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A *Streptomyces coelicolor* functional orthologue of *Escherichia coli* RNase E shows shuffling of catalytic and PNPase-binding domains

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Summary

Previous work has detected an RNase E-like endoribonucleolytic activity in cell extracts obtained from *Streptomyces*. Here, we identify a *Streptomyces coelicolor* gene, *rns*, encoding a 140 kDa protein (RNase ES) that shows endoribonucleolytic cleavage specificity characteristic of RNase E, confers viability on and allows propagation of *Escherichia coli* cells lacking RNase E and accomplishes RNase E-like regulation of plasmid copy number in *E. coli*. However, notwithstanding its complementation of *rne*-deleted *E. coli*, RNase ES did not accurately process 9S rRNA from *E. coli*. Additionally, whereas RNase E is normally required for *E. coli* survival, *rns* is not an essential gene in *S. coelicolor*. Deletion analysis mapped the catalytic domain of RNase ES near its centre and showed that regions located near the RNase ES termini interact with an *S. coelicolor* homologue of polynucleotide phosphorylase (PNPase) – a major component of *E. coli* RNase E-based degradosomes. The interacting arginine- and proline-rich segments resemble the C-terminally located degradosome scaffold region of *E. coli* RNase E. Our results indicate that RNase ES is a structurally shuffled RNase E homologue showing evolutionary conservation of functional RNase E-like enzymatic activity, and suggest the existence of degradosome-like complexes in Gram-positive bacteria.

Introduction

Ribonuclease E (RNase E), an *Escherichia coli* endoribonuclease initially discovered as a ribosomal RNA-processing enzyme (Ghora and Apirion, 1978), has since been shown to process a variety of other catalytic and structural RNAs (Gurevitz *et al*., 1983; Li *et al*., 1999; Lin-Chao *et al*., 1999; Wachi *et al*., 1999; Li and Deutscher, 2002; Ow and Kushner, 2002), degrade multiple mRNAs (for reviews, see Coburn and Mackie, 1999; Steege, 2000), remove poly(A) tails from the 3′ ends of transcripts (Huang *et al*., 1998; Walsh *et al*., 2001),and regulate the synthesis of certain plasmid DNAs (Lin-Chao and Cohen, 1991). The 1061-amino-acid RNase E protein (i.e. Rne) has a molecular weight of 118 kDa but migrates as a 180 kDa band in SDS-polyacrylamide gels (Casáregola *et al*., 1992). The catalytic function of RNase E resides in the N-terminal half of the protein (amino acid residues 1–498), which also encodes cleavage site specificity (McDowall and Cohen, 1996). An arginine-rich RNA-binding domain located between amino acids 580 and 700 can enhance cleavage of at least certain substrates (Lopez *et al*., 1999; Ow *et al*., 2000). The proline-rich/acidic C-terminal third of the RNase E protein serves as a scaffold for the formation of a multicomponent 'degradosome' complex that includes polynucleotide phosphorylase (PNPase), the RhlB RNA helicase, the ATP-generating enzyme enolase, the chaperone proteins DnaK and GroEL, polyphosphate kinase and poly(A) polymerase (Carpousis *et al*., 1994; Py *et al*., 1994; 1996; Miczak *et al*., 1996; Vanzo *et al*., 1998; Raynal and Carpousis, 1999). *In vivo*, degradosomes show proximity to the *E. coli* cytoplasmic membrane that is dependent on the N-terminal region of RNase E (Liou *et al*., 2001). An RNA degradosome-like complex has recently been identified in another bacterium, *Rhodobacter capsulatus*, although PNPase, which is a major component of the *E. coli* degradosome, was not found in this complex (Jäger *et al*., 2001).

The CafA protein, which was originally identified by its role in chromosome segregation and cell division (Okada *et al*., 1994), was subsequently found to show extensive amino acid sequence similarity to the N-terminal catalytic region of RNase E (McDowall *et al*., 1993). The discovery that CafA also has endoribonuclease activity, which is broadly similar to that of RNase E, has led to the renaming of the protein as RNase G (Li *et al*., 1999; Wachi *et al*.,

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1999; Jiang *et al*., 2000; Tock *et al*., 2000; Umitsuki *et al*., 2001; Lee *et al*., 2002). Whereas *E. coli* cells lacking RNase E are normally non-viable (Apirion and Lassar, 1978; Ono and Kuwano, 1979), overproduction of RNase G in bacteria deleted for the RNase E-encoding *rne* gene enables their survival and growth (Lee *et al*., 2002). Genes that encode putative proteins with sequence homology with regions of *E. coli* RNases E and/or G are found throughout prokaryote phylogeny (Aravind and Koonin, 2001; Condon *et al*., 2001).

Streptomyces species are morphologically, developmentally and biochemically complex soil bacteria that synthesize a variety of biologically important secondary metabolites, including a large portion of antibiotics in medical and veterinary use (Chater, 1993; Hopwood *et al*., 1995). The production of secondary metabolites generally coincides with the onset of morphological differentiation in streptomycetes, and is accomplished by a complex cascade of regulatory pathways (e.g. Chater, 1993; Huang *et al*., 2001). Earlier work aimed at understanding the role of RNA decay in the control of gene expression in *Streptomyces* species has shown that two widely studied streptomycetes, *Streptomyces lividans* and *Streptomyces coelicolor*, encode proteins that carry out RNase E-like cleavages and react with antibody generated against *E. coli* RNase E (Hagège and Cohen, 1997). More recently, the complete genomic DNA sequence for *S. coelicolor* has become available (Bentley *et al*., 2002), enabling us to identify in this species a putative open reading frame (ORF) that contains a segment showing substantial sequence similarity to the catalytic N-terminal region of *E. coli* RNase E. Here, we report that this *S. coelicolor* gene, named *rns*, encodes an endoribonuclease (RNase ES) that can functionally substitute for Rne in *E. coli*, attack oligonucleotides and other substrates at or near known RNase E cleavage sites and, like *E. coli* RNase E, interact with polynucleotide phosphorylase (PNPase) encoded by its host species to form an RNA degradosome-like complex. Notwithstanding these similarities, we find that the positions of the catalytic and PNPase-binding domains of RNases E and ES have been shuffled during evolution, so that the catalytic region of RNase ES is centrally located and PNPase-binding 'scaffold' regions are present at both ends of the protein.

Results

rns *functionally complements an* rne *null mutation*

We observed that a putative ORF in *S. coelicolor* genomic DNA includes a segment of high similarity to the catalytic domain (N-terminal 498 amino acids) of *E. coli* RNase E (Rne) and to the related *E. coli* endoribonuclease, RNase G (Rng). This ORF was amplified by polymerase chain reaction (PCR) and introduced into the multicopy pSC101-derived *E. coli* plasmid pPM30 (Meacock and Cohen, 1980) behind the IPTG-inducible *lacUV5* promoter (Silverstone *et al*., 1970; Maizels, 1973). Sequence analysis of the cloned PCR product confirmed the presence of an ORF encoding a predicted 1340-amino-acid protein (which was designated RNase ES; Rns) with a calculated molecular weight of 140 kDa. Sequence comparisons showed that the central portion of RNase ES (amino acids 563–973) has 36% identity and 58% similarity to the amino-terminal catalytic region of *E. coli* RNase E. This region of RNase ES also showed 36% identity and 56% similarity to *E. coli* RNase G. Although the N-terminal 562 amino acids of RNase ES did not show direct sequence homology with RNase E, this segment was found to contain discrete putative acidic, arginine-rich and proline-rich regions (Fig. 1), all of which are similar to motifs located in the C-terminal half of the RNase E protein (Fig. 1). Such motifs were also present in the C-terminal third of RNase ES along with a lysine-rich segment. The gene encoding the putative RNase ES ORF was designated *rns*.

PCR-amplified segments of *rns* (see Fig. 1) were expressed in *E. coli* from the *lacUV5* promoter, as described in *Experimental procedures*, and the full-length protein and specific segments of RNase ES were tested for their ability to substitute functionally for *E. coli* RNase E in an *E. coli* strain deleted for the *rne* gene. In these

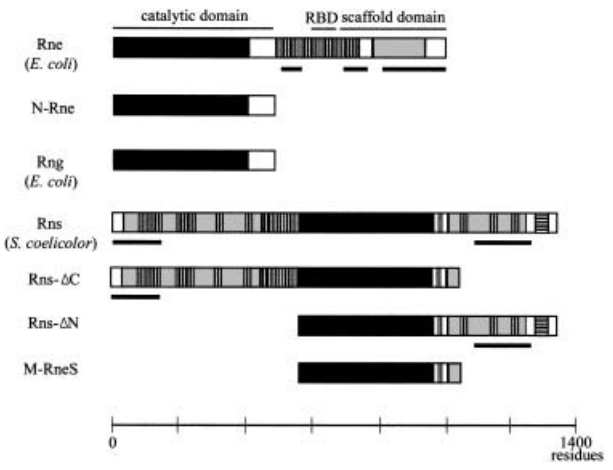

Fig. 1. Derivations of the Rne and the Rns proteins used in this study. All proteins used in this study were tagged with six histidines at the C-terminus. The catalytic domain (residues 1–498), the RNA-binding domain (RBD, residues 597–684) and the scaffold domain (residues 734–1045) in Rne are indicated (McDowall and Cohen, 1996; Vanzo *et al*., 1998). Regions containing highly conserved sequences in Rne, Rng and Rns are shown in black. Arginine-rich, lysine-rich and acidic regions are shown in vertical hatched, horizontal hatched and grey boxes, respectively, and proline-rich regions are underlined. Arginine-, lysine- or proline-rich regions contain at least 10% of each corresponding amino acid in each region, and acidic regions contain at least 20% acidic amino acids in each region.

experiments, which used a plasmid displacement procedure and recombination-deficient bacteria (Lee *et al*., 2002), pSC101-derived plasmids containing either the full-length Rns ORF or the truncated Rns ORFs shown in Fig. 1 were introduced by transformation into a chromosomally *rne*-deleted *E. coli* strain harbouring plasmid pBAD-RNE (KSL2000), which directs the synthesis of the full-length RNase E under the control of an arabinose-inducible BAD promoter. Earlier work has shown that this strain is viable when 0.1% arabinose, which induces synthesis of the plasmid-encoded RNase E at wild-type levels, is added to the medium (Lee *et al*., 2002). Cultures expressing RNase E, its N-terminal catalytic domain (N-Rne) or Rng, all of which have been shown previously to complement an *rne* deletion mutation and consequently to allow displacement of KSL2000 by a related plasmid (Lee *et al*., 2002), were included as positive controls.

Transformants that acquired the incoming plasmid were selected by culturing them in the presence of ampicillin (50 μg ml^{-1}) and adding IPTG (0, 10, 100 or 1000 μM) to induce expression of Rns, Rng or Rne proteins cloned behind the *lacUV5* promoter. After 40 generations of culture in the presence of 10 μM IPTG, most of the cells showed displacement of the resident RNase E-expressing plasmid (pBAD-RNE; KSL2000) by incoming plasmids expressing *rns* (pRNES101), *rns-ΔC* (pRNES102), *rns-ΔN* (pRNES103) or *rne* (pLAC-RNE2) (Fig. 1). The presence of IPTG inducer at a concentration of 100 μM or more was needed for displacement of the resident plasmid by incoming plasmids expressing *M-rns* (pRNES104) or, as found previously (Lee *et al*., 2002) and confirmed here, expressing *N-rne* (pNRNE5) or *rng* (pRNG3). Cells cultured in the absence of IPTG retained both resident and incoming plasmids. Bacteria expressing Rne, Rns or Rns-ΔC did not grow in the presence of 100 or 1000 μM IPTG, suggesting that the level of overexpression of these proteins induced by these high concentrations of IPTG may be toxic to *E. coli* cells.

The above results suggest that *rns* and specifically its central region, which encodes a protein region resembling the N-terminal catalytic domain of Rne, can functionally substitute for *E. coli* RNase E. Southern blotting of total DNA confirmed the absence of sequences encoding the RNase E catalytic domain in cells undergoing replacement of pBAD-RNE by M-RNase ES-expressing plasmid (Fig. 2A) and also in cells complemented by Rng. A monoclonal antibody generated against RNase E (K. J. McDowall *et al*., unpublished; Hagège and Cohen, 1997) detected no RNase E band in these cells, and also did not react with His-tagged RNase ES produced in *E. coli* (Fig. 2B, right), indicating that the epitope recognized by the antibody is not present in RNase ES and suggesting that the band found previously in RNase ES-enriched *Streptomcyes* cell fractions to react with this antibody (Hagège and Cohen, 1997) represents a cross-reacting protein. As has been observed for RNase E, the mobility of which during SDS-PAGE is dramatically affected by its proline-rich regions (McDowall and Cohen, 1996), RNase ES migrated more slowly in gels than was predicted from its calculated protein mass. *rne*-deleted *E. coli* cells complemented by *rns* or by truncated *rns* genes encoding the central region of RNase ES grew at approximately the same rate as cells complemented by the catalytic domain of *E. coli* RNase E (i.e. *N-rne*) (Fig. 2C).

Cleavage of E. coli *RNase E substrates by RNase ES*

Escherichia coli RNase E can control the copy number of ColE1-type plasmids by cleaving RNA I, an antisense regulator of plasmid DNA replication, and this property has been used to assess the biological function of RNase E *in vivo*. Induced expression of Rns or Rns-ΔC at a level that gave optimal growth of the complemented *rne*-deleted strain increased the copy number of the ColE1-type plasmid pET28a by 1.5- to twofold relative to the copy number observed in the parental strain N3433 (Fig. 3A); a similar plasmid copy number was observed in *rne*-deleted cells complemented by adventitious expression of RNase E to the level characteristic of wild-type cells. Induced expression of Rns-ΔN or M-Rns (10 μM IPTG for Rns-ΔN and 100 μM IPTG for M-Rns) at a level that optimally supported growth of the *rne*-deleted *E. coli* strain resulted in a lower pET28a copy number, implying that the N-terminal domain of RNase ES enhances its ability to cleave RNA I. An analogous effect of the C-terminal region of RNase E on that enzyme's ability to control plasmid copy number was observed (Fig. 3A), consistent with evidence that this region enhances RNase E cleavage of at least some substrates (Lopez *et al*., 1999; Ow *et al*., 2000).

His-tagged RNase ES and its truncated derivatives were affinity purified (Fig. 3B) and tested for their ability to cleave known RNase E substrates *in vitro* (Fig. 3C): chemically synthesized BR13 (McDowall *et al*., 1995) and enzymatically synthesized GGG-RNAI (Helmer-Citterich *et al*., 1988) and pM1 RNA (Lee *et al*., 2002). As shown in Fig. 3D, RNase ES or M-RNase ES cleavage of GGG-RNA I generated the same size products as cleavages by *E. coli* RNase E, its catalytic domain (N-RNase E) or RNase G (Fig. 3D). Mapping of the specific cleavage sites using 5′ ^{32}P-labelled BR13 (Fig. 3D), a synthetic oligoribonucleotide that contains the RNase E target sequence of RNA I (McDowall *et al*., 1995), showed that RNase ES and RNase E cleavages occurred at the same sites, although the relative amounts of cleavage products differed slightly for the two enzymes. All ribonucleases tested cleaved BR13 more efficiently than GGG-RNA I, as observed previously for RNase E itself (McDowall *et al*.,

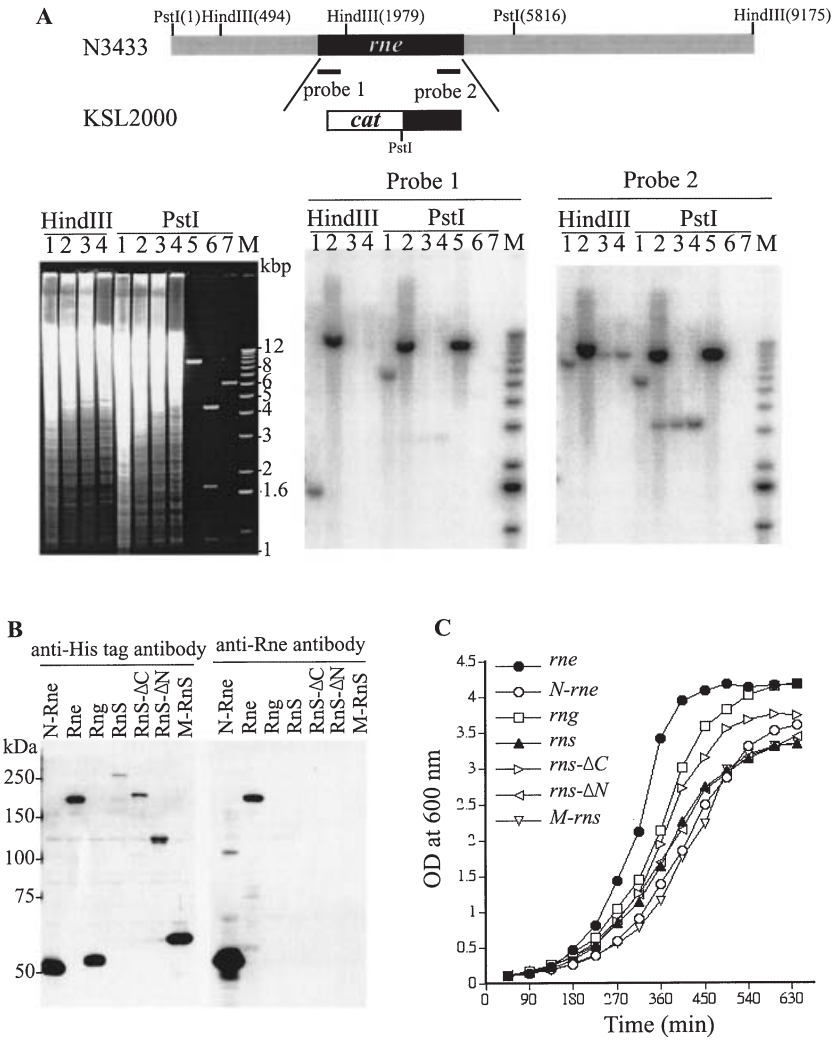

Fig. 2. Identification of the absence of the *rne* gene and its protein product in *rne*-deleted strains complemented by Rns overexpression.
A. Southern blot analysis of strains obtained by plasmid displacement experiments. Total DNA from N3433 (1), KSL2000 (2), KSL2000, of which resident plasmid (pBAD-RNE) was displaced with pRNG3 (3), or pRNES104 (4) was digested with *Hin*dIII or *Pst*I. Plasmid DNA, pBAD-RNE (5), pRNG3 (6) or pRNES104 (7) was digested with *Pst*I. DNAs were separated in a 0.8% agarose gel and stained with ethidium bromide (left). Separated DNA fragments were transferred to a nylon membrane and analysed with probe 1 (middle), stripped and reprobed with probe 2 (right). Probes 1 and 2 are complementary to *rne* coding sequences 1–1042 and 2883–3554 respectively. A 1 kbp ladder DNA (M) was obtained from Invitrogen and 5′ end-labelled with ^{32}P after being dephosphorylated by treatment with calf intestine alkaline phosphatase (CIAP).
B. Western blot analysis of strains obtained by plasmid displacement. Cultures were grown in the presence of 100 μM IPTG except that 10 μM was used for the expression of Rne, Rns, Rns-ΔC and Rns-ΔN, and harvested in mid-log phase (OD_{600} = 0.6) to obtain total protein. Loss of the resident plasmid (pBAD-RNE) in KSL2000 by introducing incoming plasmids was confirmed by Western blot analysis. The same membrane probed with anti-His-tag polyclonal antibody was stripped and reprobed with anti-RNase E monoclonal antibody.
C. Growth of *rne*-deleted cells complemented by expression of Rns and its truncated forms. Cultures were grown as described in (B), and culture samples were withdrawn at time intervals to analyse cell density and growth rate (absorbance at 600 nm).

1995). RNase ES and M-RNase ES also cleaved *E. coli* pM1 RNA, the RNA component of RNase P (Gurevitz *et al.*, 1983) and a normal substrate for RNase E (Fig. 3D). As reported previously (Lee *et al.*, 2002), this substrate was insensitive to RNase G.

Processing of 9S rRNA by Rns

RNase E was discovered initially by its ability to process 9S rRNA in *E. coli* cells, and the shift of *rne ts* bacteria to a non-permissive temperature leads to the *in vivo* accumulation of precursors of 5S rRNA (Ghora and Apirion, 1978). We found that complementation of the *rne* deletion mutant by production of sufficient Rns or M-Rns to restore cell viability did not detectably assist in the processing of 5S rRNA precursors (Fig. 4B). The 5S rRNA precursor bands accumulating in *rne*-deleted cells complemented by Rns, M-Rns or, as shown previously (Lee *et al.*, 2002), also Rng (Fig. 4B, lanes 7–9) were identical in size to, and greater in quantity than, the species accumulating in *rne*-deleted cells in which synthesis of RNase E from the pBAD-RNE plasmid (i.e. the KSL2000 strain) was turned off by shift to media lacking arabinose (Fig. 4B, lane 3). In contrast, 9S rRNA was processed normally in the chromosomal *rne* deletion mutant complemented by N-Rne (Fig. 4B, lane 6).

Together, these results argue strongly that processing of 9S rRNA is not the essential function provided by Rns complementation of *rne*-deleted *E. coli* cells. Supporting this conclusion, we found that RNase ES did not process *E. coli* 9S rRNA correctly *in vitro* (Fig. 4C). Although RNase ES cleaved *E. coli* 9S rRNA *in vitro*, the cleavage products were not identical to those generated by RNase E (Fig. 4C), and the a-b product generated by RNase ES was slightly larger than p5S (Fig. 4C; cf. Christiansen, 1988; Cormack and Mackie, 1992). Moreover, no a-b

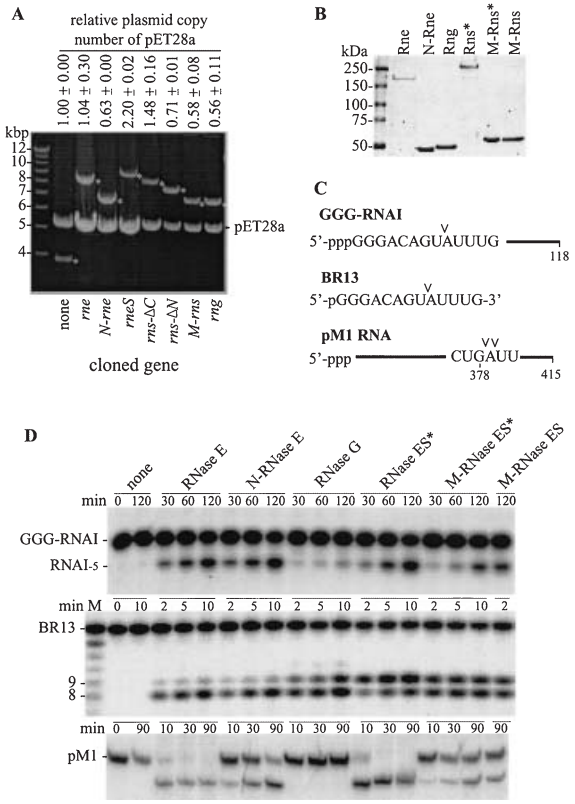

Fig. 3. Ribonuclease activity of Rns *in vivo* and *in vitro*.
A. Effects of Rns on copy number of ColE1-type plasmids. Non-ColE1-type pSC101 plasmids containing genes are indicated with *. Strains obtained from a plasmid displacement experiment were transformed with the ColE1-type plasmid pET28a, and plasmid DNA was isolated from bacteria maintained in the presence of 10 μM (Rne, Rns, Rns-ΔC and Rns-ΔN) or 100 μM IPTG (N-Rne, M-Rns and Rng). Plasmids digested with *Xba*I restriction enzyme, which has a unique cleavage site in all plasmids tested here (except pPM30, which was digested with *Eco*RI), were electrophoresed in 0.9% agarose gels and stained with ethidium bromide. Plasmid copy number was calculated relative to a concurrently present pSC101 derivative, the replication of which is independent of Rne (Lin-Chao and Cohen, 1991), by setting the molar ratio of the pSC101 derivative plasmid (pPM30) to ColE1 plasmid (pET28a) as 1. Densitometric measurements of bands corresponding to each plasmid were converted to actual ratios after normalizing the values according to the size of pSC101 derivative plasmids and are shown at the top of the gel.
B. Analysis of His-tagged affinity-purified proteins. Purified proteins were analysed on 7.5% SDS-polyacrylamide gel. * indicates proteins purified from *S. coelicolor* strains expressing Rns or M-Rns as described in *Experimental procedures*. Each His-tag affinity-purified protein (1 μg) was loaded in each lane and stained with Coomassie blue R-250. Faster migrating protein bands present in RNase E-containing and RNase ES-containing lanes were found by Western blot analysis (data not shown here) to be mostly degradation products of RNase E and RNase ES.
C. RNase E substrates analysed and their previously identified RNase E cleavage sites (Tomcsanyi and Apirion, 1985; Cormack and Mackie, 1992; Lin-Chao *et al*., 1994; McDowall *et al*., 1995).
D. Cleavage of RNase E substrates. Internally labelled RNA I or pM1 RNA (1 pmol) was incubated with no protein, 100 ng (RNase E and RNase ES) or 200 ng (RNase G, N-RNase E and M-RNase ES) of purified protein in 40 μl of 1× cleavage buffer at 37°C. Each sample (10 μl) was removed at each time point indicated and mixed with an equal volume of formamide-containing loading buffer. For 5′ end-labelled BR13 (1 pmol), half the amount of protein was used. Samples were denatured at 70°C for 10 min and loaded onto 6% (for pM1 RNA), 8% (for RNAI) or 15% (for BR13) polyacrylamide gels containing 8 M urea. The markers (M) were a 1 nt ladder generated by limited PNPase (Sigma) digestion of the substrate.

product at all was detected after digestion of *E. coli* 9S by M-RNase ES (Fig. 4C), notwithstanding its ability to substitute for RNase E functionally in *E. coli* cells, providing further evidence that 9S rRNA processing is not the basis for complementation of the *rne* deletion by *rns* derivatives.

Non-essentiality of rns *in* S. coelicolor

Whereas RNase E is normally an essential protein in *E. coli*, we found that deletion of the *rns* gene in *S. coelicolor* does not result in loss of cell viability. We constructed an *rns* deletion mutation by a gene replacement procedure that used an *S. coelicolor* strain harbouring plasmid pRNES401, which contains a temperature-sensitive replicon and a copy of *rns* with an inserted apramycin resistance marker (Fig. 5A). Bacteria grown initially at permissive temperature (30°C) were shifted to non-permissive temperature (40°C) in the presence of apramycin (50 μg ml^{-1}) to select for plasmid insertion into the chromosome, and survivors were then tested for loss of thiostrepton resistance as an indicator of double cross-over and gene replacement. PCR-amplified chromosomal DNA encompassing the *rns* region from two putative deletion strains was analysed by agarose gel electrophoresis (Fig. 5B) and sequencing (data not shown) to verify the absence of an intact *rns* gene in these actively growing bacterial cells. The *rns* deletion mutant strain A3(2)*rns* showed a 10% increase in doubling time versus the parental strain (92.9 ± 0.4 versus 99.9 ± 1.0 min) in YEME medium.

Rns protein interacts physically with S. coelicolor *homologue of PNPase*

Escherichia coli RNase E interacts with PNPase, RhlB RNA helicase, enolase, DnaK, GroEL and polyphosphate kinase and forms a scaffold for a multicomponent complex termed the 'degradosome'. A homologue of PNPase identified in *Streptomyces antibioticus*, GPSI (guanosine pentaphosphate synthetase I), has recently been purified and shown to have both polymerase and phosphorolysis activity (Jones and Bibb, 1996). To learn whether RNase ES interacts with the *S. coelicolor* homologue of GPSI, which shares 95% homology in amino acid sequence with the *S. antibioticus* enzyme, C-terminally hexahistidine-tagged RNase ES and its truncated forms were overexpressed in *S. coelicolor* and immunoprecipitated with antibody to

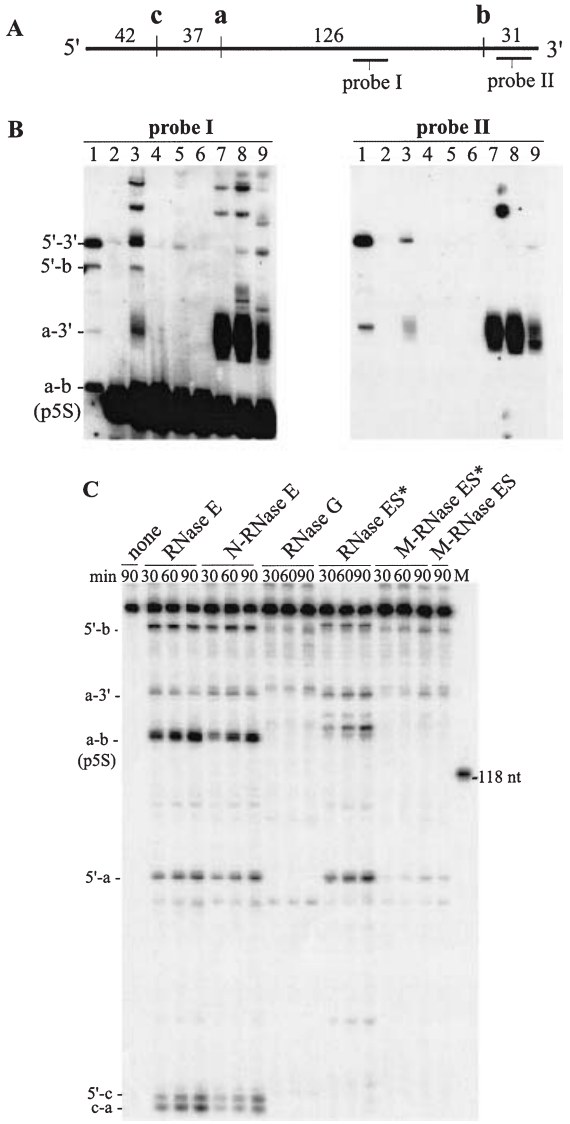

Fig. 4. Processing of *E. coli* 9S rRNA.
A. *In vitro*-synthesized transcript corresponding to *E. coli* 9S rRNA. RNase E cleavage sites are indicated as a, b and c and binding sites for probes used in (B) are shown.
B. Northern blot analysis of 5S rRNA precursors, which were identified by probing with 5S rRNA (probe I) and then retesting with 9S rRNA-specific probes that bind 3′ to residue 15 (probe II) of 9S rRNA. Lanes 2–9 contain 30 μg of total RNA prepared from N3433 plus pPM30 (2), KSL2000 with no arabinose (3), KSL2000 with 0.1% arabinose (4) and KSL2000, of which resident plasmid (pBAD-RNE) was displaced with pLAC-RNE2 (5), pNRNE5 (6), pRNES101 (7), pRNES104 (8) or pRNG3 (9). To prepare total RNA from KSL2000 (no arabinose), culture was grown to middle log phase in the presence of 0.1% arabinose, harvested, washed with plain LB medium twice and reinoculated into LB medium containing no arabinose (OD_{600} = 0.1). It was incubated further for 150 min (OD_{600} = 0.5) at 37°C and 250 r.p.m. and harvested for total RNA preparation. All other cultures were grown and harvested as indicated in Fig. 2A and B. Lane 1 contains 10 ng of 9S rRNA transcript used in (B) that was cleaved with purified RNase E as a reference.
C. 9S rRNA cleavages *in vitro*. *In vitro*-synthesized 9S rRNA transcript (1 pmol) internally labelled with [^{32}P]-UTP was cleaved as described in Fig. 3D for RNA I and pM1 RNA cleavage and loaded onto a 9% polyacrylamide gel containing 8 M urea.

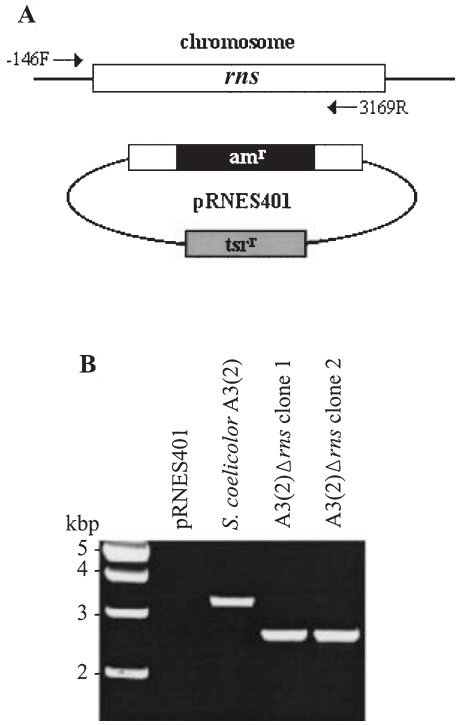

Fig. 5. Deletion of *rneS* in *S. coelicolor*.
A. Diagrammatic representation of the *S. coelicolor* chromosomal region containing *rns* and of plasmid pRNES401. The procedure for construction of the *rns* knock-out strain is indicated in *Results*, and the primers used to amplify the *rns* region from chromosome were –146F (5′ACAAGACACGGATGAGCTTC) and 3169R (5′CCTCG GCGGCGCTGCTGTA). The Rns coding region is shown by an empty bar, and antibiotic markers are indicated as *am*^{r} (apramycin resistance) and *tsr*^{r} (thiostrepton resistance).
B. PCR products were analysed by electrophoresis in 1% agarose gel and stained with ethidium bromide. The template DNA used is indicated at the top of each lane. The markers were bands of a 1 kbp DNA ladder purchased from Bio-Rad. Each PCR product was sequenced using PCR primers indicated above.

His-tag. As shown in Fig. 6A, an *S. coelicolor* protein detected by antibody to *S. antibioticus* PNPase co-immunoprecipitated with both full-length Rns and Rns-ΔC but not with M-Rns; co-immunoprecipitation with Rns-ΔN (which lacks amino acids 1–562) showed a barely detectable signal, which quantification showed was 3% of the intensity observed for Rns-ΔC (Fig. 6A, right). Far Western blotting (Fig. 6B) confirmed the ability of RNase ES and PNPase to interact and also showed that the interaction is not dependent on any RNA 'bridge' between the proteins. In these experiments, RNase ES and its truncated forms were overexpressed in *E. coli* BL21(DE3), and proteins present in cell extracts were separated from each other and from RNA by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, renatured and incubated in the presence of RNase A with purified *Streptomyces* PNPase. The membrane was then probed by

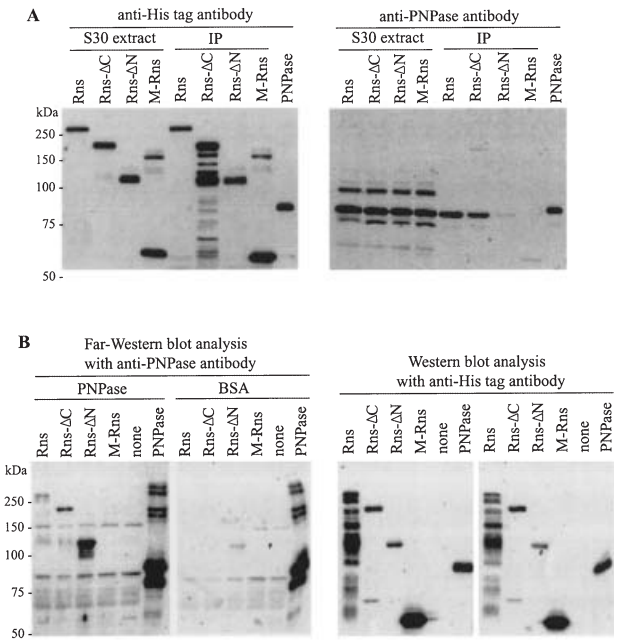

Fig. 6. Localization of *Streptomyces* PNPase homologue binding domains for RNase ES.
A. C-terminally hexahistidine-tagged RNase ES and its truncated forms were overexpressed in *S. coelicolor* and immunoprecipitated with monoclonal antibody to the hexahistidine tag. The membrane was probed with polyclonal antibody to His-tag, stripped and reprobed with polyclonal antibody to *S. antibioticus* PNPase. Faster migrating bands present in the lane containing immunoprecipitated N-Rns were observed reproducibly and were presumed to result from N-Rns protein degradation during immunoprecipitation. His-tagged affinity-purified *S. antibioticus* PNPase (20 ng) from *S. lividans* harbouring pGPSI was loaded in the last lane.
B. C-terminally hexahistidine-tagged RNase ES and its truncated forms were overexpressed in *E. coli* BL21 (DE3). Total protein extracts were separated in 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, renatured *in situ* and incubated with purified GPSI (*Streptomyces* PNPase homologue) or BSA. Blots were then probed with a polyclonal rabbit antibody raised against *Streptomyces* PNPase homologue (left). The same blots were stripped and reprobed with a monoclonal antibody to His-tag to show the overexpressed RNase ES protein and its truncated forms (right). His-tag affinity-purified *S. antibioticus* PNPase (20 ng) was loaded in the last lane.

antibody to PNPase. Signals of various strength were detected for Rns, Rns-ΔN and Rns-ΔC, but not for M-Rns (Fig. 6B), confirming the ability of full-length RNase ES and derivatives containing either terminus to bind to PNPase. Quantitative differences in relative binding abilities of Rns derivatives examined in immunoprecipitation and Far Western blotting experiments may reflect the different experimental conditions inherent in these two types of assays.

Discussion

We have shown here that *S. coelicolor* encodes a high-molecular-weight ribonuclease that cleaves known RNase E substrates (e.g. pM1, RNAI, BR13; Fig. 3C and D) at the same sites as RNase E, confers viability on an *rne*-deleted *E. coli*, regulates the copy number of ColE1-type plasmids in *E. coli* and contains proline-rich/arginine-rich acidic 'scaffold' regions that affect endonucleolytic activity and bind to an *S. coelicolor* homologue of PNPase in a degradosome-like structure. These properties indicate that RNase ES is functionally related to RNase E, rather than to CalfA/RNase G, which lacks a degradosome 'scaffold' (Fig. 1), does not cleave pM1 RNA normally (Fig. 3D) and fails to regulate plasmid copy number (Fig. 3A). However, the catalytic and scaffold regions of RNases E and ES have been structurally shuffled during bacterial evolution, so that the catalytic domain of RNase ES resides near the centre of the molecule, and the PNPase-binding domains are at the N- and C-terminal ends (cf. the N-terminal catalytic region and C-terminal scaffold region of RNase E). The ability of RNase ES to substitute functionally for RNase E despite its inability to process *E. coli* 9S rRNA accurately provides further evidence for the recent conclusion (Lee *et al.*, 2002; Ow and Kushner, 2002) that 9S rRNA processing is not the basis for RNase E essentiality to *E. coli* survival and growth. Moreover, as deletion of the *rns* gene does not affect the viability of *S. coelicolor*, its activity also cannot be required to generate 5S rRNA or other essential product(s) in its native host. As we can identify only one site in the *S. coelicolor* chromosome that encodes an RNase E/G-like protein, we conclude that *S. coelicolor* lacks a separate RNase G gene. Certain other bacterial genomes also appear to lack sequences encoding either RNase E-like or RNase G-like proteins (Table 1), further demonstrating that this class of enzymes is not uniformly required for bacterial survival and growth.

Putative proteins containing evolutionarily conserved sequences resembling those present in the catalytic domains of *E. coli* endoribonucleases RNase E and/or RNase G are encoded by the chromosomes of many bacterial species and a few eukaryotes (Table 1). Our discovery of a functionally similar, structurally shuffled orthologue of *E. coli* RNase E in *S. coelicolor* prompted us to examine the structural arrangement of protein domains in other RNase E/G-like proteins that we detected by analysis of the genomic DNA sequence in other organisms. This analysis suggested that RNase E/G-like proteins can be classified into four groups based on the position of their putative catalytic region and the presence or absence of an ancillary region containing acidic/arginine-rich/proline-rich 'scaffold' sequences (Fig. 7A). In type I enzymes, the catalytic domain is located near the N-terminal end, and the 'scaffold' is in the C-terminal half, as in *E. coli* RNase E. In type II enzymes, the C-terminal 'scaffold' is generally shorter, and arginine-rich/acidic/proline-rich segments of varying

Table 1. RNase E/G homologues.

Organism	Size (amino acids)	Taxon
Type I		
>*Escherichia coli*	1061	Proteobacteria γ
>*Salmonella typhimurium*	1067	Proteobacteria γ
>*Salmonella enterica* ssp.	1132	Proteobacteria γ
>*Yersinia pestis*	1221	Proteobacteria γ
>*Haemophilus influenzae*	951	Proteobacteria γ
>*Pasteurella multocida*	1004	Proteobacteria γ
>*Vibrio cholerae*	1052	Proteobacteria γ
>*Pseudomonas aeruginosa*	1057	Proteobacteria γ
>*Xylella fastidiosa*	1132	Proteobacteria γ
>*Ralstonia solanacearum*	1014	Proteobacteria β
>*Neisseria meningitidis*	919	Proteobacteria β
>*Xanthomonas campestris* pv.	1187	Proteobacteria γ
>*Xanthomonas axonopodis* pv.	1221	Proteobacteria γ
Buchnera aphidicola	902	Proteobacteria γ
Buchnera sp. APS	902	Proteobacteria γ
Mycobacterium tuberculosis (CDC1551)	621	Actinobacteria
Nostoc sp.	687	Cyanobacteria
Synechocystis sp.	674	Cyanobacteria
Type II		
Caulobacter crescentus	898	Proteobacteria α
Brucella melitensis	891	Proteobacteria α
Mesorhizobium loti	984	Proteobacteria α
Agrobacterium tumefaciens	973	Proteobacteria α
Sinorhizobium meliloti	924	Proteobacteria α
Rickettsia prowazekii	683	Proteobacteria α
Rickettsia conorii	690	Proteobacteria α
Chlorobium tepidum TLS	560	Chlorobi
Arabidopsis thaliana	575	Eukaryota
Type III		
Streptomyces coelicolor	1340	Actinobacteria
Corynebacterium glutamicum	1021	Actinobacteria
Mycobacterium tuberculosis (H37Rv)	953	Actinobacteria
Mycobacterium leprae	924	Actinobacteria
Type IV		
>*Escherichia coli*	489	Proteobacteria γ
>*Salmonella typhimurium*	489	Proteobacteria γ
>*Salmonella enterica* ssp.	489	Proteobacteria γ
>*Yersinia pestis*	489	Proteobacteria γ
>*Haemophilus influenzae*	491	Proteobacteria γ
>*Pasteurella multocida*	491	Proteobacteria γ
>*Vibrio cholerae*	489	Proteobacteria γ
>*Pseudomonas aeruginosa*	485	Proteobacteria γ
>*Xylella fastidiosa*	497	Proteobacteria γ
>*Ralstonia solanacearum*	489	Proteobacteria β
>*Neisseria meningitides*	497	Proteobacteria β
>*Xanthomonas campestris* pv.	499	Proteobacteria γ
>*Xanthomonas axonopodis* pv.	500	Proteobacteria γ
Chlamydophila pneumoniae	515	Chlamydiales
Chlamydia trachomatis	512	Chlamydiales
Chlamydia muridarum	512	Chlamydiales
Bacillus halodurans	500	Firmicutes
Bacillus anthracis str. A2012	377	Firmicutes
Clostridium perfringens	480	Firmicutes
Clostridium acetobutylicum	481	Firmicutes
Listeria monocytogenes	453	Firmicutes
Listeria innocua	453	Firmicutes
Thermoanaerobacter tengcongensis	472	Firmicutes
Thermotoga maritime	454	Thermotogae
Fusobacterium nucleatum ssp.	458	Fusobacteria
Aquifex aeolicus	458	Aquificae
Methanopyrus kandleri AV19	484	Archae
Pyrococcus horikoshii	472	Archae
Pyrococcus abyssi	481	Archae
Pyrococcus furiosus DSM 3638	469	Archae
Halobacterium sp. NRC-1	471	Archae
Sulfolobus tokodaii	424	Archae
Aeropyrum pernix	467	Archae
Pyrobaculum aerophilum	443	Archae
Guillardia theta, chloroplast	429	Eukaryota
Nephroselmis olivacea, chloroplast	375	Eukaryota
Porphyra purpurea	511	Eukaryota

Sequences of proteins containing homologous regions to the first 420 amino acids of *E. coli* RNase E or *E. coli* RNase G were obtained from the NCBI database by BLAST search and aligned using the CLUSTAL W program. Aligned sequences were classified by relative arrangements of putative scaffold versus putative catalytic domains as shown in Fig. 7A. Organisms encoding both RNase E-like and RNase G-like proteins are indicated with an arrowhead (>).

length interrupt the S1 domain (Bycroft *et al*., 1997) of the catalytic region. Most of the RNase E/G-like gene sequences identified in Actinobacteria, including *rns* of *S. coelicolor*, encode a centrally located catalytic region (type III). Type IV RNase E/G homologues resemble *E. coli* RNase G, lack an arginine-rich/acidic/proline-rich scaffold region and have sizes ranging from 375 to 515 amino acids, in contrast to putative RNase E-like proteins (types I–III), which range from 575 to 1340 amino acids in length. Notwithstanding the different positions of the non-catalytic regions found in various RNase E-like proteins, the occurrence of motifs in these regions has been remarkably conserved. Archaea, certain bacteria and a few eukaryotes encode RNase G-like proteins, and some groups of bacteria, in particular Proteobacterial species, encode both an RNase G-like protein and a putative type I RNase E-like enzyme (Table 1). In contrast, we did not observe genes specifying RNase G-like proteins in sequenced genomes of bacterial species encoding types II and III RNase E-like enzymes. Collectively, these data argue that the functionally related genes *rne* and *rns* are orthologues and also suggest that *E. coli* RNases E and G are paralogous.

Classification of RNase E/G-like proteins by the relative positions of possible catalytic versus possible scaffold regions of these proteins (Table 1) provides a phylogenic tree that is in good agreement with phylogenic classification made on the basis of small-subunit ribosomal RNA (Fig. 7B and C). This finding suggests that RNase E/G homologues are ancient proteins that have evolved closely with ribosomal RNA in bacterial organisms. Given our observation that RNase ES does not accurately process 9S rRNAs from *E. coli* despite its ability to confer viability on *E. coli* cells deleted for *rne*, it is perhaps not surprising that RNase E/G ribonucleases and the rRNA segments that they attack appear to have evolved congruently.

Interestingly, insertions into the S1 domain of type II RNase E-like proteins occur immediately adjacent to the α-helix, which separates β-strands 4 and 5 of the S1 domain from other β-strands in the S1 structure determined for the *E. coli* PNPase (Bycroft *et al*., 1997) and modelled recently for *E. coli* RNase E (Diwa *et al*., 2002). Although the functional significance of these highly conserved arginine-rich acid insertions into the S1 domain is unknown, we speculate that they may affect interactions between the S1 domain and RNAs, and may also provide additional binding sites for 'degradosome' proteins.

Experimental procedures

Strains, plasmids and culture conditions

Construction of an *rne*-deleted *E. coli* strain KSL1000 (*rne*::*cat*, *recA*::*Tn10*) and pBAD-RNE, pLAC-RNE, pNRNE5

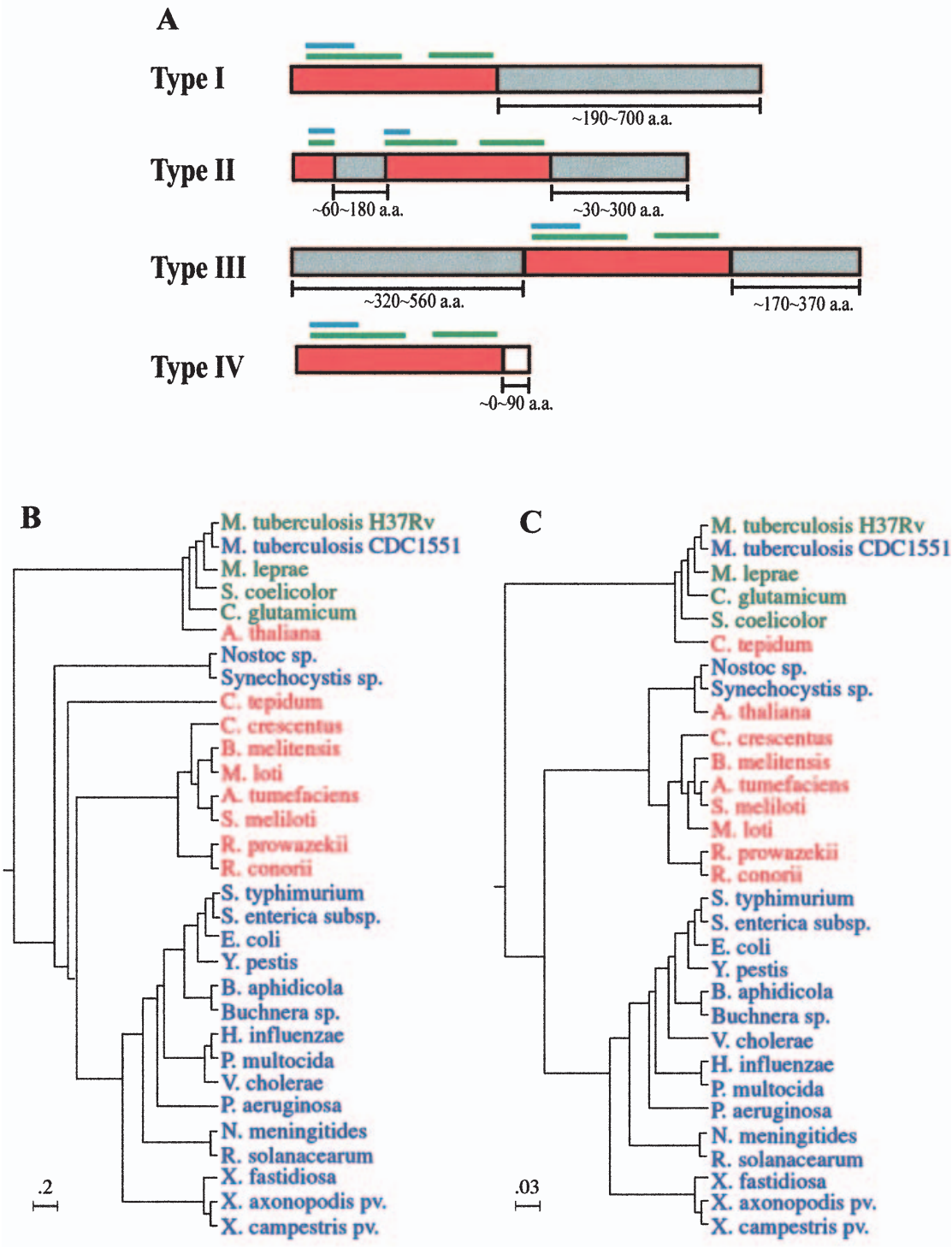

Fig. 7. Classification of RNase E/G homologues.
A. Sequences of putative proteins showing homology to the N-terminal catalytic domain of RNase E or RNase G were obtained from the National Center for Biotechnology Information and aligned using the CLUSTAL W program. The alignment information was used to classify these RNase E/G homologues by the relative positions of possible catalytic (in red) versus possible scaffold (in grey) regions. S1 domain and high sequence similarity regions (HSR; Kaberdin *et al*., 1998) 1 and 2 are indicated by blue and green bars respectively.
B. Distribution of organisms containing RNase E-like enzymes classified as type I–III in Table 1 based on RNase-E like protein sequences.
C. Distribution of organisms containing RNase E-like enzymes classified as type I–III in Table 1 based on small-subunit ribosomal RNA sequences.
The evolutionary trees were drawn with CLUSTAL W program. The evolutionary distance scale is as shown, with the length of the bar representing 20 changes every 100 amino acids (B) or three changes every 100 nt (C). Organisms containing RNase E-like proteins classified to type I, II and III are indicated in blue, red and green respectively.

and pRNG3 plasmids has been described previously (Lee *et al*., 2002). Full-length *rns* and its truncated forms, *rns-ΔC*, *rns-ΔN* and *M-rns*, were PCR amplified from *S. coelicolor* chromosome, cloned into pNRNE5 by replacing *N-rne* using unique *Not*I and *Spe*I sites that resulted in pRNES101-104 respectively. PCR primers used are 5′rns-wt (5′-ATGGATCCGCGGCCGCATATGCTCGAACCGACCGAACCCACC, first eight codons of *rns* are underlined), 5′rns-CR (5′-ATGGATCCGCGGCCGCATATGGTCGAGCGCGTCATGGTCGTCCGC (codons from 536 to 543 of *rns* are underlined), 3′rns-wt (5′-TCTAGACTAGTGAATTCA*GTGGTGGTGGTGGTGGTG*GCCCTCGTCGGTGGCGGCCGAGAC (sequence complementary to last eight codons of *rns* is underlined, and sequence complementary to hexahistidine is in italic) and 3′rns-CR (5′- TCTAGACTAGTGAATTCA*GTGGTGGTGGTGGTGGTG*GGCCGCCTCGGCCTCGGCGGCGCT (sequence complementary to codons from 1053 to 1060 of *rns* is underlined, and sequence complementary to hexahistidine is in italic). Primers used to amplify the full-length *rns*, *rns-ΔC*, *rns-ΔN* and *M-rns* are 5′rns-wt and 3′rns-wt, 5′rns-wt and 3′rns-CR, 5′rns-CR and 3′rns-wt, and 5′rns-CR and 3′rns-CR respectively. Plasmids pRNES201-204 and pRNES301-304 were constructed by subcloning *Nde*I–*Eco*RI fragments from pRNES101-104 into the same sites in pET21a (Novagen) and in *Streptomyces* plasmid pIJ6021 (Takano *et al*., 1995) respectively. pRNES201Δapr was constructed by replacing the *Age*I–*Bsr*GI fragment in the *rns* coding region of pRNES201 by the *Eco*RV–*Sma*I fragment containing the apramycin resistance gene from pULVK2A (Kumar *et al*., 1994). The *Age*I and *Bsr*GI ends of the vector fragment had been filled using Klenow fragment of DNA polymerase I. pRNES401 was constructed by cloning the *Hin*dIII–*Xba*I fragment containing the *rns* gene flanked by the apramycin resistance gene from pRNES201Δapr into the same sites of pHZ132 (Hu *et al*., 1994). pHZ132 carries a temperature-sensitive replicon from pSG5 and is unstable at high temperature (40°C) in *S. coelicolor*.

pGPSI was constructed by cloning the *Eco*RI–*Nde*I fragment containing the GPSI gene from pET-GPSI into the same sites of a *Streptomyces* vector, pIJ6021. pET-GPSI is a derivative of pJSE371 (Jones and Bibb, 1996) that expresses a C-terminally hexahistidine-tagged form of GPSI and was constructed by cloning a PCR fragment containing sequences of the C-terminal part of GPSI with a hexahistidine tag into *Eco*RI–*Sfi*I sites of pJSE371. The PCR primers used were GPSI-1785F (5′-CATCGGCCCGAAGCGACAGA) and GPSI-3′His (5′-GCGGATCCGAATTC*TCA***GTGGTGGTGGTGGTG GTG**CTGCTCGGCGTCGTC (complementary sequences for stop codon and hexahistidine tag are in italic and in bold type respectively).

S. lividans 1326 and *S. coelicolor* A3(2) were used as the *Streptomyces* host for expression of GPSI protein and Rns protein as well as its truncated forms respectively. Conditions for culture and transformation of *Streptomyces* were as described previously (Kieser *et al*., 2000) unless otherwise indicated.

Protein work

All ribonucleases were purified using the His•Bind purification kit (Novagen) as described previously (Lee and Cohen, 2001) except that RNase ES and M-RNase ES were purified from KSL1000 harbouring pRNES101 and pRNES104 as well as *S. coelicolor* harbouring pRNES301 and pRNES304. *Streptomyces* cultures were grown to early log phase, induced with thiostrepton (5 μg ml^{-1}) for 12 h and harvested for protein purification and immunoprecipitation. The protein concentration was calculated using Coomassie brilliant blue G250 as described previously (Sedmak and Grossberg, 1977) and using bovine serum albumin (BSA) as a standard. For immunoprecipitation of RNase ES and its truncated forms, preinduced *S. coelicolor* cells harbouring pRNES301-304 were lysed in a prechilled buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1% NP-40 (1% Nonidet P-40 lysis buffer) by passing them through a French Press at 15 000 p.s.i. twice. After clearing the cell debris at 39 000 *g* for 20 min at 4°C, the supernatant was collected. Aliquots of the lysates were precleared by incubation with normal mouse IgG and protein A-agarose (Santa Cruz Biotechnology) for 30 min at 4°C. The precleared lysates were then incubated with 1 μg ml^{-1} anti-mouse tetra His monoclonal antibody (Qiagen) for 1 h and subsequently incubated with protein A-agarose for an additional 1 h at 4°C. The immune complexes were centrifuged for 5 min at 2000 *g* and washed five times with 1% Nonidet P-40 lysis buffer. The proteins were run on a 7.5% SDS-polyacrylamide gel, and gels were electroblotted to a nitrocellulose membrane and probed as described previously (Hagège and Cohen, 1997). Anti-GPSI antibody was a gift from Dr G. Jones and was diluted 1:10 000 for Western blot analysis.

For Far Western blotting, total protein was prepared from *E. coli* strain BL21 (DE3) harbouring pET21a or pRNES 201-204, which were grown in LB medium to the mid-log phase (OD_{600} = 0.4) and induced for 1 h with 1 mM IPTG. The procedure for Far Western blotting was as described previously (Vanzo *et al*., 1998), and the concentration of probe protein used was 500 ng ml^{-1} Hepes hybridization buffer (250 ng cm^{-2} membrane).

Synthesis of RNA and in vitro *cleavage assay*

Synthesis of ^{32}P-labelled RNA I, BR13, *E. coli* 9S rRNA and pM1 and RNase E cleavage assay have been described previously (Lee *et al*., 2002).

Northern blot analysis

The procedure for Northern blot analysis has been described previously (Lee *et al*., 2002).

Southern blot analysis

Purified total DNA was digested with *Hin*dIII or *Pst*I. A 1 kb DNA ladder (Invitrogen) was end-labelled with ^{32}P by T4 kinase after dephosphorylation with CIAP. Samples of a labelled 1 kb DNA ladder, *Hin*dIII- or *Pst*I-digested total DNA and *Pst*I-digested plasmids pBAD-RNE, pRNG3 and pRNES104 were separated in a 0.8% agarose gel. Separated DNA fragments were transferred to a Zeta-Probe® (Bio-Rad) nylon membrane and hybridized with probes specific to *rne* coding sequences 1–1042 and 2883–3554 (probes 1 and

2 respectively; see Fig. 2B). Probes were synthesized using a High Prime kit (Roche Diagnostics) according to the manufacturer's instructions. DNA templates were prepared by digesting pBAD-RNE with *Hin*dIII and *Not*I for probe 1 or *Sph*I and *Xmn*I for probe 2.

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