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Production of an *E. coli* Toxin Protein; Colicin A in *E. coli* Using an Inducible System

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Colicins are bacterial toxins that kill *Escherichia coli* and related cells; their mode of action is of interest in protein import and toxicology. Colicins translocate across the *E. coli* outer membrane and periplasm by interacting with several receptors. This translocation process involves interaction of the colicin with the outer membrane porin OmpF and subsequently with the integral membrane protein TolA. The N-terminal domain of colicin N is involved in the import process. Our aim was to produce a large quantity of colicin A for functional and structural studies. It is a prerequisite to have a correctly folded and stable protein for these studies. The commonly utilised expression system uses the Lex A promoter, which requires induction with toxic mitomycin C, though the yield is low. Here we present the production of an E. coli toxin and its immunity protein in *E. coli* using a safe inducible promoter.

Key Words: Protein production, Toxin, colicins, Immunity protein.

Introduction

The ultimate aim of biochemistry is to decipher how events occur inside of living cells or organisms. The living cell must also be regarded as the ideal model system for any biochemical application, as most physiological targets are found inside living cells. The cell envelope of the Gram-negative bacterium *Escherichia coli* is a complex structure and many of the proteins found in the bacterial envelope are involved in import mechanisms. A variety of bacterial toxins have taken advantage of some of these envelope proteins to enter the bacteria. Pore-forming toxins present the important challenge of the translocation process, and the study of the membrane insertion mechanism of pore-forming colicins has also become a very important subject. How these proteins pass through or integrate into membranes is still one of the fundamental unsolved problems in biochemistry.

Colicins are plasmid-encoded toxin proteins produced by immune *E. coli* that are active against sensitive *E. coli* and closely related cells. Their toxic activities are of various types; some colicins form ion

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channels in the cytoplasmic membrane of sensitive cells, while others act as nucleases that degrade DNA or 16s RNA in the cytoplasm, and one, colicin M, inhibits the biosynthesis of murein[1]. Their toxic activities against target cells are known to occur in three distinct stages. The first step is receptor recognition and binding, where colicins bind to a specific receptor at the cell surface[2]. This is followed by the translocation step, where colicins cross both the outer membrane and periplasm to reach their cellular target [1−2]. The final stage is the killing action, where colicins exert their lethal effects either by **1**. Formation of a pore in the cytoplasmic membrane[3], **2**. DNAse or RNAse activity or **3**. Inhibition of murein biosynthesis in the cytoplasm (for review see reference 1).

Colicins have three linearly organised functional domains, each domain being implicated in a specific stage of colicin activity. The central domain (R-domain) is responsible for the receptor-binding activity. The N-terminal domain (T-domain) is involved in translocation. The C-terminal domain (P-domain) carries the lethal activity, and this domain either forms a voltage gated pore in the cytoplasmic membrane or digests nucleic acids in the cytoplasm[4−6].

Figure 1. The domain structure of colicins. These domains (T, R and P) deviate from the domain sizes of colicin N determined by El-Kouhen[7]. The N-terminal domain is involved in the translocation step and is called the T domain. The central domain of colicin N is termed the R domain and it contains information required to bind to the receptor. The C-terminal section is required for the lethal activity of pore formation and it is called the P domain.

Colicin A and colicin N are members of a group A (Tol dependent) pore-forming colicin whose target receptor is the *E. coli* outer membrane protein OmpF[8]. Colicin translocation requires three members of the Tol locus: Tol A, Tol Q and Tol R[10]. Tol Q and Tol R are integral membrane proteins of the *E. coli* inner membrane and there is no evidence that they reach across the periplasm to the outer membrane. However, Tol A is thought to span the periplasm by means of its extended central domain (Tol A-II), which links the N-terminal Tol A-1 domain in the cytoplasmic membrane to the C-terminal Tol A-III domain[11]. The interaction between Tol A-III and the N-terminal domain of colicins is thought to be the principle event of translocation[12]. All the translocation domains of colicins interact with periplasmic receptors and can be divided into two groups: those that bind to the Tol proteins and those that bind to the Ton proteins. This division was identified early in the history of colicin research and the Tol proteins were named because mutations on Tol proteins render cells "tolerant to colicin". This is still, along with resistance to filamentous phage, the clearest phenotype of Tol protein mutants[10]. The Ton proteins such as Ton B, on the other hand, have a clear role in active transport across the outer membrane[23]. The tol system consists of the proteins Tol A, B, Q and R. Tol A is analogous to Ton B in that it consists of a cytoplasmic transmembrane domain (domain I) joined to a C-terminal, possibly outer membrane domain (domain III) by an extended region of repetitive secondary structure (domain II)[24]. In the case of Tol A, this linker region is proposed to be α-helical, but it may fold back upon itself several times rather than being a straight rod-like structure. If the structure were straight this 230 amino acid residue section would be much longer than the width of the periplasm[25]. Group A colicins are known to bind to Tol A-III via their N-terminal translocation domains. In the case of colicin A, the area of interaction has been defined using deletion mutants[26]. For colicin N , site-directed mutagenesis and biophysical measurements such as calorimetry and fluorescence were used on the isolated N-terminal domain to define a series of residues required for Tol A binding[9]. Colicin N

is composed of a largely unstructured T- domain linked by a glycine-rich sequence to a central R-domain containing a six-stranded β-sheet structure. The R-domain is connected to the P-domain (a 10 α-helical structure) by means of a 65Å α-helix[7−9]. While receptor binding and pore formation have been extensively studied, much remains unknown about the translocation step.

The main defence against pore-forming colicins for *E. coli* strains are the colicin immunity proteins encoded by the immunity gene. Immunity proteins for pore-forming colicins are integral cytoplasmic membrane proteins with three or four predicted transmembrane helices and are highly specific for a particular colicin. It is predicted that the inactivation of pore-forming colicin by their immunity proteins occurs in the membrane just prior to channel opening[18]. It has been shown that the immunity protein of colicin A interacts with the pore forming domain in the inner membrane[8]. The immunity protein thus does not appear to stop the colicin from binding and translocating across the outer membrane, but prevents it from forming a pore once it is inserted into the cytoplasmic membrane[23].

The production and purification of recombinant proteins is an important step in the biochemical analysis and functional characterisation of proteins. A widely used strategy for protein isolation involves expression of the protein in bacterial cells. The production of *E. coli* toxins in *E. coli* often leads to low-level production and extensive proteolysis that limit the application of this approach to stable toxin proteins. The natural expression system of colicin A must be induced by mitomycin C, which is a potent carcinogen. In the course of our attempts to express this colicin without using mitomycin C induction, we encountered significant difficulties, especially low-expression yield and proteolysis of colicin in *E. coli.*

To solve these problems, a high-level expression system was constructed and the overproduction of colicin A in *E. coli* is presented in this paper. This system additionally contains an immunity protein gene against colicin A and allows for the production of both colicin A and an immunity protein against it.

Experimental

Bacterial Strains and Plasmids

Construction of pISACOLA was completed in two steps. First, DNA encoding the colicin A and immunity protein was amplified using two oligonucleotides with 18bp matching sequence to colicin A and immunity proteins.

1. ColAXhoI-Sense: TTTTTCTCGAGCATGCCTGGATTTAATTATGG
2. ImmAMluI-reverse: TTTTTTTTACGCGTCTCCGGTAACGATAGATG

Plasmid pCA31 carrying wild-type colicin A and immunity protein was used as a template [27]. Second, the PCR product (DNA fragment encoding colicin A and immunity protein) was gel purified and ligated into purified, digested vector plasmid pET8C [28] using *Xho* I and *Mlu* I restriction sites. The pET8C vector introduces a methionine, six histidines and two serine (NH2-MHHHHHHSS-) linkers at the N-terminus of any gene inserted between *Xho* I and *Mlu* I restriction sites [28]. This 6 His and 2 Ser linker facilitates purification by nickel affinity chromatography [13]. The resulting histidine-tagged colicin A protein is under the control of a T7 promotor on the vector. This final construct (pISACOLA) was used initially to transform *E. coli* DH5α cells with ampicillin selection. Successful transformants were selected on the basis of ampicillin resistance, mini-prep restriction digest analysis or analytical PCR, and a subsequent plasmid preparation was used for DNA sequencing or to transform *E. coli* BL21 (DE3) cells. DNA sequencing of this plasmid showed that both colicin A and immunity protein were correctly inserted. A circular plasmid map of this

construct is shown in Figure 2.

Protein Purification

E. coli BL21(DE3) cells were transformed with pISACOLA and grown in 3 ml O/N cultures of LB medium containing ampicillin (200 μg/ml). A 3 ml O/N culture was introduced into 500 ml of LB containing ampicillin (200 μg/ml) and grown at 37 ∘C. The cells were induced at OD_{600} 0.8-0.9 with isopropyl β-D-thiogalactopyranoside (1 mM IPTG), and grown for a further 3 h. The cells were harvested and resuspended in 20 mM phosphate, 300 mM NaCl, pH 7.4, buffer containing RNAse (20 μg/ml) and DNAse (20 μg/ml). The cells were lysed by French press and the supernatant was obtained by centrifugation at 40,000 rpm (125,800 x g) in a Beckman ultra-centrifuge with a 45Ti rotor for 1 h at 4 ∘C. The fusion protein was found to be soluble, with none remaining in the cell membrane pellet. The N-terminal 6 X Histidine-tag facilitated purification of the fusion by means of a Qiagen Ni-NTA (nickel nitrilotriacetic acid) affinity column[14]. The toxin protein was washed onto the column with 50 mM phosphate, 300 mM NaCl, pH 8.0 buffer, additionally washed with the same buffer containing 30 mM imidazole, and eluted in 300 mM imidazole, pH 7.0 using 5 ml of buffer for each fraction. The toxin was analysed for purity by 12% SDS-PAGE (Figure 3). A sample of was taken from each fraction and after the addition of 5 μl of sample buffer, the 10 μl protein samples loaded on to the 12% SDS-PAGE gel. SDS-PAGE was carried out as described by Laemmli[20]; the reservoir buffer was 0.125 M Tris, 0.96 M Glycine and 0.5% SDS pH 8.3 and the sample buffer was 0.25 M Tris, 8% SDS, 0.02% Bromphenol blue and 40% Glycerol pH 6.8. Electrophoresis was carried out at a constant current of 24 mA After electrophoresis, proteins in the gel were visualised by staining with 0.20% Coomassie Brilliant Blue in a solution of 30% isopropanol and 10% acetic acid and then destained overnight in aqueous acetic acid/isopropanol (10% acetic acid, 10% isopropanol). The protein concentration of purified colicin A toxin was determined from ultraviolet absorption at 280 nm, using the extinction coefficient for A_{280}: 51,350 $M^{-1}cm^{-1}$, calculated from the number of tryptophan and tyrosine residues in the sequence. The validity of calculated A_{280} as an accurate measure of protein concentration has been verified previously [19].

Circular Dichroism (CD) Spectroscopy

Far-UV-CD spectroscopy was carried out on a Jobin-Yvon CD6 spectra polarimeter. Measurements were performed at 20 °C using a 0.2 cm path length cuvette (Hellma), and protein concentration of 0.5 mg/ml. The protein was in 50 mM phosphate, 300 mM NaCl, pH 7.4, buffer, and this was also used for baseline subtraction. The CD signal measured was mean residue $\Delta\varepsilon$ ($M^{-1}cm^{-1}$).

Colicin Toxicity

An *in vivo* cell survival assay using fluorescent probes has been previously described[31]. *E. coli* BE3000 cells were grown in LB medium to an OD_{600} of 0.5 (5×10^8 cells/ml), washed in water and reconstituted in 10 mM phosphate, pH 7.4, to a concentration of 5×10^9 cells/ml, and stored on ice. Measurements of toxicity were determined by the fluorescence colicin depolarisation of *E. coli* cells[29−30] followed by a change in ANS fluorescence using an SLM 8100 spectrofluorometer operating in ratio mode with spectral band-widths of 8 nm for both excitation and emission, excitation wavelength was 360 nm and emission was measured between 400 and 550 nm. For measurement, 0.3 ml of cells was mixed with 2.7 ml of phosphate buffer, and 150 μl

of ANS (8-anilino-1-napthalenesulphonic acid) was added after 100 s (1 μM) and left to equilibrate. Five microlitres of colicin A, 10 μg/ml was added after 300 s. The fluorescence increase was monitored for 2000 s.

Results and Discussion

Details on the construction of pISACOLA plasmid are given in Figure 2 and the experimental section. The pET8c vector was used to clone the genes encoding colicin A and immunity protein of colicin A. This vector is under the tight control of the T7 promoter that facilitates the expression of toxic proteins[13−15,16]. Our construct also includes an N-terminal poly-histidine tag (6 histidines) for simple and efficient protein purification. DNA sequencing of this constructed plasmid (Figure 2) confirmed that the desired inserts were successfully cloned into a pET8C expression vector and also that the constructs were in the correct reading frame.

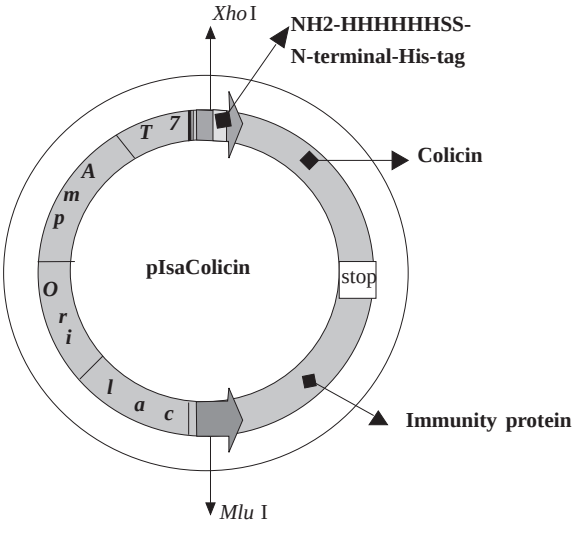

Figure 2. Circular plasmid map of the pISAColA constructed to produce colicin A and its immunity protein. *Xho* I and *Mlu* I restriction enzymes were used and their sites are shown. Amp: ampicillin resistance gene and T7: promoter from T7 phage, Ori: origin of replication, lac: lac operon.

The fusion protein was purified from the *E. coli* cell lysate as described in the experimental section. Ni-NTA agarose resin was used to purify the fusion protein from the supernatant of *E. coli* cell lysate after ultra-centrifugation. Proteins containing the histidine affinity tag bind to Ni-NTA agarose resin with a greater affinity than other *E. coli* proteins. Non-specifically bound proteins were washed through the column without affecting the binding of His-tagged fusion protein. Elution of the fusion protein from the column was achieved by imidazole, which competes with the His tag for interaction with the Ni-NTA resin.

Eluted samples were analysed on SDS-PAGE and showed a strong protein band with a molecular mass of around 63 kDa. The calculated molecular weight of the fusion protein using ProtParam tool (Swiss Institute of Bioinformatics) was 62,992 Da which is very close to the experimental molecular mass, extinction coefficient of this fusion at 280 nm is 51,350 $M^{-1}cm^{-1}$ and its theoretical pI is 7.78 (these data were calculated using ProtParam tool, its web address is http://expasy.proteome.org.au/cgi-bin/protparam). The protein

yields were calculated using UV absorbance at 280 nm. One litre of BL21 (DE3) *E. coli* cell culture gave more than 30 mg (35-40 mg) of highly pure toxin protein.

Figure 3. Colicin A expression in *E. coli* BL21 (DE3) cells. 1: Supernatant after ultra-centrifugation (before Ni-NTA resin). 2: Flow through from the Ni-NTA resin. 3: Elution fraction 1 in 300 mM imidazole, 50 mM phosphate and 300 mM NaCl. 4: Molecular weight markers (66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20.1 kDa). 5: Elution fraction 2. 6: Elution fraction 3. 7: Elution fraction 4. 8: Elution fraction 5.

The event central to the translocation process is the interaction of colicin T-domain with Tol A-III. Studies of colicin A have shown that translocation is accelerated in urea, indicating that colicins are likely to unfold to expose the T-domain allowing interaction with Tol A[2]. The structure of the T-domain of any colicin before and after interaction with Tol A is still unknown. Despite obtaining crystals of residues 36-397 of colicin N[6], the final solved structure lacked the N-terminal 90 residues and gave no structural information on the N-terminal domain structure of colicin N (residues 1-66). The only member of the colicin family whose full-length structure is known is colicin Ia[17]. While its pore-forming domain adopts a similar 10 $\alpha-$helical fold to colicin N, its N-terminal domain appears largely unstructured.

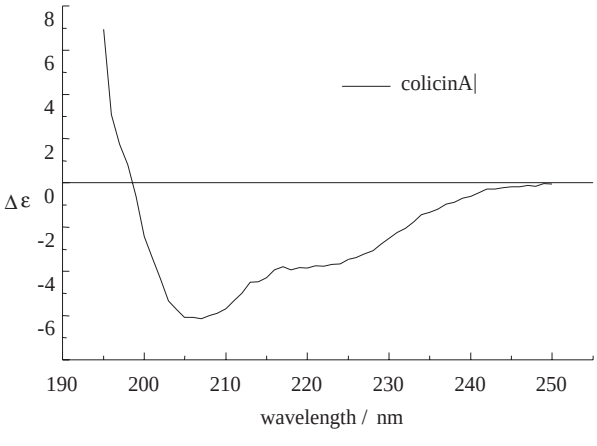

Figure 4. Circular dichroism spectrum of colicin A produced by the T7 promoter system described in the text. Colicin A in at a concentration of 0.5 mg/ml phosphate buffer was placed in a 0.2 mm pathlength cuvette. The spectra were collected at 20 °C in a Jobin-Yvon CD6 spectra polarimeter.

The strategy of the high level production of colicin A is based on the production of two different proteins together in *E. coli*. It is already known that one of them is the colicin A toxin protein and the other one is an immunity protein against the same toxin. The activity of the immunity proteins of the

channel-forming colicins is poorly understood. The channel domain of colicin A is inhibited by its immunity protein through direct interaction in the *E. coli* inner membrane but the ability of a single channel-forming colicin to kill a cell is prevented only by the immunity protein that is coded by the same plasmid[18]. The protective mechanism of the immunity protein is a unique aspect of the interaction of colicins with cell membranes and immunity protein toxin interaction. Immunity proteins recognise their target colicin most probably through direct interaction and inhibit its killing activity, or they may also interact with the inner membrane components of the translocation machinery to prevent the insertion of colicin A into the inner membrane[1].

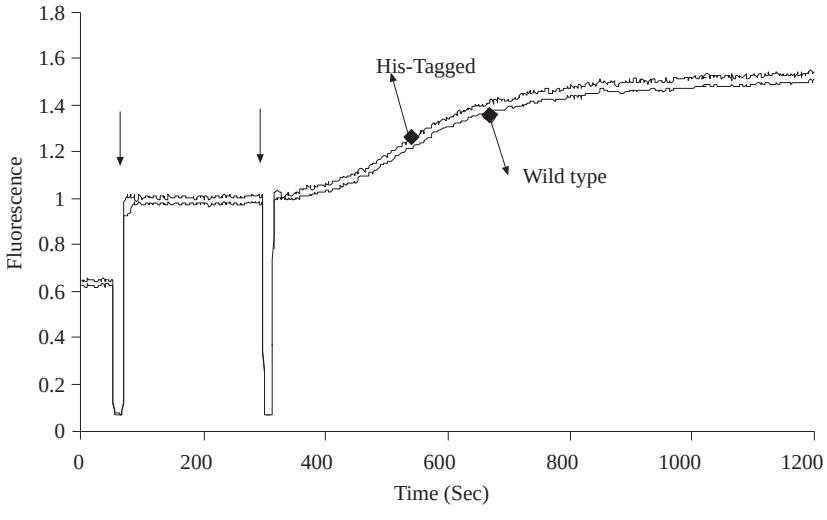

Figure 5. Effect of the addition of full length histidine tagged colicin A and wild-type colicin A on the change in fluorescence of potential sensitive probe ANS. The fluorescence, measured at 480 nm, of the sensitive BE300 cells was allowed to stabilise. ANS added at the first arrow and caused a jump in fluorescence. Both wild-type and histidine tagged recombinant (His-Tagged) colicin A toxin were added at the second arrow at a ratio of 400 colicins per cell. After translocation the colicin A toxin causes membrane depolarisation by channel formation in the inner membrane of *E. coli*.

As mentioned earlier, the gene construct consists of two proteins originating from different parent molecules. The plasmid we describe here (pISACOLA) permits the production of colicin A under the tight control of the T7 promoter system. It was prepared to allow constitutive expression of the col A immunity protein and thus obtain a high level of the inducible expression of colicin A. The addition of the histidine tag (6 His) was used for the purification of this fusion protein by a single affinity chromatography step, but it also reduces proteolysis of the flexible N terminal region of this protein. The pET8c construct gives high yields of stable protein and it is a critical step for the crystal structure studies. Structural and biophysical knowledge of this protein will aid our understanding of colicin translocation through the cell envelope of Gram-negative bacteria. The secondary structure of colicin A was determined by circular dichroism spectroscopy. The results (circular dichroism spectrum of recombinant colicin A gave the ratio of 38% helix, 26% beta and other 36%) indicate the toxin to be a fully folded α helix rich protein in agreement with measurements previously published for the wild-type protein[21]. The toxicity tests show that there was no difference between wild-type colicin A and our recombinant colicin A toxin. The toxicity of full length histidine tagged colicin A was confirmed using the potential sensitive fluorescent probe ANS[29]. After receptor binding and translocation the colicin causes membrane, depolarisation by channel formation in the inner membrane as

shown in Figure 5. This behaviour of His-tagged colicin A is identical to that native non His-tagged colicin A toxin[30].

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