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Biofilm Formation and Antibiotic Resistance in *Salmonella* Typhimurium Are Affected by Different Ribonucleases

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Biofilm formation and antibiotic resistance are important determinants for bacterial pathogenicity. Ribonucleases control RNA degradation and there is increasing evidence that they have an important role in virulence mechanisms. In this report, we show that ribonucleases affect susceptibility against ribosome-targeting antibiotics and biofilm formation in *Salmonella*.

Keywords: *Salmonella*, biofilms, antibiotics, RNases, RNA

Salmonella infections are a serious medical and veterinary problem worldwide and there is an increasing need for new strategies for prevention and control [23]. In this study, we wanted to combine our knowledge on ribonucleases (RNases) with the evidence that changes in RNA metabolism can affect virulence.

Ribonucleases are the enzymes that mature and degrade transcripts, ultimately regulating RNA levels in the cell. Endoribonucleases cleave RNA internally, while exoribonucleases degrade the RNA molecule from one extremity. In *Salmonella*, the main endoribonucleases are RNase E and RNase III [4, 32]. RNase E is an essential endoribonuclease that cleaves single-stranded RNA. The C-terminal of RNase E includes binding sites for other proteins, forming the degradosome, a multiprotein complex involved in RNA degradation. RNase III is a ubiquitous enzyme, specific for double-stranded RNA. The main exoribonucleases in *E. coli* and *Salmonella* are PNPase, RNase R, and RNase II, which degrade RNA from the 3′-extremity [3, 9, 32]. RNases are key factors in the control of important cellular processes since they determine the final levels of every transcript. Some RNases are up-regulated under stress situations and have been reported to be involved in virulence processes in pathogenic organisms (reviewed in [3, 4, 19, 32]). Development of bacterial resistance to antimicrobial drugs is an ever-increasing clinical problem and RNases could be potential novel targets for therapeutic intervention [10].

We have constructed *S.* Typhimurium SL1344 isogenic mutants deficient in the main ribonucleases (Table 1). These mutants were tested against a panoply of antimicrobial agents representatives of different antibiotic classes (Table 2). The minimum inhibitory concentration (MIC) was determined by the microdilution method as previously described [2]. It is important to note that the growth pattern of the mutant strains was very similar to that of the wild type [38], which excludes any influence of the growth rate in the observed MICs. The results showed that among the diverse classes of antibiotics tested, the susceptibility of the RNase mutant strains was only affected by ribosome-targeting agents (Table 2). Notably the RNase III mutant strain (Δ*rnc)* was more susceptible to kanamycin, spectinomycin, tobramycin, and chloramphenicol. The first three are broad-spectrum antibiotics that belong to the aminoglycoside class. This class of antibiotics interferes with protein synthesis by selectively binding to the bacterial ribosome [21]. Chloramphenicol also inhibits protein synthesis and

Table 1. List of strains and plasmids used in this study.

Strains	Relevant Markers/Genotype	Source/Reference
S. Typhimurium, SL1344	Str^{R}*hisGrpsLxyl*	[16]
CMA-537 (*rne537*)	SL1344 *rne-537* (Δ*rne*::Cm^{R})	[39]
CMA-539 (Δ*pnp*)	SL1344 *pnp-539* (Δ*pnp*::Cm^{R})	[39]
CMA-551 (Δ*rnc*)	SL1344 *rnc-14*::ΔTn*10* (Tc^{R})	[40]
CMA-700 (Δ*rnb*)	SL1344 *rnb* (Δ*rnb*::Cm^{R})	This study
CMA-701 (Δ*rnr*)	SL1344 *rnr* (Δ*rnr*::Cm^{R})	This study
E. coli, DH5α	*rec*A1 *end*A1 *gyr*A96 thi-*hsd*R17 *sup*E44 *rel*A1 _*lac*ZYA-*arg*FU169 f80dLacZDM15	New England Biolabs
Plasmids	**Comment**	**Source/Reference**
pWSK29	Low-copy plasmid (Amp^{R})	[42]
pSE420	IPTG-inducible plasmid (Amp^{R})	Invitrogen
pSVA-5 (p*pnp*)	pSE-420 expressing PNPase	[39]
pSVA-7 (p*rnc*)	pWSK29 expressing RNase III	[38]
pSVA-8 (p*rne*)	pSE-420 expressing RNase E	[38]
pSVA-9 (p*rnb*)	pSE-420 expressing RNase II	This study
pSVA-10 (p*rnr*)	pSE-420 expressing RNase R	This study

Table 2. MIC ranges of antibiotics (μg/ml).

Strains	Aminoglycosides			Phenicol	Macrolide	Fluoroquinolone		Quinolone	β-Lactam
	KAN	SPT	TOB	CHL	ERY	NOR	OFL	NAL	AMP
wt	4	64	4	8	128	1	0,5	8	2
rne537	4	32	4	8	256	1	0,5	8	2
Δ*rnc*	2	32	2	4	128	1	0,5	8	2
Δ*rnb*	4	64	4	8	128	1	0,5	8	2
Δ*rnr*	4	32	4	8	128	1	0,5	8	2
Δ*pnp*	4	64	4	8	128	1	0,5	8	2

The values presented are the result from at least three independent experiments.
KAN, kanamycin; SPT, spectinomycin; TOB, tobramycin; CHL, chloramphenicol; ERY, erythromycin; NOR, norfloxacin; OFL, ofloxacin; NAL, nalidixic acid; AMP, ampicillin.

affects the assembly of ribosomal subunits [31]. RNase III is involved in the primary processing of rRNA (reviewed in [3]) and it was reported to co-purify with ribosomes [1]. Such interaction was suggested to facilitate ribosomal biogenesis [1]. These observations provide a possible explanation to answer why antibiotics that affect ribosome assembly are more efficient in the absence of RNase III. Furthermore, neomycin and paromomycin, two other aminoglycosides, were recently reported to promote a reduction in the 30S and 50S ribosomal subunit amounts in an RNase III *E. coli* mutant [11].

The RNase R mutant also showed higher susceptibility to spectinomycin, an antibiotic that inhibits the elongation cycle of translation [5, 26] (Table 2). *Trans*-translation releases stalled ribosomes from non-stop mRNAs, and RNase R is known to be involved in the decay of these defective transcripts [27]. In agreement with our results, deficiencies in *trans*-translation increase susceptibility to protein synthesis inhibitors in *Salmonella* [8, 20, 41].

RNase E is essential and therefore we have used a mutant in which the protein is missing the C-terminal and cannot form the degradosome (the allele was called *rne537*). This RNase E mutant also showed a higher sensitivity to spectinomycin. The significant contribution of RNase E and the degradosome to the ribosome biogenesis and quality control may be a plausible explanation [7, 12, 25]. Surprisingly, the RNase E mutant had a higher resistance to erythromycin. Erythromycin is a macrolide that affects

protein synthesis and inhibits 50S subunit formation [31, 36] and the absence of the degradosome might somehow contribute to stabilize one or more transcripts involved in conferring erythromycin resistance.

The different susceptibilities of the ribonuclease mutants are indicative that they use different cellular targets, and strengthen the idea that RNA degradation pathways contain a certain degree of specificity and are not fully equivalent.

As a response to environmental pressures, bacteria can aggregate on abiotic and biotic surfaces, forming a biofilm. Biofilm development and antibiotic resistance are intimately connected, since the biofilm matrix can delay the penetration of antimicrobial agents [15, 22, 35, 43]. Biofilm formation is a highly regulated process that includes an array of key regulators, including ribonucleases and small noncoding RNAs [6, 17, 33, 38]. Ribonucleases in turn have been shown to regulate these functional RNAs [18, 37, 39, 40]. Therefore, we have investigated whether ribonucleases affect biofilm properties. The extracellular matrix of biofilms is composed of curli fimbriae, cellulose plus other polysaccharides, and proteins. We have monitored curli fimbriae and cellulose biosynthesis by assessing the growth and morphology of the different *Salmonella* strains on Congo Red Luria agar plates, as previously described [14, 28, 29]. A PNPase mutant had previously been reported to display a different colony morphotype compared with the wt on these plates [30], and was used as a control. The expression of cellulose was also evaluated by fluorescence analysis on Calcofluor agar plates [34].

Among the strains analyzed, the endoribonuclease mutants showed the major differences in colony morphotype (Fig. 1). The wild-type strain presented dark red and rough colonies as previously reported for this strain [14]. On the other hand, mutant Δ*rnc* gave rise to smooth and pale colonies with a red circle around them (Fig. 1A). It is known that strain SL1344 produces a low amount of cellulose [14]. However, in the Calcofluor agar plates, the Δ*rnc* mutant fluoresced with even less intensity than the wt (Fig. 1B), which might indicate that cellulose biosynthesis is compromised. Deficiencies in the production of curli fimbriae can neither be ruled out. Complementation with RNase III *in trans* partially restored the wt morphotype. Furthermore, the overexpressing strain also exhibited a morphotype distinct from that of the wt, indicating that RNase III has an important role in biofilm development. The ability to bind Congo Red dye seems to be also compromised in the *rne537* mutant (Fig. 1A), revealing that expression of the extracellular matrix components (cellulose and/or curli fimbriae) might also be affected. The brighter fluorescence displayed in Calcofluor plates by the overexpressing strain (Fig. 1B) indicates a possible role of RNase E in modulating the amount of cellulose in the cell. Concerning exoribonucleases, the lack of RNase II seems to raise the cellular production of cellulose (Fig. 1B), indicating that this enzyme may also modulate the levels of genes involved in cellulose production.

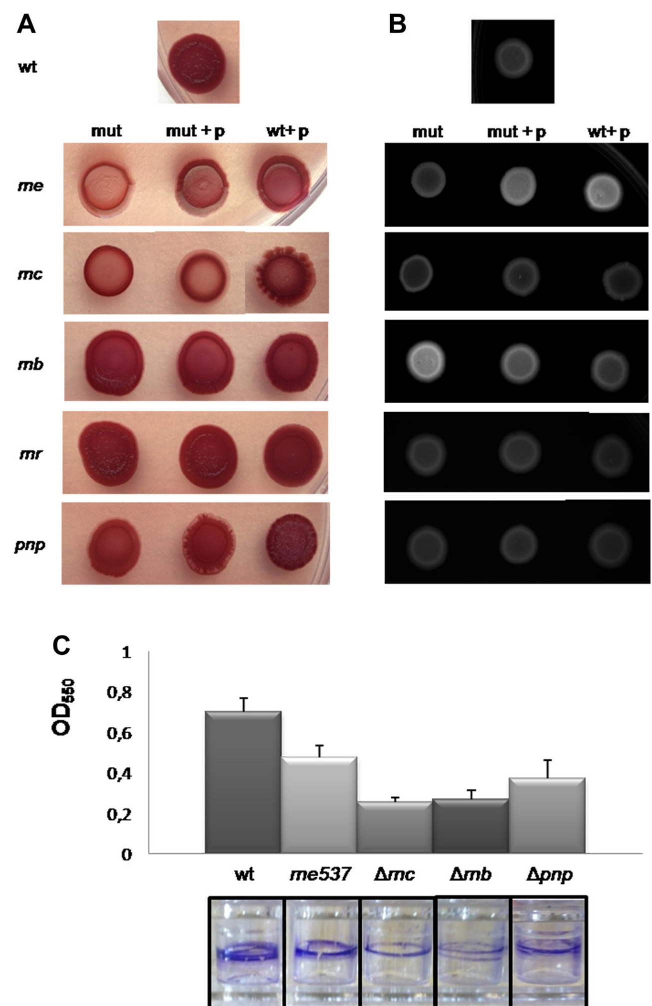

Fig. 1. Biofilm properties of different *Salmonella* strains on (**A**) LB plates without NaCl containing Congo red or (**B**) LB plates with Calcofluor. Strains are indicated on the left side of the image. On the top, mut represents the different ribonuclease mutant strains; mut+p is the complemented mutant and wt+p is the overexpressing strain. (**C**) Effect of the lack of some ribonucleases on biofilm development in microtiter plates in Iso-Sensitest medium (Oxoid). The thickness of biofilms in cultures of different strains was measured by determining optical density at 550 nm (OD_{550}) after staining with crystal violet. Error bars represent standard deviations.

As expected, the PNPase mutant showed an altered morphotype on Congo Red plates (Fig. 1A). CsgD, a master activator of biofilm development [44], was reported to be substantially reduced in the absence of PNPase in *Salmonella* [30].

The mutant strains that showed higher deviations on Congo Red and Calcofluor tests were further analyzed regarding biofilm formation in polystyrene microtiter plates, as described by Merrit *et al*. [24]. This assay measures the ability of bacteria to attach to the wells of a microtiter dish in the interface between the air and the liquid medium. All the mutants tested showed a reduced ability to form biofilm, but Δ*rnc* and Δ*rnb* were the mutants in which less biofilm was formed (Fig. 1C). The deficiency of biofilm formation observed in Δ*rnb* was somehow surprising when considering the higher levels of cellulose produced by this strain. However, it has been shown in *E. coli* that cellulose overproduction negatively affects curli-mediated surface adhesion and cell aggregation, thus acting as a negative determinant for biofilm formation [13]. Consistent with this, the higher cellulose produced by the RNase II mutant could account for a decrease in adhesion and thus biofilm formation.

This study underlines the importance of RNases in antibiotic susceptibility and biofilm formation, two important factors in bacterial survival. In particular, endoribonucleases E and III seem to affect these important functions. The involvement of these two ribonucleases in ribosomal biogenesis can be the basis of the higher sensitivity to ribosome-targeting antibiotics observed in the respective mutant strains. Mutants of these RNases are also known to exhibit a reduced motility, and a strong variation of CsgD mRNA levels [38], which may explain the deficiency in biofilm production. The simultaneous contribution of RNase III to biofilm development and antibiotic susceptibility reinforces the view that this enzyme is an important global regulator [4]. The evolutionary conservation of this ribonuclease in bacteria confirms its biological importance in the cell.

It was recently proposed that RNases could be attractive novel therapeutic targets [10]. Further investigation on their mode of action may be applied in the design of new strategies to combat pathogenic bacteria.

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