RELEASE OF THE PERIPLASMIC RIBONUCLEASE I INTO THE MEDIUM FROM *ESCHERICHIA COLI* TREATED WITH THE MEMBRANE-ACTIVE POLYPEPTIDE ANTIBIOTIC POLYMYXIN B

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1. Introduction

Evidence for the periplasmic or surface localisation of enzymes in gram negative bacteria is based on the following observations: (1) selective release of enzymes (e.g. ribonuclease) during spheroplast formation [1,2]; (2) selective release of enzymes (e.g. ribonuclease I) by the osmotic shock procedure [3]; (3) inactivation of these proteins by specifically designed inhibitors whose active groups are able to penetrate the cell envelope but not the cytoplasm [4]; (4) electronmicroscopic localisation by means of histochemical methods [5]. Nothing, however, is known about the mechanism by which these enzymes are bound to the cell. In this communication, we present evidence that the periplasmic ribonuclease I (EC 2.7.7.h) and other proteins are selectively and almost quantitatively released from *Escherichia coli* by polymyxin B. Since the basic polypeptide antibiotic polymyxin B acts on susceptible microorganisms by electrostatic interactions of its free aminogroups with acidic phosphate residues of cell wall and membrane phospholipids [6], the reported data strongly suggest that these lipids play an important role in binding the periplasmic proteins to the cell wall. The results have been orally presented [7].

2. Methods

A wildtype *E. coli* was used [8]. Cells were grown with constant shaking at 37° in a medium of the following composition: meat extract, 1 g; peptone from meat, 10 g; NaCl, 5 g per l of deionized water; pH 7.0. The ribonuclease I-activity was determined according to Neu and Heppel [9]. β-Galactosidase was measured in induced cells as described by Malamy and Horecker [1]. The protein content was estimated by the Lowry procedure [10]. The osmotic shock was performed as described by Nossal and Heppel [3]. Ribosomes containing ^{14}C-labeled RNA were prepared from ^{14}C-uracil grown cells of the ribonuclease I-lacking *E. coli* MRE 600 [11] using the procedure of Kurland [12]. Heptose was assayed for by a modified Dische procedure [13] with synthetic L-glycero-D-mannoheptose [14] as standard. Polyacrylamide electrophoresis was performed at pH 8.9 using a gel containing 7.5% acrylamide [15] in a DESAGA vertical gel plate electrophoresis apparatus. All experiments were done in plastic tubes and with plastic pipettes in order to avoid binding of ribonuclease I [9] and polymyxin B onto glass. Polymyxin B sulfate (sterile powder) was generously supplied by Pfizer GmbH (Karlsruhe, Germany).

3. Results

Fig. 1 demonstrates that little of the cellular RNA is degraded in *E. coli* having a polymyxin B-destroyed permeability barrier (8 μg polymyxin B/ml are sufficient to kill more than 99.9% of the cells), whereas the RNA of externally added ribosomes is mostly disintegrated. This degradation is dependent on the amount of polymyxin B added,

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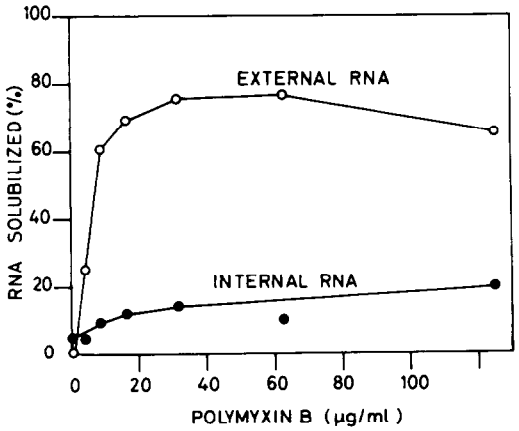

Fig. 1. Depolymerization of ribosomal RNA in polymyxin B-treated cells of *Escherichia coli* (● - - - ●) and in externally added ribosomes (○ - - - ○). The incubation mixture contained early stationary phase cells (0.09 mg protein) in 0.2 ml of 0.14 M NaCl - 0.001 M EDTA, pH 7.5, 5 μl of ribosomes (0.6 absorbance units, 260 nm), and 0.01 ml of polymyxin B. For the determination of internal RNA degradation, ^{14}C-uracil grown cells (55,000 cpm) and unlabeled *E. coli* MRE 600 ribosomes were used. For the determination of externally degraded ribosomal RNA unlabeled cells and ^{14}C-uracil labeled *E. coli* MRE 600 ribosomes (33,000 cpm) were mixed. Incubation was 40 min at 37°. The reaction was stopped by addition of ice cold 5% trichloroacetic acid. Acid insoluble counts were collected by filtering the samples through nitrocellulose filters (0.45 μm pore size) and the remaining radioactivity was determined by liquid scintillation spectrometry of the dried filters in a toluene based scintillation liquid. In terms of absorbance units, the amount of externally added ribosomes was equal to the amount of ribosomes in the cells.

polymyxin B itself having no ribonuclease activity. Since ribonuclease I is the only known enzyme in *E. coli* which is able to degrade ribosomal RNA into acid soluble products in the presence of ethylenediaminetetraacetate (EDTA), the observed activity must be due to the ribonuclease I. This is further substantiated by the lack of this activity in *E. coli* MRE 600, a ribonuclease I-less strain [11]. That the observed ribonuclease I-activity is almost quantitatively in the medium and not at the surface of polymyxin-treated cells, is shown in table 1. This ribonuclease release was complete after 4 min of incubation. From the presented data it is clear that the release is specific, cytoplasmic enzymes (β-galactosidase) being associated with the sedimented cells. The

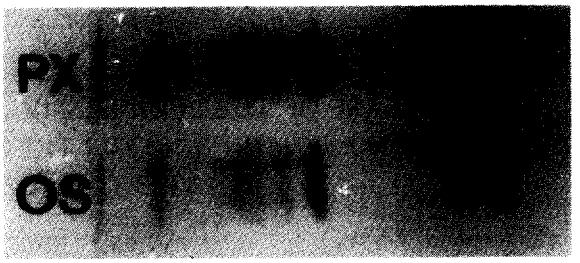

Fig. 2. Polyacrylamide electrophoresis of the osmotic shock fluid (OS) and of the polymyxin B supernatant (PX) of *Escherichia coli*. The material was taken from the experiments described in table 1. About 250 μg of protein were applied to the gel. The start position is at the left.

release of lipopolysaccharide (LPS), measured as heptose, is probably due to the presence of EDTA [16] since periplasmic proteins are released in the absence of EDTA from *Salmonella typhimurium* by polymyxin B without loss of LPS (M.Teuber, unpublished results). The amount of protein released is in good agreement with data reported for osmotic shock fluids or spheroplast supernatants [2,3]. The low protein content of the osmotic shock fluid in our experiments is due to the fact that the cells were not sufficiently preconditioned by washing with ice cold Tris-buffer [3]. It should be emphasized, however, that the specific activity of ribonuclease I is nearly the same in the shock fluid and the polymyxin supernatant. In fig. 2, the protein patterns obtained by polyacrylamide electrophoresis of the osmotic shock fluid [3] and of the polymyxin supernatant are shown. The striking similarity of these patterns is good evidence that other periplasmic proteins are released in addition to ribonuclease I. The assignment of these protein bands to known enzymic activities is currently under investigation.

4. Discussion

The reported results clearly demonstrate the usefulness of polymyxin B as an important and convenient tool for cell wall and membrane studies [17,18]. It now should be possible to determine the factors which are responsible for the binding of the periplasmic proteins to the cell wall. On the basis

Table 1
Protein, heptose, ribonuclease I and β-galactosidase content of different fractions from *E. coli*.

Fraction	Protein	Heptose	Ribonuclease I		β-Galactosidase	
	(mg/ml)	(μg/ml)	(u/ml)	spec. act. (u/mg prot.)	(u/ml)	spec. act. (u/mg prot.)
Untreated cells	0.86	2.25	0.87	1.01	3.01	3.49
Polymyxin supernatant	0.12	1.52	0.73	6.08	0.01	0.08
Osmotic shock fluid	0.03	ND	0.23	7.7	ND	ND

The polymyxin supernatant was obtained from early stationary phase cells by incubation with 30 μg polymyxin B/ml for 20 min at 37° in 0.14 M NaCl - 0.001 M EDTA, pH 7.5. Cells were removed by centrifugation at 20,000 *g*. 1 ribonuclease I unit = 0.1 absorbance unit (260 nm) becoming acid soluble per min at 37°, measured with sRNA from *E. coli* B (Calbiochem) as substrate. 1 β-galactosidase unit = 1 μmole *O*-nitrophenyl-β-D-galactopyranoside hydrolysed per min at 24°. ND = not determined.

of the known mode of action of polymyxin B [6, see introduction] and of data presented in this communication, it may well be the phospholipids and not the lipopolysaccharide of the cell wall-membrane which play the essential role for keeping these proteins bound to the cell. In addition, the described observations provide new evidence for the periplasmic or surface localisation of the ribonuclease I *in vivo*. The system is now being studied in detail.

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