INACTIVATION OF BACTERIAL RIBOSOMES *IN VIVO* AND *IN VITRO* BY CLOACIN DF13

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1. Introduction

Cloacin DF13 is a bacteriocin produced by *Enterobacter cloacae* DF13 [1]. Treatment of sensitive bacteria with cloacin DF13 results in a leakage of potassium ions and in the inactivation of ribosomes [2]. In this communication we describe that this inactivation can also be accomplished *in vitro*. The ability of cell-free extracts of the sensitive strain *Ent. cloacae* 02 to support poly U-directed poly phenylalanine synthesis is strongly reduced upon incubation with purified cloacin DF13. The inactivation of ribosomes is accompanied by a specific cleavage of 16S rRNA near its 3′-terminus, producing a RNA fragment of 48 nucleotides. An identical fragment is split off upon treatment *in vivo* with cloacin DF13.

Ribosomes from thecloacinogenic strain *Ent. cloacae* DF13 and from an insensitive strain of *Escherichia coli* can also be inactivated *in vitro* and a similar RNA fragment is produced. Ribosomes from the taxonomically unrelated *Bacillus licheniformis* are refractory to the action of cloacin DF13.

2. Materials and methods

Cloacin DF13 was obtained from *Enterobacter cloacae* DF13 by induction with mitomycin C and purified by precipitation with ammonium sulphate and chromatography on QAE-Sephadex [3].

Broth-grown cells of the sensitive strain *Ent. cloacae* 02, the cloacinogenic strain *Ent. cloacae* DF13, which is immune to cloacin-action, and the insensitive strains *E. coli* MRE 600, *B. licheniformis* and *Saccharomyces carlsbergensis* S74, N.C.Y.C were used for the preparation of cell-free extracts (S-30), ribosomes and 'supernatant factors' [2]. ^{32}P-labeled ribosomes were prepared from cells grown in a low-phosphate medium containing 0.05 M Tris–HCl (pH 7.3), 0.04 M NH_4Cl, 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.02 mM $FeSO_4 \cdot 7H_2O$, 0.5% glucose, 20 μg histidine/ml and 100 mg Standard Bouillon (Oxoid) in the presence of 0.1 mCi [^{32}P]orthophosphate per ml of medium (carrier free, Philips Duphar, Petten, The Netherlands). For growth of *B. licheniformis* the medium was supplemented with 0.03 M KCl, 6.0 mM glutamic acid and another 100 mg Standard Bouillon. ^{32}P-labeled cells were lysed according to the method of Godson and Sinsheimer [4].

For isolation and 'fingerprinting' of the RNA-fragment, the fragment was extracted from the 10% polyacrylamide gel in 0.01 M Tris–HCl (pH 7.0) supplemented with 1 M NaCl, dialyzed against distilled water and lyophilized. Digestion with T_1-ribonuclease (Sankyo Co. Ltd., Tokyo, Japan) was performed in 0.01 M Tris–HCl (pH 7.5), 1.0 mM EDTA for 30 min at 37°C at enzyme/substrate ratio of 1:20. The digestion products were separated in two dimensions by electrophoresis according to Sanger et al. [5]. Sequence analysis of the oligonucleotides was performed as described previously [6].

Table 1
Influence of cloacin DF13 on the poly U-directed polyphenylalanine synthesizing activity of ribosomes from different species.

Expt.	Source of ribosomes	Source of 'supernatant factors'	Cloacin DF13	Incorporation activity (cpm)	(%)
1	*Ent. cloacae* 02	*Ent. cloacae* 02	–	28.956	100
			+	7.241	25
		Ent. cloacae DF13	–	26.336	91
			+	25.476	88
2	*Ent. cloacae* DF13	*Ent. cloacae* DF13	–	34.584	100
			+	35.123	102
		Ent. cloacae 02	–	31.576	91
			+	8.423	24
3	*Ent. Cloacae* 02 treated*	*Ent. cloacae* 02 treated*	–	3.458	12
			+	3.521	12
4	*E. coli*	*E. coli*	–	36.377	100
			+	10.845	30
5	*B. licheniformis*	*B. licheniformis*	–	10.420	100
			+	11.736	113
6	*S. carlsbergensis*	*S. carlsbergensis*	–	6.431	100
			+	6.279	98

2.5 A_{260} units of ribosomes in TMK-buffer (0.01 M Tris–HCl pH 7.8, 0.01 M magnesium acetate and 0.06 M KCl) were mixed with 10 μg of purified cloacin DF13 in TMK-buffer and supplemented with 'supernatant factors' so that the mixture represents the state identical to the crude extracts (S-30). The polyphenylalanine synthesizing activity of the mixtures was then assayed as described previously [2]. Specific activity of phenylalanine was 477 mCi/m mol (The Radiochemical Centre, Amersham, England).

* Isolated from cells which have been treated *in vivo* for 30 min with an excess of crude cloacin DF13.

3. Results and discussion

The poly U-directed polyphenylalanine synthesizing activity of ribosomes from the sensitive strain *Ent. cloacae* 02 can be inhibited drastically by purified cloacin DF13 (table 1, expt. 1). No inhibition is observed when the same ribosomes are supplemented with the 'supernatant factors' from the cloacinogenic strain or when crude preparations of cloacin DF13 are used [2]. This suggests the presence of an inhibitor of cloacin DF13-action in cloacinogenic cells; the nature of this inhibitor will be described more fully in a separate report. Consistent with these observations, purified cloacin DF13 was found to be inactive in a cell-free protein synthesizing system prepared from *Ent. cloacae* DF13; inhibition by cloacin DF13 is seen, however, when 'supernatant factors' from the sensitive strain are used (table 1, expt. 2). Obviously, ribosomes from sensitive as well as immune cells can be inactivated by cloacin DF13 *in vitro*.

Ribosomes from *Ent. cloacae* 02 treated *in vivo* with cloacin DF13 have been found to be unable to support protein synthesis in a cell-free system [2] and the addition of purified cloacin DF13 causes no further inhibition (table 1, expt. 3). This suggests that the mechanism of inactivation of ribosomes *in vivo* is the same as that occurring *in vitro*.

To test the specificity of this inactivation, cell-free protein synthesizing systems were derived from a number of organisms, which are insensitive to the action of cloacin DF13 *in vivo*. It appears that ribosomes of *E. coli*, a genus belonging to the same family of bacteria as *Enterobacter*, can be inactivated *in vitro* by cloacin DF13, while ribosomes of the taxonomically unrelated *B. licheniformis* and the eukaryotic 80S ribosomes of *S. carlsbergensis* are refractory to the action of cloacin DF13 (table 1, expts. 4, 5 and 6).

As far as can be judged from analysis by polyacrylamide gel electrophoresis and column chromatography [2], no difference exists in the ribosomal proteins

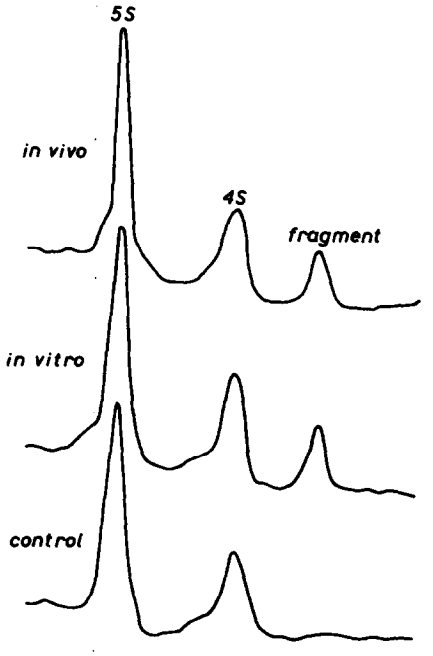

Fig. 1. Polyacrylamide gel electrophoresis of cloacin DF13-fragment RNA produced *in vivo* and *in vitro*: a) Exponentially growing cells were treated with an excess of crude cloacin DF13. After 45 min of incubation at 37°C the ribosomes were isolated; b) 5 A_{260} units of ribosomes in TMK-buffer were incubated with 10 μg purified cloacin DF13 for 30 min at 37°C. In both cases sodium dodecylsulphate (final concn. 1%) was added before extraction of the RNA with phenol. Samples were then analyzed on 9 cm long 10% polyacrylamide gels for 3.5 hr at 2 mA per gel [7]. The figure shows tracings of the gels made with a Joyce-Loebl U.V. scanner.

from control and *in vivo* inactivated ribosomes. The 16S rRNA of inactivated ribosomes exhibits however a slightly enhanced electrophoretic mobility in composite polyacrylamide-agarose gels, suggesting a change in this RNA molecule (experiment not shown). Analysis of the low molecular weight RNA's of *in vivo* inactivated 70S ribosomes on 10% polyacrylamide gels shows that in addition to 5S rRNA an RNA fragment, with a mobility slightly greater than that of tRNA, is present (cf. fig. 1). A similar fragment was extracted from 70S ribosomes of *Ent. cloacae* or *E. coli* upon treatment with cloacin DF13 *in vitro*, whether the relevant 'supernatant factors' were present or not. No fragment could be extracted from ribosomes of *B. licheniformis* or *S. carlsbergensis*. The appearance of this RNA fragment, which probably is derived from

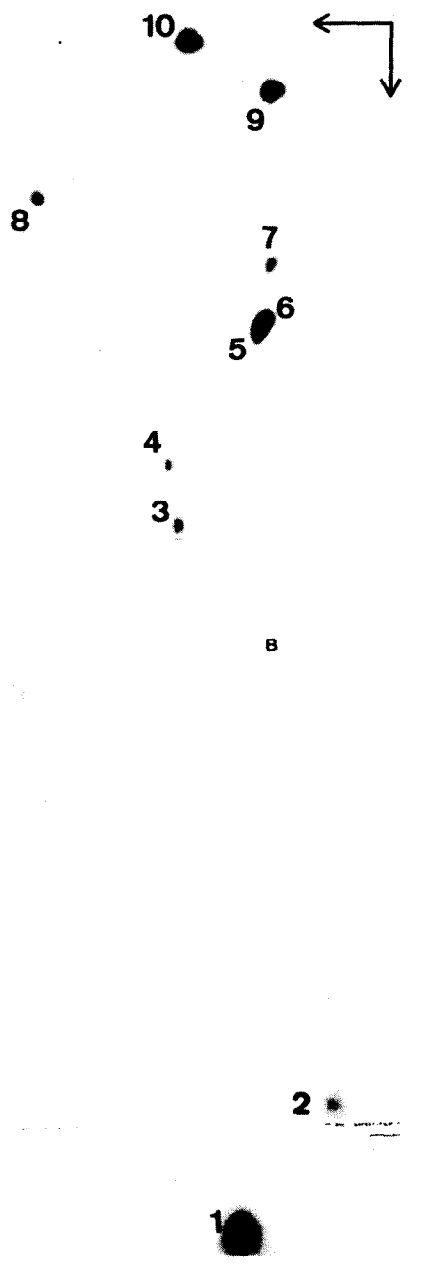

Fig. 2. Two-dimensional separation of the products obtained by digestion with T_1-ribonuclease of the RNA fragment produced upon incubation of 70 S ribosomes of *E. coli* with purified cloacin DF13. 5 μg of ^{32}P-labeled RNA fragment (1.2×10^6 cpm) plus 15 μg carrier RNA were digested with T_1-ribonuclease and the products were separated on cellulose acetate (pH 3.5, 7 M urea) and DEAE-cellulose paper (7% formic acid). After radioautography, all spots were excised and counted according to the method of Clausen [8]. The numbering of the spots corresponds to that in table 2. (B is the position of the blue marker; the first dimension is from right to left, the second one downwards).

the 16S rRNA, fully parallels the inactivation of the ribosomes.

To elucidate the origin of this RNA fragment *in vivo* as well as *in vitro*, ribosomes were used from cells labeled with [^{32}P]orthophosphate. After gel electrophoresis the RNA fragment was isolated from the gel and subjected to digestion with T_1-ribonuclease. The digestion products were separated by electrophoresis in two dimensions (cf. fig. 2). The 'fingerprints' of the fragments isolated from ribosomes of different origin were completely identical as indicated by base composition of the digestion products.

Sequence analysis was performed on all oligonucleotides in a 'fingerprint' of the RNA fragment derived from cloacin DF13-treated ribosomes of *E. coli*. The results are summarised in table 2. Spot no. 9 contains a 3′-OH terminus and is identical to the 3′-terminal oligonucleotide of 16S rRNA as has been reported by Ehresmann et al. [9]. Also the oligonucleotides no. 5 and no. 7 have unequivocally been localized near the 3′-terminus of 16S rRNA [9, 10]. Oligonucleotide no. 10 has the same base sequence as oligonucleotide no. 9 except for the 3′-terminal adenosine residue. This homology and also the observation that the molar yield of spot no. 10 varies at expense of that of spot no. 9, indicates spot no. 10 to be a degradation product of spot no. 9. The results justify the conclusion that the RNA fragment is derived from 16S rRNA by a specific cleavage at 48 nucleotides from its 3′-terminus. The sequence data are consistent with the primary structure of this part of the 16S rRNA molecule as given by Fellner et al. [10]. These data also suggest that the base sequences at the 3′-termini of 16S rRNA's from *E. coli* and *Ent. cloacae* are identical.

Our results parallel the observations with colicin E3, a bacteriocin produced by *E. coli*. Colicin E3, however, is different from cloacin DF13 in amino acid composition [3, 11], and is inactive on our indicator strains. Unlike colicin E3, cloacin DF13 induces a leakage of potassium ions, a decrease in cellular ATP level and is inactive under strictly anaerobic conditions [12].

However, both bacteriocins have a similar effect on protein biosynthesis [13]. Colicin E3 also induces *in vitro* inactivation of ribosomes accompanied by the cleavage of 16S rRNA at the 3′-terminus [14]. Cloacin DF13 appears to produce an identical fragment. Its specificity towards bacterial ribosomes *in vitro* is a little different: ribosomes of *Bacillus*, which are significantly different from *E. coli* ribosomes [15], can be inactivated by colicin E3 [16] but not by cloacin DF13.

Table 2
Oligonucleotides found after digestion with T_1-ribonuclease of a small RNA fragment in cloacin DF13-treated ribosomes of *Escherichia coli*.

Spot no.*	Sequence
1	G_p (6 residues)
2	C–G_p
3	U–C–G_p
4	U–A–G_p
5	m_2^6A–m_2^6A–C–C–U–G_p**
6	U–A–A–C–C–G_p
7	(mU, A, A)–C–A–A–G_p***
8	U–U–G_p
9	A–U–C–(C, U–C, A–C, U–U–C)–A_{OH}
10	A–U–C–(C, U–C, A–C, U–U–C)$_p$

* The numbering refers to fig. 2.
** In alkaline digests m_2^6A–m_2^6A$_p$ was found.
*** mU is a 'fast U' on electrophoresis on Whatman 3 MM at pH 3.5.

The presented data show that the inactivation of ribosomes by cloacin DF13 is always associated by a specific cleavage of 16S rRNA near the 3′-terminus, suggesting this to be the cause of the inactivation.

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