COLICIN E3-INDUCED *IN VITRO* INACTIVATION OF RIBOSOMES FROM COLICIN-INSENSITIVE BACTERIAL SPECIES

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1. Introduction

Colicin E3 inactivates 30 S ribosomal subunits in sensitive bacteria [1] by causing a specific cleavage of 16 S ribosomal RNA [2, 3]. An RNA fragment about 50 nucleotides long ("E3-fragment") is cleaved from the 3′-terminus [2]. Recently, the same ribosome inactivation and 16 S RNA cleavage have also been demonstrated *in vitro* by incubating 70 S ribosomes with purified E3 [4, 5].

Colicin E3 is a bacteriocin [6–8]. One unique feature of bacteriocins in general is their narrow killing spectrum [7]. Thus colicin E3, like other related colicins, is active against bacterial strains which belong to the genera *Escherichia* and *Shigella* (both are in the family *Enterobacteriaceae*), but not most of the bacterial species which belong to other bacterial genera or families. Presumably, these resistant bacterial species do not possess specific receptors needed for the adsorption of colicins to the cells, and this may be the basis of the narrow killing specificity. In fact, mutants of *E. coli* can easily be obtained that are resistant to colicins because of the absence of the receptors [6–8]. As expected, *in vitro* experiments have shown that ribosomes from such E3-resistant mutant cells are inactivated *in vitro* as are those from sensitive cells [5]. We have now tested E3-sensitivity of the ribosomes from other bacterial species which are taxonomically unrelated to *E. coli* and are resistant to E3. *Bacillus stearothermophilus* and *Azotobacter vinelandii* were selected for this purpose. Both belong to the bacterial families different from *Enterobacteriaceae*, and, in addition, are known to be significantly different from *E. coli* in their ribosomal components [9]. The results show that E3 causes specific *in vitro* inactivation of ribosomes from these bacterial species. With radioactive *B. stearothermophilus* ribosomes it has been demonstrated that the inactivation involves a specific cleavage of 16 S RNA analogous to that observed with *E. coli* ribosomes.

2. Materials and methods

Bacillus stearothermophilus strain 799 and *Azotobacter vinelandii* strain OP were used. *Bacillus* belongs to the family *Bacillaceae*. *Azotobacter* belongs to the family *Azotobacteriaceae*. The resistance of both strains to E3 was confirmed by cross streaking them with colicin E3 on nutrient agar plates. In the case of *B. stearothermophilus*, this was done at 60°. It was shown that colicin E3 is sufficiently stable at 60°, and the observed resistance is not due to heat inactivation of E3 at 60°. The growth of *B. stearothermophilus* cells and the isolation of ribosomes from the cells were described previously [9, 10]. *Azotobacter vinelandii* cells were grown as described by Wilson [11]. Ribosomes were isolated by the same method as used for *E. coli* [9, 12]. Poly-U-dependent polyphenylalanine synthesis was performed according to the method described previously using an *E. coli* "enzyme" preparation containing soluble factors and aminoacyl tRNA synthetases [12].

Colicin E3 was prepared from *E. coli* strain CA38

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Table 1
E3-induced inactivation of ribosomes from *B. stearothermophilus* and *A. vinelandii*.

Exp.	Source of ribosomes	E3	Immunity substance	Activity assay (cpm)	Activity assay (%)
(a)	*B. stearothermophilus*	–	–	882	100
		–	+	859	98
		+	–	263	30
		+	+	818	93
(b)	*A. vinelandii*	–	–	744	100
		–	+	833	111
		+	–	84	11
		+	+	711	96

The reaction mixtures contained in 50 μl: ribosomes (170 μg in (a) and 133 μg in (b)), E3 (5 μg in (a) and 1.25 μg in (b)), and the "immunity substance" (13 μg), as indicated; and in addition, Tris (pH 7.6) 10^{-2} M, $MgCl_2$ 10^{-2} M, NH_4Cl 0.03 M, β-mercaptoethanol 6×10^{-3} M. After incubation at 37° for 1 hr, aliquots (containing 2.5 A_{260} units of ribosomes in (a) and 2.0 A_{260} units in (b)) were taken and poly-U-dependent polyphenylalanine synthesizing activity of the ribosomes was assayed [11]. The specific activity of phenylalanine was 10 μg/μmole and the incubation was done at 37° for 15 min.

according to the method of Herschman and Helinski [13].

The "immunity substance" [4, 5] was extracted from the colicinogenic strain CA38 with 1 M NaCl and purified by DEAE-Sephadex chromatography and Sephadex G-50 gel filtration. The preparation is free from colicin E3 and about 90% pure as judged by polyacrylamide gel electrophoresis at pH 8.7. A preliminary report on the purification of the immunity substance has been described [14] and details of the method will be published elsewhere.

The E3-induced *in vitro* RNA cleavage reaction was followed using ^{32}P-ribosomes as described [5]. Analysis of the [^{32}P]RNA fragment produced by E3 was done by the fingerprinting technique of Sanger [15, 16].

3. Results

70 S ribosomes isolated from *B. stearothermophilus* and *A. vinelandii* were incubated with purified E3 in the presence and absence of the purified "immunity substance". The ribosomes were then assayed for poly-U-dependent polyphenylalanine synthesis. Table 1 summarizes the results. It can be seen that colicin E3 inactivates ribosomes from both *A. vinelandii* and *B. stearothermophilus*. This inactivation is not due to non-specific damage to the ribosomes because the "immunity substance" protects the ribosomes from inactivation, as in the case of E3-induced inactivation

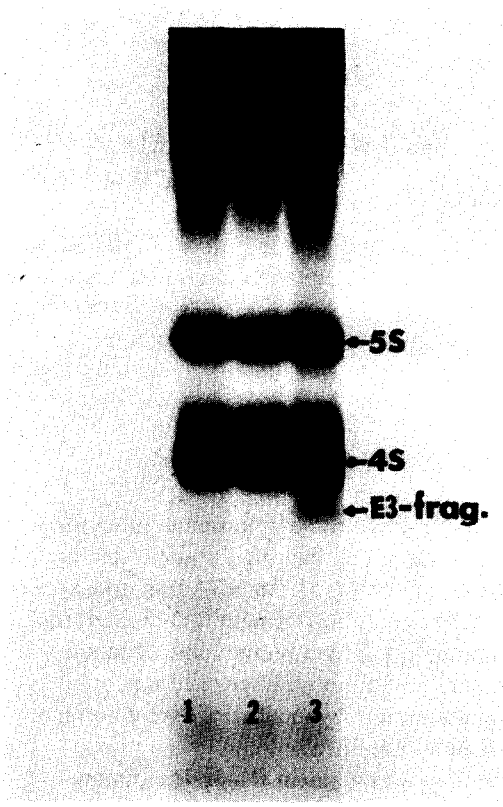

Fig. 1. Polyacrylamide gel electrophoresis of E3 fragment produced from *B. stearothermophilus* ribosomes. ^{32}P-labelled 70 S ribosomes (24 μg) were incubated with E3 (0.6 μg) in the presence (sample 1) and absence (sample 3) of the "immunity substance" (13 μg). Control ribosomes (sample 2) were incubated without E3. Other components in the reaction mixtures are described in the legend to table 1. After incubation at 37° for 60 min, samples were mixed with sodium dodecyl sulfate (final conc. 0.2%) and EDTA (final conc. 2 mM), incubated at 37° for 15 min, and applied to an 8% polyacrylamide gel. Electrophoresis was carried out at 200 V for 4 hr. A photograph of the radioautogram of the gels is shown.

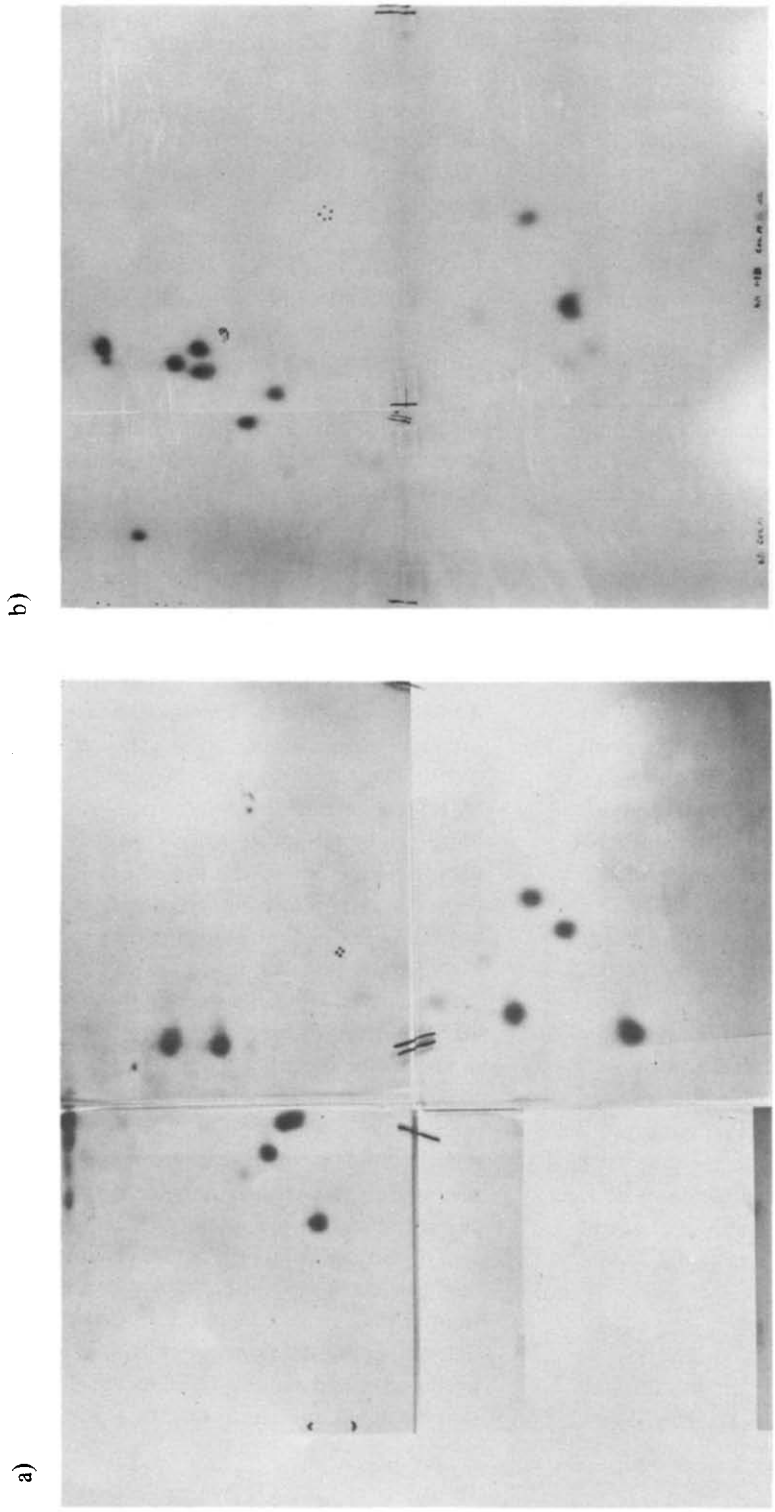

Fig. 2. T1 RNAase fingerprint of E3 fragment RNA's produced from *B. stearothermophilus* ribosomes (a) and *E. coli* ribosomes (b). The [^{32}P]E3-fragment RNA produced after incubation of [^{32}P]30 S subunits (50 μg) and non-radioactive 50 S subunits (100 μg) (both from *B. stearothermophilus*) with E3 (5 μg) was extracted from the polyacrylamide gel, digested with T1 RNAase and fingerprinted according to the methods of Sanger [15, 16]. Electrophoresis was performed in the first dimension, from right to left, at pH 3.5 in 6 M urea, and then in the second dimension, from top to bottom, in 7% formic acid. The fingerprint of the E3-fragment produced from ^{32}P-*E. coli* ribosomes *in vitro* [14] is shown for comparison (b). The experimental procedures were as those used for the *Bacillus* E3-fragment. Following electrophoresis, the sheets were cut into 4 pieces for convenience in exposing them to X-ray film.

of *E. coli* ribosomes [4, 5]. The "immunity substance" by itself does not affect ribosome activity (table 1).

In order to see if the observed inactivation is also due to a specific cleavage of 16 S RNA, we treated ^{32}P-labelled 70 S ribosomes from *B. stearothermophilus* with E3 and looked for small RNA fragments corresponding to the "E3-fragment" found after the treatment of *E. coli* ribosomes with E3 [2, 5]. The RNA extracted from the E3-treated [^{32}P]ribosomes was examined by polyacrylamide gel electrophoresis after sodium dodecyl sulfate treatment. As shown in fig. 1(3), a small RNA fragment was observed which had a faster mobility than transfer RNA's. The fragment was not observed in the absence of E3 or in the presence of both the "immunity substance" and E3. The fragment is derived from 16 S RNA, since the fragment with the same mobility was observed when a mixture of [^{32}P]30 S and non-radioactive 50 S subunits was incubated with E3 (data not shown). The presence of 50 S subunits is required for the E3-induced cleavage of 16 S RNA in the *Bacillus* system in a fashion similar to that reported previously for *E. coli* ribosomes [17, 18].

Fingerprint analysis of the *Bacillus* E3-fragment was performed using [^{32}P]30 S subunits and non-radioactive 50 S subunits with E3, because contamination of [^{32}P]E3-fragments with [^{32}P]transfer RNA was much less in this case compared to the preparations obtained using [^{32}P]70 S ribosomes. The [^{32}P]*Bacillus*-E3-fragment was isolated from the gel, digested with T1 RNAase, and analyzed by the fingerprinting technique of Sanger (fig. 2(a)). The oligonucleotide pattern was different from the pattern of the E3-fragment obtained from *E. coli* ribosomes (fig. 2(b)). The simple oligonucleotide pattern suggests that the fragment is homogeneous. Preliminary experiments (J. Dahlberg and J. Sidikaro, unpublished experiments) have shown that the fragment is derived from the 3′-end of 16 S RNA, probably by a single endonucleolytic cleavage, as in the case of *E. coli*.

4. Discussion

The cleavage of 16 S RNA by E3 *in vitro* is an interesting biochemical reaction. Protein-free 16 S RNA cannot be cleaved by E3 [4, 5]. Moreover, both 30 S and 50 S subunits must be present for the cleavage reaction to occur [17, 18]. In addition, certain antibiotics known to interact with 30 S subunits block the E3-induced cleavage reaction, suggesting that E3 requires a specific ribosome conformation in order to cause the cleavage (see discussion in [19]). Despite such a stringent "substrate specificity", the present results show that E3 is able to cause specific inactivation of bacterial ribosomes which are quite different from *E. coli* ribosomes. 16 S RNA of *B. stearothermophilus* has a base composition different from *E. coli* 16 S RNA [20], and DNA–RNA hybridization-competition experiments indicate a large difference in nucleotide sequence between the two 16 S RNA's [9]. *Bacillus* 30 S ribosomal proteins are also quite different from *E. coli* 30 S ribosomal proteins, as judged by polyacrylamide gel electrophoresis, amino acid composition, and immunochemical techniques ([9, 21, 22] and unpublished experiments in our laboratory). *E. coli* and *B. stearothermophilus* ribosomes certainly have different heat stabilities [9, 23]. Yet, the 16 S RNA in *B. stearothermophilus* can also undergo a specific nucleolytic cleavage. Similarly *Azotobacter* ribosomes can be specifically inactivated by E3. In contrast, cytoplasmic ribosomes from yeast appear to be refractory to E3 (our unpublished experiments). The results indicate that these ribosomes from distantly related bacterial species share a common structural feature necessary for E3 to cause the cleavage reaction. As shown in this paper, the fragments cleaved from *B. stearothermophilus* and *E. coli* 16 S RNA's by E3 are similar in size but are different in nucleotide sequence. However, identification of the exact nucleotide sequence around the cleavage point has not been done in either case. Further comparative studies on the cleavage reaction using these bacterial ribosomes may help identify the common structural feature required for the colicin E3 action.

The narrow killing spectrum is a unique feature of bacteriocins in general [7]. The present results show that, in the case of colicin E3, this specificity is not determined by the target of colicins, the ribosomes in this case, and support the concept that the specificity is based on surface structures in various bacterial species.

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