Ribosomal-RNA patterns of *Escherichia coli*, *Salmonella typhimurium* and related Enterobacteriaceae

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Summary. rRNA sequences are usually highly conserved among species. In Enterobacteriaceae we have shown that *Salmonella typhimurium* does not have an equivalent to the 23S rRNA of *Escherichia coli* but its 23S rRNA is cleaved *in vivo* into two smaller species. This cleavage appears to be a result of a difference between the *S. typhimurium* and *E. coli* rRNA sequences rather than to differences in ribonuclease activity. We have surveyed a wide range of Enterobacteriaceae for the presence or absence of 23S rRNA and found this rRNA species to be present in all strains of *E. coli*, *Shigella* and *Citrobacter* and all salmonellae examined except *S. typhimurium*. All *S. typhimurium* cultures, isolated at different times and from several different countries, lack an intact 23S rRNA. Thus, the presence or absence of this rRNA species is an excellent diagnostic characteristic for *S. typhimurium*.

Introduction

Although different genera of the family Enterobacteriaceae often show only distant genetic and phenetic relatedness, several genes, such as those specifying the ribosomal RNAs, are highly conserved among species (Kohne, 1968; Brenner and Falkow, 1971). Thus, the isolated genes specifying rRNA show $\geq 90\%$ relatedness by hybridisation analysis among species such as *Escherichia coli* and *Proteus mirabilis* (Kohne, 1968) in marked contrast to the poor (8%) re-association kinetics of total DNA (Brenner, 1978).

Early studies established the apparently universal occurrence of 23S, 16S and 5S components of prokaryotic rRNA (Brenner *et al.*, 1969). Thus, an observation by Winkler (1979) that at least one apparently wild-type strain of *Salmonella typhimurium* lacked intact 23S rRNA was somewhat unexpected. We have therefore surveyed a collection of naturally occurring isolates of *S. typhimurium*, other serotypes of *Salmonella* and other Enterobacteriaceae, to determine how widespread this variation in rRNA might be and whether it was restricted to the strain studied by Winkler. The results of this kind of survey may, of course, have considerable potential for diagnostic purposes. Furthermore, we have investigated the cause of this difference in rRNA patterns and have shown that

it results from differences in the rRNA genes themselves, rather than from differences in specific ribonucleases in the different species. The evolutionary implications of this finding are considered.

Materials and methods

Bacteria

The 65 strains of Enterobacteriaceae examined were: 15 clinical and laboratory-derived strains of *Escherichia coli*; 1 strain each of *Shigella dysenteriae*, *Sh. boydii* and *Sh. flexneri* and 2 strains of *Sh. sonnei*; two strains of *Citrobacter freundii*; 14 strains of *S. typhimurium* isolated from a diverse range of animal and environmental sources and 2 laboratory-maintained strains LT2 and LT7 (Lilleengen, 1948) that had been investigated by Winkler (1979); 7 strains of *S. paratyphi B*; 15 other strains of *Salmonella* from subspecies I representing 12 different O-antigen groups and 15 host-specific or non-specific serotypes (*S. alachua*, *S. anatum*, *S. choleraesuis*, *S. enteritidis*, *S. hadar*, *S. heidelberg*, *S. infantis*, *S. kedougou*, *S. livingstone*, *S. paratyphi A*, *S. pomona*, *S. rubislaw*, *S. senftenberg*, *S. virchow* and *S. waycross*); and 5 strains of *Salmonella* representing serotypes from subspecies II–V (*S. II manombo*, *S. III arizonae* [44: z₄, z₂₄; -], *S. III arizonae* [48: i; z₃₅], *S. IV houten* and *S. V bongor*). The designation of serotypes of *Salmonella* in subspecies I–V follows recommended guidelines (Lindberg and Le Minor, 1984). Strains were stored on Dorset's egg slopes at room temperature until they were subcultured on MacConkey's Agar (Oxoid) for testing.

Media and bacterial growth

L-broth (Miller, 1972) contained (/L), Bacto-Tryptone (Difco) 10 g, Yeast Extract (Oxoid) 5 g, and NaCl 5 g. A single colony of each strain was inoculated to 5 ml of L-broth and incubated overnight at 37°C with vigorous shaking at 100 rpm. The overnight culture was diluted 1 in 50 in fresh L-broth (10 ml) and the cells were grown to late exponential phase.

Preparation of RNA for electrophoresis

Cultures, grown as described above, were chilled and the cells pelleted by centrifugation at 6000 *g* for 10 min. Where appropriate (*vide infra*), cultures of different strains were mixed in equal volumes before centrifugation. Bacterial cell pellets were washed in cold Tris-EDTA buffer, pH 8.0 (Maniatis *et al.*, 1982) and lysed by the addition of 800 µl of lysis buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 350 mM NaCl and SDS 2% w/v) and boiled for 1 min. After mixing, 200 µl of equilibrated phenol (Maniatis *et al.*, 1982) was added and the lysate heated at 90°C, with mixing, for a further 30 s. Samples were chilled and extracted with 200 µl of chloroform: isoamylalcohol (24:1). After centrifugation (12 000 *g* for 4 min), the supernate was removed and subjected to a further hot phenol-chloroform extraction and two extractions with chloroform-isoamylalcohol; the nucleic acids were precipitated twice with ethanol. Samples were resuspended in 50 µl of TE buffer, denatured by addition of 20 µl of gel-running buffer (5 × strength), 100 µl of deionised formamide and 34 µl of deionised formaldehyde (36% w/v), and heated at 55°C for 15 min with occasional mixing. One-tenth volume of loading dye (glycerol 50% v/v with 1 mM EDTA and bromophenol blue 0.4% w/v) was added and the RNA separated by electrophoresis.

Formaldehyde-agarose gels

Denaturing agarose gels 1.2% w/v (Maniatis *et al.*, 1982) were prepared in gel-running buffer (0.04 M MOPS, pH 7.0, 10 mM sodium acetate and 1 mM EDTA). After cooling the dissolved agarose to 55°C, deionised formaldehyde was added to a final concentration of 2.2 M and ethidium bromide at 0.5 µg/ml. Gels were electrophoresed in the gel-running buffer described above at 5 V/cm for c. 2 h.

Transformation of *S. typhimurium* strain LT2 with *rrnB* genes of *E. coli*

Plasmid pC6 encodes the entire *E. coli rrnB* operon and is similar to pKK3535 (Brosius *et al.*, 1981) but with the insert in the opposite orientation. This plasmid was kindly provided by Dr C. Squires. The entire nucleotide sequence of the *rrnB* operon is known (Brosius *et al.*, 1981). To introduce this plasmid into *S. typhimurium*, pC6 DNA was first transformed into the restriction-deficient strain LR5000 by the CaCl₂ procedure (Leder-

berg and Cohen, 1974) selecting for ampicillin resistance. DNA purified from this strain was subsequently transformed into the wild-type strain LT2 of *S. typhimurium*.

Results

Absence of 23S rRNA from *Salmonella*

When RNA preparations from cultures of 15 strains of *E. coli*, 5 strains of *Shigella* and 2 strains of *Citrobacter* were analysed by electrophoresis in denaturing formaldehyde-agarose gels, they displayed typical “*E. coli*” rRNA patterns with both 23S and 16S components (fig. 1). In contrast, all 16 strains of *S. typhimurium* examined, irrespective of their source or year of isolation, exhibited a very different pattern (the “*S. typhimurium*” pattern). Although the 16S rRNA band of *S. typhimurium* strains always migrated at the same rate as the 16S band of *E. coli*, the strains of *S. typhimurium* lacked a band of mobility equivalent to the 23S rRNA of *E. coli* (fig. 1). In place of the 23S rRNA band, two bands of mobility corresponding to 1.7 and 1.1 kilobases were observed. Within the genus *Salmonella*, all *S. typhimurium* strains gave the *S. typhimurium* rRNA pattern whilst all 20 strains from *Salmonella* subspecies I–V gave the *E. coli* pattern. Thus, the absence of a 23S rRNA seems to be specific to *S. typhimurium*. The only exceptions to this finding were 2 strains of *S. paratyphi B* which showed the *S. typhimurium* pattern although 5 other strains of that serotype gave the normal *E. coli* pattern. The taxonomic significance of this observation is discussed below.

In *S. typhimurium* strains, the 23S rRNA is replaced by two smaller rRNA species which were shown to be equivalent to 23S rRNA because they hybridised in “Northern” blots with plasmid pC6 DNA, encoding the 23S rRNA gene of *E. coli* (data not shown). To show that cleavage of 23S rRNA into two fragments reflected the situation *in vivo*, and was not due to cleavage during RNA extraction from *S. typhimurium* by nucleases active during the extraction process, equal volumes of cultures of *E. coli* and *S. typhimurium* were mixed immediately before cell lysis and RNA extraction. The mixed culture gave a mixed rRNA pattern (fig. 2) showing that *Salmonella* nucleases were not active during extraction.

The difference in rRNA patterns between *E. coli* and *S. typhimurium* might reflect a difference in intracellular RNAases, such that *S. typhimurium* contains an RNAase that cleaves 23S rRNA but is absent from *E. coli*. Alternatively, the nucleases of

Fig. 1. Agarose gel showing rRNA patterns from various Enterobacteriaceae. The 23S and 16S rRNA species are indicated. Details of the strains are given in the text.

the two species may be the same, but a sequence difference in the rRNA makes *S. typhimurium* 23S rRNA far more susceptible to cleavage. To address this question plasmid pC6, encoding the *E. coli* 23S rRNA, was transformed into *S. typhimurium* strain LT2 and the rRNA extracted and separated by electrophoresis (fig. 3). The *E. coli* 23S rRNA remained intact when expressed in *S. typhimurium* demonstrating that the cleavage of *S. typhimurium* 23S rRNA resulted from sequence differences in the rRNA molecules rather than a difference in the specificity of the nucleases in the two species.

Discussion

The introduction of numerical taxonomy and the technique of DNA-DNA re-association has re-

sulted in the description of many new species and genera in the family Enterobacteriaceae, which now includes more than 125 hybridisation groups (Brenner, 1983; Farmer *et al.*, 1985; Ewing, 1986). It has also indicated the need for a reappraisal of some of the accepted relationships implied from earlier taxonomic schemes which were devised on the basis of only a few phenotypic traits. It might be useful, therefore, to consider the genetic relatedness of the Enterobacteriaceae used in this study.

First, although the four species of *Shigella* were originally delineated from *E. coli* on the basis of their pathogenicity, and have been maintained as separate entities in the interests of clinical microbiology and epidemiology (Ewing, 1986), they nevertheless constitute with *E. coli* a single genetic species (i.e., they show $\geq 75\%$ DNA relatedness

Fig. 2. Agarose gel showing rRNA from: track 1, *S. typhimurium* strain LT2; track 2, *S. typhimurium* strain LT2 extracted together with *E. coli* strain K12; track 3, *E. coli* strain K12. The *E. coli* 23S and 16S rRNA species are indicated.

under stringent conditions; Brenner *et al.*, 1973). Second, the taxonomy of *Salmonella* has been similarly problematic. In this genus, strains differentiated by serotyping of O and H antigens were originally given species ranking; the eventual result of that approach was that >2000 serotypes masqueraded as species. Modern methods of taxonomy have now established that all strains of salmonellae and arizonae (the latter previously differentiated from the former by a few biochemical characters) constitute a single genetic species comprising seven

Fig. 3. Agarose gel showing rRNA from: track 1, *S. typhimurium* strain LT2; track 2, *S. typhimurium* strain LT2 transformed with plasmid pC6 encoding the *E. coli* 23S rRNA. The 23S and 16S rRNA species are indicated.

subspecies (Le Minor *et al.*, 1982 and 1986). The citrobacters, close relatives of the salmonellae, used to be included with them in the tribe Salmonelleae but are now known to have diverged sufficiently from them to warrant distinct taxonomic status in the tribe Citrobacterae (Ewing, 1986). Furthermore, citrobacters afford a useful link between escherichiae and salmonellae because, in terms of DNA relatedness, species of *Citrobacter* are as closely related (40–50%) to *E. coli* as they are to *Salmonella* (Crosa *et al.*, 1973; Brenner, 1978).

Our findings show that strains of *E. coli*, *Shigella*, *C. freundii* and most serotypes of *Salmonella* give a similar rRNA pattern in which both 16S and 23S species of rRNA are represented. This commonality amongst four genera of Enterobacteriaceae which, in terms of DNA relatedness, might be considered to be no more than moderately related, only serves

to highlight the unusual rRNA pattern of strains of *S. typhimurium*. The 14 naturally occurring strains of *S. typhimurium* examined by us were isolated between 1950 and 1985 from diverse human, animal and environmental sources in 12 different countries worldwide. They represent 14 of the phage-type/biotype groups described by Anderson *et al.* (1978). Genetic data reported elsewhere (Old, 1984) suggest that strains of *S. typhimurium* of all phage-type/biotype groups evolved from a common, archetypal bacterium. The present findings, reporting an absence of 23S rRNA from all strains of *S. typhimurium* examined by us, support this hypothesis. In contrast, our observation that some strains of *S. paratyphi B* possess the *E. coli* rRNA pattern, whereas others possess the *S. typhimurium* pattern, does not fit the classical taxonomic grouping. This finding implies that certain members of the *S. paratyphi B* serotype would best be classified as *S. typhimurium*. The need to reassess the classification of *S. paratyphi B* is not inconsistent with the known heterogeneity of strain characters in that serotype and is discussed more fully by Barker *et al.* (1988).

The finding that a wide range of *Salmonella* strains lacks a 23S rRNA species is somewhat surprising in view of the conserved nature of rRNA sequences among species and the essential role of

this RNA in ribosome function. We have shown that the lack of 23S rRNA in *S. typhimurium* is not an artefact of rRNA isolation. Furthermore, when the *E. coli rrnB* gene is expressed in *S. typhimurium* the 23S rRNA remains intact, implying that the difference between *E. coli* and *S. typhimurium* is not a specific ribonuclease activity. Rather, there must be a sequence difference in the 23S rRNA from the two species. This is an intriguing possibility in view of the fact that the rRNAs of *E. coli* and *S. typhimurium* are transcribed from seven independent *rrn* operons (Lehner *et al.*, 1984). Since all the 23S rRNA molecules of *S. typhimurium* are cleaved, this implies that each of the seven *rrn* genes has acquired the same alteration. Presumably, gene conversion events have ensured that, within a species, the seven genes are maintained in the same genetic configuration. This provides what is probably the best evidence for gene conversion in prokaryotes and defines an interesting evolutionary system for studying the mechanisms of gene conversion.

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