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RESEARCH ARTICLE

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Colicin S8 export: extracellular and cytoplasmic colicin are different

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Abstract The properties of colicin S8 are different for the cytoplasmic, periplasmic and extracellular protein. Interactions with its specific receptors reflect this. Active cell extracts separate into a non-anionic along with an anionic fraction by DEAE-Sephacell chromatography. Previously, we have purified cell-associated colicin S8 as an aggregation of highly related polypeptides; cytoplasmic colicin S8 seems to be post-translationally processed into an aggregation of polypeptides of molecular mass ranging from 45,000 Da to 60,000 Da. We suggest that a conformational change to colicin S8 may occur related to the export process.

Keywords *Escherichia coli* · Colicin export · Colicin S8 · Bacteriocins · Specific receptors

Introduction

Colicin S8, a bacteriocin, is synthesized when *Escherichia coli* cells harboring Col S8 plasmid are treated with agents such as mitomycin C or ultraviolet light [8, 26]. The protein thus synthesized is exported across both inner and outer membranes and released into the medium [25]. Colicins do not contain a signal sequence and their release is allowed by colicin lysis proteins [5], which might use the general export pathway of the cell that uses signal peptides to direct proteins to the cell envelope [25]. These proteins increase envelope permeability, and colicins are released from colicinogenic cells as water-soluble polypeptides [3]. However, in order to be transferred across a membrane, a soluble protein might alter its shape to suit distinct cell environments. There are reports of multiple forms of colicins. For example, colicins K [10] and V [15] were purified as a complex of protein with bacterial antigen O. More recently, colicins A, E1 and Ia [13, 14, 19] have been found in vitro in at least two different membrane-associated states. In addition, it has been suggested that multiple contacts with periplasmic-exposed Tol proteins is a prerequisite for group A colicin translocation [13]. Thus, the existence of colicins in more than one conformation seems to be a common phenomenon.

Colicins that are members of the ion-channel-forming family of colicins [17] are proteins that undergo a transition from soluble to transmembrane configuration. In fact, a conformational change has been proposed for colicin A upon release from the cytoplasm to the medium [14]. Channel-forming colicins undergo a remarkable series of conformational gyrations during their journey from the extracellular milieu to the periplasmic membrane [11]. Conformational changes of the same sort have been demonstrated for the alfa-toxin of *Staphylococcus aureus*, which, like colicin A, can form channels in biological membranes [20]. An improved model for pore-forming colicin import proposes unfolding of the colicin initiated very early in the translocation process by its binding to the receptor [7].

We previously reported purification of the colicin S8 that remains cell-associated after induction of producing cells [4], and demonstrated that at least two electronegatively different proteins with colicin activity exist, both being composed of extremely related polypeptides. The experiments presented here suggest a difference in conformation between cytoplasmic, periplasmic and extracellular colicin S8. We suggest that a conformational change of colicin S8 might be involved in the export of colicin S8. Thus, our previous finding that colicin S8 exists as an aggregation of polypeptides could be of physiological significance.

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Materials and methods

Preparation of colicin active fractions

E. coli K-12 VE831 [8] was used as an indicator strain and for control experiments, and VE832 (VE831 containing pColS8) was used as a source for colicin. Bacteria were grown at 37°C in LB medium [21]. Where appropriate, colicin detection was in solid medium. For colicin production, growth was carried out with high-speed rotational shaking during incubation using 2-l flattened flasks of 25 cm bottom diameter containing 1-l culture. Induction was at an absorbance of 0.4 at 600 nm, with mitomycin C added at 0.4 μg/ml (final concentration). Induced cells were harvested by centrifugation (6,000 *g* for 10 min) from 1 l cultures. After resuspending in 200 ml washing buffer (0.01 M Tris-HCl pH 8.0) the cells were gently shaking for 3 h at 37 C in the dark. Cells were again harvested, and the supernatant saved as ML (wash). Cold-shock treatment was as follows: pelleted cells were resuspended (at 40 ml/g bacteria) in 0.05 M EDTA, 20% saccharose, 0.03 M Tris-HCl, pH 7 buffer. After incubation at room temperature for 15 min and centrifugation, cells were immediately treated with 1:1 (v/v) 0.5 mM $MgCl_2$ at 0°C for 15 min. Cells were pelleted, supernatant was saved, cold shock treatment repeated once and supernatants pooled and saved at −20°C as CS (cold-shock) (this protocol was obtained from P. Garcia in our laboratory). Finally, cells were resuspended in 100 ml 0.01 M Tris-HCl pH 8 and sonicated for three intervals of 60 s using an MSE probe sonicator. Absorbance was reduced to 10%. Cellular debris was separated by centrifugation at 5,000 *g* for 15 min and the supernatant was sterilized with chloroform and saved as SON (sonicate). Frozen SON (−20°C) remained active for several months. A cocktail of protease inhibitors: 10 mM leupeptin, 50 mM phenylmethylsulfonyl fluoride and 100 mM N-a-*p*-tosyl-L-lysine chloromethyl ketone (Sigma, St. Louis. Mo.) was used in all preparations.

Titration and chromatography

Stab test, quantitative and qualitative titers were carried out as described previously [9]. Internal colicin was tested with strain BCM97 [8].

Chromatographic separation on DEAE-Sephacell column

DEAE-Sephacell (10×200 mm, Pharmacia) was equilibrated with 20 mM Tris-HCl, pH 8.0 buffer and loaded with colicin active fractions. After elution, a 0–1 M NaCl gradient was applied (continuously or discontinuously) to the column at 30 ml/h at 4°C. The effluent was monitored by the absorbance at 280 nm using a UV-M detector.

Resistant isogenic strains isolation

E. coli K12 C600, C10 strain from Nágel de Zwáig [22] was used. It was laid over stabs of producing colicin strain BS81a [8] and also over spots of several colicin active fractions. After 24 or 48 h, colonies growing within growth inhibition "halos" were picked up, purified and tested for both T6 bacteriophage and colicin K resistance. Those isolates showing no "halo" and sensitivity to T6 bacteriophage were classified as "tolerant" [23] while no "halo" and T6 resistance corresponded to "resistant".

Statistical analysis

Cluster analysis with Euclidean relative distance and group average linkage was used for displaying homogeneous groups and the differences between them [12]. TWINSPAN, a FORTRAM program to arrange multivariate data in an ordered two-way table by classification of the individuals and their attributes. This analysis was performed by M. Fariñas, at the ICAE (Instituto de Ciencias Ambientales y Ecológicas, Venezuela).

Maxicell experiments

Maxicells were prepared using the method of Sancar et al. [24]. Strains were colS8::Tn3 mutants obtained as described by Garcia and Dyke [8].

Results and discussion

Induction and release of colicin S8

We have shown before [4] that, under the conditions used, optimum induction of colicin S8 is obtained following 60 s exposure to UV-irradiation or addition of mitomycin C (0.4 μg/ml). Once the cells are induced and incubated in the dark at 37°C, production proceeds at nearly the same rate for at least 3 h. Under these conditions, the culture supernatant contained 42% of the total colicin produced (Table 1). A significant amount remained cell-associated (58%), as was determined after sonication of the cells. Most (26%) of the cell-associated colicin could be released by cold-shock treatment compared to 16% released previously by washing the cells. These results indicate that colicin S8 has a periplasmic stage on its way out of the cell. When these cells were sonicated, we found that a small amount of colicin S8 had remained inside (16%).

Chromatographic separation on a DEAE-Sephacell column

Colicin S8 active fractions from different cell compartments can be separated into two major activity peaks after cationic exchange chromatography (Fig. 1). The first active fraction did not associate with the DEAE-Sephacell matrix and thus eluted with the elution buffer. This fraction appears to predominate in ML, where there is 10 times the amount present in SON and 100 times that in CS. In fact, it predominates in SON if we consider the origin of the ML fraction: induced cells continuously producing for 3 h. The second active fraction was eluted from the column in the presence of 0.2 M NaCl. Most of it is present in ML and CS preparations, as only a residual 10% was detected in SON. Both fractions, ML and CS, could contain the colicin released from the cytoplasm. The non-anionic colicin (eluted with the elution buffer) must be the synthesized colicin S8 as it is the main component in the SON fraction.

Biological heterogeneity of active fractions

Colicin S8 shares receptors with colicin K and T6 [1]. We selected some resistant isogenic isolates (see Materials

Table 1 Colicin S8 activity distribution in cell fractions. *ML* Wash fraction,*CS* cold-shock fraction, *SON* sonicate fraction

Fraction	Volume (ml)	Total proteins (mg)	Colicin activity (total U)	Specific activity	Total colicin produced (%)
Supernatant	1,000	1,920	4.74×10^{12}	2.4×10^{8}	42
ML	100	5,200	1.82×10^{12}	3.5×10^{8}	16
CS	100	3,400	2.96×10^{12}	8.7×10^{8}	26
SON	100	5,700	1.82×10^{12}	3.2×10^{8}	16

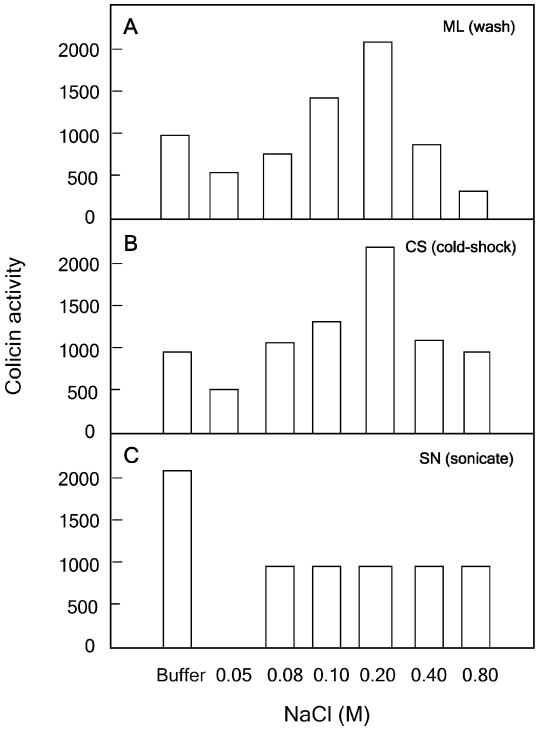

Fig. 1A–C Cationic exchange chromatography of colicin S8 active fractions. Colicin active fractions obtained from cellular cultures were each loaded onto a DEAE-Sephacell column and eluted first with buffer and then with a discontinuous 0–1 M NaCl gradient. **A** *ML* (wash), **B** *CS* (cold-shock), and **C** *SON* (sonicate). Colicin activity is given in arbitrary units according to the qualitative test described elsewhere [9]

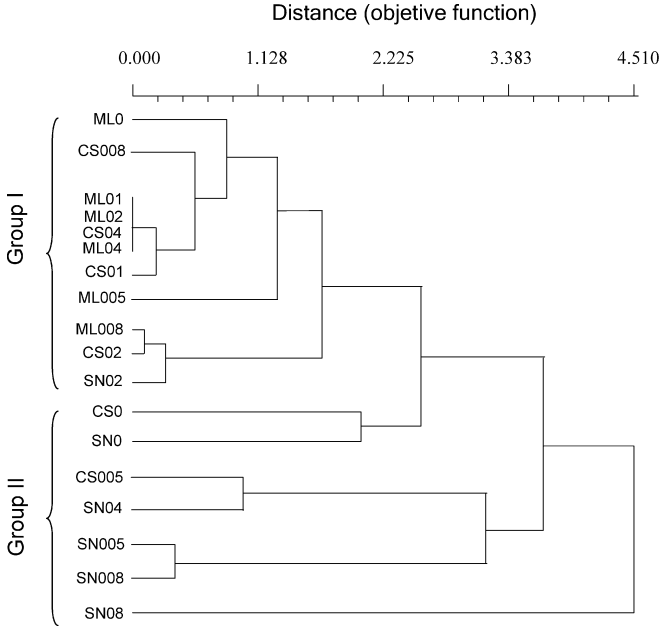

Fig. 2 Dendrogram of subfractions according to their activity towards resistant isogenic strains. Colicin active subfractions collected from the cationic exchange chromatography of colicin S8 active fractions were tested against 17 independently isolated resistant strains. A relative euclidean, group average dendrogram of subfractions was constructed. Group I is composed of type 1 and type 1-2 isolates, and group II of type 2 isolates. *ML* Wash fraction, *CS* cold-shock fraction, *SN* sonicate fraction (see text). NaCl concentration: *0* 0 M, *005* 0.05 M, *008* 0.08 M, *01* 0.1 M, *02* 0.2 M, *04* 0.4 M, *08* 0.8 M

and methods) that showed three types of behavior: "no halo", "turbid halo" and "half turbid-half transparent halo". The "no halo" phenotype could correspond to resistance (no receptors) or tolerance (receptors but no uptake system to enter the external membrane). Thus, specific surface receptors and specific components of the uptake system used by colicin S8 must be modified in order to respond differently to colicin S8 active fractions.

Cellular subfractions obtained after chromatographic separation in DEAE-Sephacell were tested for their activity against a selection of 17 strains from the resistant isogenic isolates. Subfractions and isolates were sorted out by a cluster analysis according to similarities in their response. Isolates fall into three different patterns of behavior. The first type of response is shown by those isolates reacting similarly to subfractions from ML and CS: type 1. CS and SON show a different type of response: type 2. There is a third group of isolates showing a mixture of these two patterns: type 1-2. This mixed type 1-2 is probably due to some colicin remaining on the surface after cold shock treatment. Isolates could be classified into two major groups when a dendrogram was produced using these data (Fig. 2): group I looks more homogeneous and is composed of isolates characterized as type 1 and type 1-2, and group II is composed of isolates of type 2. These results suggest one predominant conformation of colicin S8 in ML and CS fractions (exported colicin), with another conformation present in SON (cytoplasmic colicin). These two conformations would differ in electrical charge. In addition, our results indicate that colicin S8 is active prior to export.

Table 2 Activity of internal and external colicin S8 upon incubation with chloroform for the times indicated

pColS8	Inhibition "halo"			
	5 min	10 min	15 min	20 min
col+, lys+, imm+[a]	+	+	+	+
col+, lys–, imm+[b]	–	–	–	+

[a]Strain VE832 containing a wild type colS8 plasmid (see Materials and methods)
[b]BCM97 containing a lysis defective plasmid (see Materials and methods)

Activity of the internal colicin

Mutant BCM97 synthesizes some colicin but does not release it, as it is probably a lysis-deficient mutant (lys^-), according to the insertion location on the genetic map of pColS8 [8]. After long incubation with chloroform, a "halo" of colicin activity is observed (Méndez Ivonne, personal communication), indicating that chloroform has allowed the internal colicin to leave the cell, and that this colicin is totally active (Table 2). Thus, conformational change is not a requirement for colicin S8 activity.

Polypeptides synthesized by the pColS8 plasmid

Minicell preparations of colicin-producing plasmids give a 60 kDa band on SDS-PAGE whereas colicin-deficient mutants do not [8]. A similar experiment was carried out using the maxicell expression system (Fig. 3). The ColS8 DNA expressed several polypeptides of 60, 50, 48 and 45 kDa while ColS8::Tn1 mutants failed to produce any of these. In fact, maxicells and minicells are different expression systems. Two notable properties of maxicells are the absence of the cell wall and the presence of the chromosome along with the plasmid. As in minicells, there is one only replicon: the plasmid. We conclude that: (1) the 50, 48 and 45 kDa polypeptides are post-translational products of the 60 kDa polypeptide, and (2) minicells cannot process the 60 kDa polypeptide because of chromosomal and/or cytoplasmic factors that are either missing or not concentrated enough.

In a previous study [4] we purified colicin S8 as an aggregation of highly related polypeptides of molecular mass ranging from 47,000 Da to 63,000 Da. In the present article we propose that colicin S8 is synthesized as a polypeptide of about 56,000 Da, and that it is active on the membrane in a dimeric form (110,000 Da) and then modified (specifically or unspecifically) by proteolysis, multimer formation and/or conformational changes. We propose either that colicin S8 is synthesized as an active protein and undergoes a conformational change upon release to the medium, or that it is specifically modified inside the cytoplasm to produce exportable colicin S8.

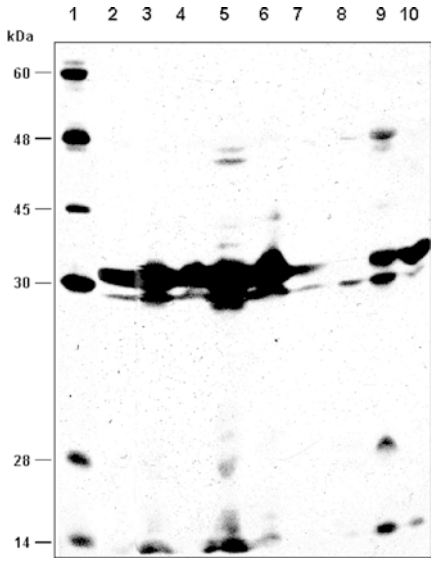

Fig. 3 Polypeptides synthesized by pColS8 in maxicell preparations. Maxicells were prepared and labeled using the method of Sancar et al. [24]. SDS-polyacrylamide gels were prepared according to Tyler and Sherratt [26]. Lanes: *1* Polypeptides synthesized in a maxicell preparation by wt pColS8, *2–7* mutant hybrid plasmids obtained in Garcia and Dyke [8]: col^+, imm^+, Ap^r mutant BCM62 (*2*); col^-, imm^+, Ap^r mutant B12-26-1 (*3*); col^-, imm^+, Ap^r mutant BCM25 (*4*); col^-, imm^-, Ap^r mutant BCM114 (*5*); col^-, imm^+, Ap^r mutant BCM920 (*6*); Δ col, imm^-, Ap^r mutant BCM147 (*7*); *8* col^-, imm^+, Ap^r mutant BCM195; *9* col^+, imm^+, Ap^r mutant B12-5-6; *10* col^+, imm^+, Ap^r mutant BCM213. Molecular weight markers are indicated on the left side. The picture is a composition of the same gel with exposure to X-ray film for 12 h for *lane 1* and 48 h for all the others

In the first case, a model has been presented regarding colicin A [16], a monomer with hydrophilic residues exposed on the molecule surface turning partly inside out before forming dimers with the hydrophobic residues on the outer surface of cytoplasmic colicin. Extracellular colicin does not form dimers. Our second suggestion is that a conformational/structural change may be caused by processing of cytoplasmic colicin S8. Differences in the export mechanism could form the basis for this model. In contrast to colicins, which differ widely in their structure and mode of action, colicin lysis proteins are highly homologous in gene organization, primary structure and function [18]. However, some differences have been shown between two lysis proteins: CalE1 (colicin E1 lysis protein) and CelA (colicin A lysis protein) [2]. CelA is strongly dependent on both the SecA and the SecY (PrlA) proteins for its own export and for promoting export of other proteins, whereas Cal seems to be less dependent upon these two proteins. Thus, differences in the mechanism of colicin export may be related to conformational structural changes of the colicin molecule. The change may be similar in nature to the unfolding of colicin A during its translocation into the cell [6]. Our proposal here is that there is a difference between cytoplasmic and extracellular colicin S8, and that that difference could be of physiological significance.

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