaryotes are similar to bacterial ribosomes, higher eukaryotes show sensitivity to streptomycin and 2-deoxystreptamines but insensitivity to macrolides and lincosamides (Kurtz, 1974).

Macrolides and lincosamides show little toxicity in humans while the therapeutic use of aminoglycosides (streptomycin and 2-deoxystreptamines) is limited by ototoxicity and nephrotoxicity (for review see Mingeot-Leclercq and Tulkens, 1999). Although the basis for the selective drug related toxicity is unknown, several lines of evidence suggest links to the mitochondrial genome (Prezant *et al.*, 1993).

Mutational genetic, biochemical and structural studies have characterized the importance of specific nucleotides for the high-affinity binding of ribosomal drugs to their respective rRNA target site (for review see Noller, 1991; Garrett *et al.*, 2000). Ribosomal drugs apparently work by altering the balance between conformational states of the ribosome as it goes through the process of translation. Streptomycin, in particular, affects a ribosomal accuracy switch; a structural rationale for the properties of streptomycin to affect both initial tRNA selection and proof-reading was offered by the finding that it makes the transition of the ribosome to a restrictive state more difficult by preferential stabilization of a ribosomal ambiguity (ram) state (Carter *et al.*, 2000).

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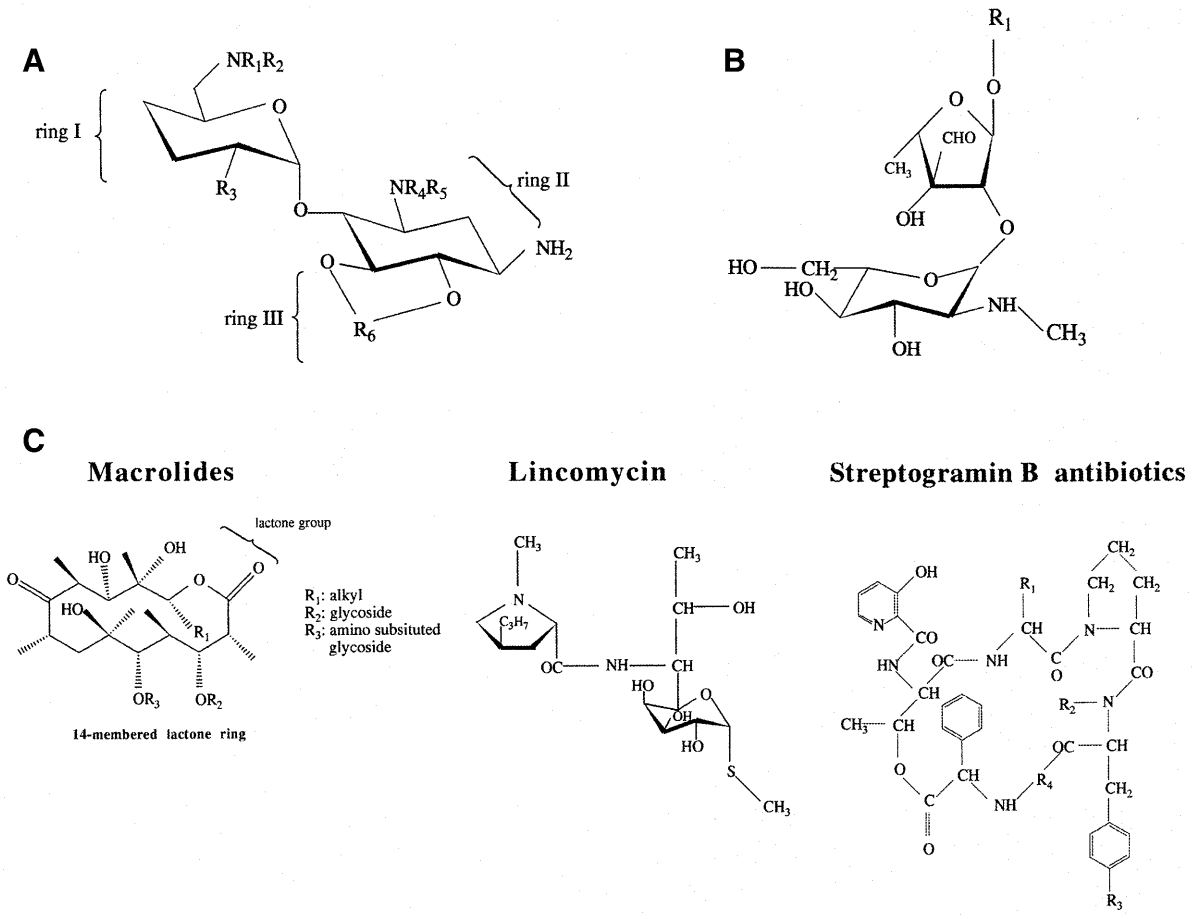

Fig. 1. Chemical structures of ribosomal antibiotics. (**A**) Common elements of 2-deoxystreptamine aminoglycosides to which expression of 1408G confers resistance. Rings I and II are common moieties of the aminoglycosides. Ring I carries an N6 amino group, ring II carries N1 and N3 amino groups, the linkage of ring III can vary. In the neomycin class ring III is connected to position 5 of ring II (4,5-disubstituted ring II), in the kanamycin class ring III is linked to position 6 of ring II (4,6-disubstituted ring II: gentamicin, amikacin, tobramycin, kanamycin). (**B**) Structure of aminoglycosides of the streptomycin family to which expression of RpsL 87 Gly confers resistance. Streptomycins are aminoglycoside antibiotics characterized by the presence of a cyclitol (R_1). (**C**) Chemical structures of antibiotics to which expression of 2058G confers resistance. Macrolides are composed of a minimum of two amino (R_3) and/or neutral sugars (R_2) attached to a lactone ring of variable size, most commercially available macrolides contain a 14-atom lacton ring (clarithromycin, roxithromycin, erythromycin) or a 15-atom lacton ring (azithromycin) structure; lincosamides (lincomycin, clindamycin) are alkyl derivates of proline and are devoid of a lactone ring; streptogramin B antibiotics (quinupristin, pristinamycin I, virginiamycin S, streptogramin B) are cyclic hexadepsipeptides, R_4 usually is pipecolic acid or one of its derivatives.

To establish the link between antibiotic action, ribosomal *in vitro* assays and binding affinity as determined by defined sequence alterations, we decided to investigate bacterial mutations causing drug resistance *in vivo*. Our results lead us to conclude that investigation of bacterial drug resistance mutations allows the definition and understanding of the basis for the specificity of ribosomal antibiotics, in particular the importance of mitoribosomal sequences in influencing toxicity.

RESULTS

The structure of the ribosomal antibiotics investigated is given in Figure 1. To determine which specific differences between eukaryotic and bacterial ribosomal components mediate the selectivity and toxicity of ribosomal drugs, bacterial organisms with acquired mutational drug resistance (e.g. Finken *et al.*, 1993; Sander *et al.*, 1996; Wang and Taylor, 1998) should be most helpful. The identification of resistance-associated mutations and the comparison with their eukaryotic mitochondrial and cytoplasmic homologues should help to identify those residues likely to be involved in determining the selectivity and toxicity of ribosomal drugs.

By mutational analysis in *Escherichia coli* a number of nucleotides within the small subunit rRNA *(E. coli* numeration is used for all sequences discussed) have been implicated in streptomycin resistance, i.e. 507C → U, 523A → C, 525C → U, 912C → U (Montandon *et al.*, 1986; Powers and Noller, 1991; Lodmell and Dahlberg, 1997). Alteration of position 523A → C, 525C → U, 526C → U or 912C → U is found in streptomycin-resistant chloroplasts (Harris *et al.*, 1989). *In vivo* acquired drug resistance in bacterial pathogens is associated with mutational alterations of positions 501C → U, 522C → U, 523A → C, 526C

Table I. Identity of single positions determining drug susceptibility

Drug	Region	Position	Eubacteria	Drug suscept.	Chloroplasts	Drug suscept.	Higher eukaryotes: Mitochondrial	Drug suscept.	Cytoplasmic	Drug suscept.
Streptomycin	530-loop (ssrRNA)	523	A	**S**[a]	A	**S**	C	**S**	A	**R**[b]
	915-region (ssrRNA)[c]	912	C		C		C		U	
	rpsL	42	Lys		Lys		Lys		Lys	
		87	Lys		Lys		Gln		Gly	
2-deoxy-streptamines	Decoding region (ssrRNA)	1408	A	**S**	A	**S**	A	**S**	G	**R**
Macrolides Lincosamides Streptogramin B	Peptidyltransferase region (lsrRNA)[d]	2058	A	**S**	A	**S**	G	**R**	G	**R**

Drug susceptibility patterns are shown in bold type.
[a]S, susceptible.
[b]R, resistant.
[c]ssrRNA, small subunit rRNA.
[d]lsrRNA, large subunit rRNA.

→ U, 912C → A/G/U, 913A → G, 915A → C (e.g. Finken *et al.*, 1993). (For the secondary structure of rRNA regions associated with drug resistance see supplementary material).

Inspection of eukaryotic mitochondrial and cytoplasmic rRNA sequences reveals that only positions 523 and 912 differ between bacterial and eukaryotic ribosomes; 523 is C in mitochondrial rRNA and 912 is U in cytoplasmic rRNA (see Table I and supplementary Figure 1). The paradoxical finding that rRNA mutations associated with drug resistance are found both in mitochondrial ribosomes (streptomycin-susceptible) and in cytoplasmic ribosomes (streptomycin-resistant) prompted us to re-investigate the contribution of the respective mutations to drug resistance. Introduction of the 523A → C mutation into *Mycobacterium smegmatis* leads to low-level drug resistance (Table II), while we were unable to retrieve streptomycin-resistant transformants from cells carrying the 912C → U mutation, indicating that a 912C → U mutation does not confer streptomycin resistance in the system used.

In addition to mutations in *rrs*, alteration of ribosomal protein S12 (RpsL) is a major cause of streptomycin resistance in prokaryotes, in particular involving a lysine at amino acid position 42 or 87 (Funatsu and Wittmann, 1972; Finken *et al.*, 1993). The homologous human ribosomal proteins carry Lys-42, but Gln-87 (mitoribosomes) or Gly-87 (cytoribosomes) (see supplementary Figure 2). To test the hypothesis that Gly-87 mediates streptomycin resistance in eukaryotic cytoribosomes, genetic transfer experiments were carried out in *M. smegmatis*. Replacement of the chromosomal wildtype (wt) *rpsL* in *M. smegmatis* by a mutant *rpsL* carrying the mitochondrial Gln-87 conferred resistance to low drug concentrations; introduction of the mutant *rpsL* Gln-87 into bacteria carrying a chromosomal resistant *rpsL* (42 Lys → Arg) resulted in merodiploid strains with decreased streptomycin resistance (Table II). In contrast, replacement of the chromosomal wt *rpsL* by a mutant *rpsL* Gly-87 mediated high levels of drug resistance and introduction of the mutant *rpsL* Gly-87 did not restore a streptomycin-susceptible phenotype in merodiploid strains carrying *rpsL* 42 Lys → Arg (Table II). These results demonstrate that Gln-87 mediates low-level drug resistance while the cytoribosomal Gly-87 confers resistance to high drug concentrations.

2-deoxystreptamine aminoglycosides bind directly to the decoding region of the ribosomal A site (Moazed and Noller, 1987). Despite a multitude of nucleotides critical for drug-binding, i.e. 1407C/1494G, 1408A, 1491G, 1493A, 1495U (De Stasio *et al.*, 1989; Fourmy *et al.*, 1996), drug resistance in bacterial pathogens with *in vivo* acquired ribosomal resistance is confined to mutation of a single nucleotide position, i.e. 1408A → G (e.g. Sander *et al.*, 1996). Introduction of the 1408A → G mutation into the small subunit rRNA of *M. smegmatis* confers resistance to 2-deoxystreptamines (Table II), suggesting that bacterial susceptibility to this class of aminoglycosides is largely determined by the presence of an adenosine at nucleotide 1408 (Recht *et al.*, 1999). To investigate whether the susceptibility of a ribosomal system is associated with the nature of the nucleotide at position 1408, *in silico* sequence comparisons were carried out and revealed a mitochondrial adenosine versus a cytoplasmic guanosine at the corresponding sequence position (see Table I and supplementary material), indicating that the identity of the 1408 nucleotide determines drug susceptibility.

Macrolides, lincosamides and streptogramin B antibiotics inhibit protein synthesis by binding to the 50S ribosomal subunit, where the drugs interact with bases in the peptidyl transferase loop of 23 S rRNA (Rodriguez-Fonseca *et al.*, 1995). A 2058A → G or 2611C → G alteration (resulting in disruption of base pair interaction with 2057G) is found in macrolide resistant yeast mitochondria (Sor and Fukuhara, 1984), a 2057G → A, 2058A → G, 2059A → G or 2611C → G substitution in drug resistant chloroplasts (Harris *et al.*, 1989). Bacterial

Table II. Substitutions in ribosomal components and minimal inhibitory concentrations

Drug	Genotype	MIC	Relative resistance[a]
2-deoxystreptamines (Amikacin)	16S rRNA 1408A (wt)	0.6 mg/L	–
	16S rRNA 1408G[b]	>500 mg/L	>800
Streptomycin	16S rRNA 523A, RpsL 42-Lys + 87-Lys (wt)	1.0 mg/l	–
	16S rRNA 523C[b]	16 mg/L	16
	RpsL 42-Arg[c]	>512 mg/L	>500
	RpsL 87-Gln[c]	8 mg/L	8
	RpsL 87-Gly[c]	128 mg/L	128
	RpsL 42-Arg + 42-Arg[d]	>512 mg/L	>500
	RpsL 42-Arg + 87-Gln[d]	32 mg/L	32
	RpsL 42-Arg + 87-Gly[d]	>512 mg/L	>500
	RpsL 42-Arg + 87-Lys (wt)[d]	2 mg/L	2
Macrolides (Clarithromycin)	23S rRNA 2058A (wt)	0.06 mg/L	–
	23S rRNA 2058G[b]	128 mg/L	>2000

[a]Relative resistance is the value of the MIC of mutant cells divided by the MIC of wild-type cells.
[b]rRNA mutations were introduced into *M. smegmatis* as described in Methods.
[c]RecA-mediated gene conversion was used to introduce the mutation into the chromosomal *rpsL* gene of *M. smegmatis* mc^2 155.
[d]Merodiploid strains of *M. smegmatis SMR5 recA*$^-$ expressing chromosomal resistant *rpsL* (42-Arg) and recombinant *rpsL* (42-Arg, 87-Lys; 42-Lys, 87-Gln; 42-Lys, 87-Gly; 42-Lys, 87-Lys).

pathogens with *in vivo* acquired macrolide resistance show an alteration of 2057G, 2058A or 2059A. Site-directed mutagenesis of 2057G, 2058A or 2059A confers drug resistance in the context of a bacterial ribosome (Douthwaite and Aagaard, 1993; Wang and Taylor, 1998). Introduction of a 2058A → G mutation results in macrolide resistance in *M. smegmatis* (Table II; see also Sander *et al.*, 1997). Sequence comparisons demonstrate that mitochondrial and cytoplasmic rRNAs of higher eukaryotes carry a guanosine at position 2058 of 23 S rRNA but a 2057G and 2059A nucleotide (see Table I and supplementary material), indicating that in both ribosomal systems resistance correlates with the identity of nucleotide 2058.

DISCUSSION

Drugs affecting protein synthesis discriminate between bacterial and eukaryotic ribosomes to various extents. It has been hypothesized that the natural resistance or sensitivity of a ribosomal system (bacterial, chloroplast, protozoan, eukaryotic mitochondrial, eukaryotic cytoplasmic) to a ribosomal drug is determined by the sequence of its rRNA (Sor and Fukuhara, 1984; Beckers *et al.*, 1995). Chemical footprint experiments and high resolution crystal structure data suggest that a multitude of different nucleotides participate in binding of a drug to its target region within the rRNA (Carter *et al.*, 2000; Nissen *et al.*, 2000; for review Noller, 1991); correspondingly and in general, a number of different nucleotide substitutions are associated with acquired drug resistance in bacteria. Despite this complexity the natural resistance or sensitivity of a ribosomal system is likely to be governed by the presence of a single critical nucleotide or amino acid position.

A particularly informative example is the aminocyclitol streptomycin. Sequence comparisons of bacterial drug resistance mutations indicate that only 16S rRNA position 912 and RpsL position 87 are informative for the cytoplasmic ribosome, i.e. these positions differ between bacterial and eukaryotic cytoplasmic ribosomes. *rrs* nucleotide 912, although not directly involved in streptomycin binding, is in close proximity to the streptomycin binding site (Carter *et al.*, 2000). Nucleotide 912 is supposed to participate in a conformational switch during mRNA decoding (Lodmell and Dahlberg, 1997), in which *rrs* 912 is either base paired to *rrs* 888G (restrictive state) or 885G (error prone). *rrs* 912C → U is expected to facilitate the conformational switch but should not alter the equilibrium between the restrictive and error prone state. Introduction of the reciprocal mutation (912U → C) in yeast, which are naturally resistant to streptomycin, increased sensitivity to the antibiotic (Chernoff *et al.*, 1994). However, we were unable to retrieve streptomycin-resistant transformants from cells carrying the 912C → U mutation. These data are in accord with previous findings which failed to isolate streptomycin resistant *E. coli* from cells carrying a multicopy plasmid with a mutated 912C → U *rrn* B operon (Morgan *et al.*, 1988). Although these differences might reflect organism-specific characteristics, our results suggest that it is not the 16S rRNA position 912 which determines streptomycin resistance in eukaryotes, but rather the glycine at position 87 of the RpsL homologue.

The results presented here support the hypothesis that the specificity and selectivity of drugs targeting the ribosome are largely determined by the presence of a defined nucleotide or amino acid position within a ribosomal component. The *in vivo* toxicity of ribosomal drugs is thus most likely to be a consequence of the susceptibility of the eukaryotic mitoribosome.

These conclusions are based on the following observations: (i) resistance to drugs inactive against eukaryotic cytoplasmic ribosomes can be transferred to prokaryotes by alteration of a defined rRNA nucleotide or ribosomal protein amino acid to its respective eukaryotic homologue in the context of a bacterial ribosome; and (ii) susceptibility of mitochondrial ribosomes correlates with the presence of a resistant eukaryotic or susceptible bacterial sequence position. In support of these observations are archaebacterial ribosomes, which are naturally resistant to streptomycin and macrolides (Cammarano *et al.*, 1985) and show a genotype consistent with these conclusions: RpsL Gly-87; 23S rRNA 2058G.

Given that mitoribosomes carry a bacterial 1408A it is unlikely that further modifications of 2-deoxystreptamine class antibiotics, as defined by conserved ring I (N6 substituted) and ring II, will result in drugs with less toxicity. These findings explain in part the unsuccessful attempts in the past of developing more specific and less toxic aminoglycosides by trial and error approaches. Macrolides, lincosamides and streptogramin B antibiotics might offer more fruitful avenues for further modifications, as both the mitoribosomes and the cytoribosomes of higher eukaryotes carry a resistant 2058G. On the basis of these considerations it can be predicted that development of antibiotics resulting in derivatives capable of binding to a prokaryotic target structure despite a resistance mutation (e.g. 2058G, 1408G) will be compromised by considerable toxicity.

The *in vitro* and *in vivo* selection of drug resistant bacteria, mapping of resistance conferring mutations and comparison to eukaryotic (mitochondrial and cytoplasmic) ribosomal nucleic acid and protein sequences may offer an important strategy to predict the specificity and toxicity of future antibiotics targeting the bacterial ribosome. High resolution crystal structures of ribosomal subunits complexed with antibiotics (Carter *et al.*, 2000; Nissen *et al.*, 2000) provide a fundamental framework to understand the mode of action of resistance mutations, e.g. resistance mutations leading to decreased drug binding, destabilization of the ram state by streptomycin resistance mutations exhibiting a hyperaccurate phenotype. Now that the structure of the ribosome is emerging it should be possible to develop new ribosomal antibiotics endowed with less toxicity and greater specificity for bacterial ribosomes by rationale drug design, i.e. by developing drugs whose binding is determined by sequence positions that are different in bacteria compared to both eukaryotic cytoribosomal and mitoribosomal components.

METHODS

Bacterial strains and mutagenesis. Single rRNA allelic strains of *M. smegmatis* were used to facilitate introduction of mutations: mc^{2}155 rrnB$^-$ (Sander *et al.*, 1996), mc^{2}155 rrnB$^-$*rpsL*$^{3+}$ (carrying three chromosomal *rpsL* genes, the construction of this strain is to be described elsewhere). rRNA mutations were introduced into plasmid pMV361-H-rRNA (Sander *et al.*, 1996) by PCR-mediated site-directed mutagenesis; RecA-mediated gene conversion was used to transfer the mutation into the chromosomal rRNA gene (Prammananan *et al.*, 1999). All mutant rRNAs were expressed from the chromosomal *rrnA* promoter; analysis at the DNA and RNA level was performed as described (Sander *et al.*, 1997) to confirm that the cells were homozygous for the mutated rRNA.

The wildtype *rpsL* of *M. smegmatis* was cloned as a 0.9 kb fragment under control of its own promoter into integrative vector pMV361; the respective mutations were introduced by PCR-mediated mutagenesis. RecA-mediated gene conversion by selective plating (20 mg/L streptomycin) was used to transfer the respective resistance mutation into the chromosomal *rpsL* of parental *M. smegmatis* mc^{2}155 (streptomycin-susceptible). *Mycobacterium smegmatis* SMR5 recA$^-$ (# 1473, streptomycin-resistant and carrying a chromosomal *rpsL* 42-Lys → Arg mutation) was used for construction of merodiploid strains. For construction of merodiploid strains a derivative of pMV361 was used which, after integration into the bacterial chromosome, is stably maintained even in the presence of negative selection pressure (B. Springer, manuscript submitted).

Determination of minimum inhibitory concentrations (MIC). Single colony cultures, grown in BHI media, were used for MIC tests in a microtiter plate format [starting culture (200 µl) of 0.025 OD_{600} bacterial cells]. The following drugs were added in 2-fold series of dilutions: amikacin, gentamicin, tobramycin, streptomycin, clarithromycin.

The MIC is the drug concentration at which the growth of the cultures was completely inhibited after 72 h incubation at 37°C (*M. smegmatis* has a generation time of 3 h, the incubation period used corresponds to 24 generations).

Comparative sequence analysis. Bacterial, chloroplast, mitochondrial and eukaryotic rRNA sequences were extracted from the DDBJ/EMBL/GenBank database. Secondary structure models (http://www.rna.icmb.utexas.edu; Gutell, 1996) were used for alignment of the relevant sequence positions.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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