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Colicin A and colicin E1 lysis proteins differ in their dependence on *secA* and *secY* gene products

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The export of colicin A and of colicin E1 is not equally affected in both *secA* and *secY* mutants of *Escherichia coli*: release of colicin A occurs slowly while that of colicin E1 is blocked. Processing and functioning of Cal, the colicin A lysis protein, seem to be slightly or not at all modified in these mutants, whereas synthesis and assembly of CelA, the colicin E1 lysis protein, are highly inhibited. These variations observed in the dependence of the two lysis proteins on *secA* and *secY* gene products are interpreted as being either the cause or the consequence of the differences observed in their rate of biogenesis.

Lysis protein; Colicin; SecA protein; SecY protein; Protein secretion; *Escherichia coli*

1. INTRODUCTION

Colicin lysis proteins are lipopeptides encoded by various colicinogenic plasmids present in some strains of *Escherichia coli* [1]. They are membrane proteins, being preferentially found in the outer membrane [2,3,4]. Like all bacterial lipoproteins, they are synthesized as precursors. The precursor is modified by a diglyceride before being matured by cleavage of the signal peptide by the signal peptidase II specific for lipoproteins [5]. Thus they could be expected to use the general export pathway of the cell that uses the signal peptide to direct proteins to the cell envelope [6].

On the other hand, colicin lysis proteins are secretion factors, as they allow colicins that do not contain a signal sequence to cross both inner and outer membranes and to be released in the spent medium [7]. This release has been shown not to be colicin-specific [8]. Many proteins are released together with the colicin. The colicin lysis proteins have been used to promote export of engineered proteins [9,10,11].

The general export machinery of the cell includes various gene products [6]. The proteins encoded by the *secA* and *secY* (*prlA*) genes have been shown to be essential for protein export across the cytoplasmic membrane [12,13]. Both are required for the translocation of the murein-lipoprotein, Lpp [14,15], that is processed and matured as a colicin lysis protein [1]. However, small proteins such as the M13 coat protein assemble spontaneously into the cytoplasmic membrane without the aid of any protein [16,17]. It seemed interesting therefore to study the role of *secA* and *secY* gene products in the assembly and functioning of the colicin lysis proteins. The study was performed with thermosensitive mutants on two lysis proteins. The colicin A and colicin E1 lysis proteins were chosen as they differ in their kinetics of processing and maturation, despite extensive sequence similarity [18]. The colicin E1 lysis protein, CelA, is processed and matured as fast as Lpp, whereas the colicin A lysis protein, Cal, is matured slowly and is observed together with every intermediate form. It is shown that the two lysis proteins differ also greatly in their dependence on the SecA and SecY proteins: CelA, the colicin E1 lysis protein 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, the colicin A lysis protein, seems to be less dependent upon these two proteins.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

Escherichia coli K12 strains HJM114 (F' *lac, pro* [*lacpro*]) and its derivatives CJ105 (*secAts*) and CJ107 (*secYts*) [14], were obtained from A. Kuhn by V. Geli.

Plasmids pColA9, pAT1, pCK4 [19] and pColE1 [20] have been described. Plasmid pCTB113 was from J.M. Masson [11].

2.2. *Growth conditions*

Strains were grown in LB medium or in M9 medium supplemented with thiamin (1 μg/ml), lactate (0.4%, v/v), Casamino acids (0.01%, w/v), and required amino acids (20 μg/ml). Cultures were incubated with good aeration. Mitomycin C (Sigma) was used at 300 ng/ml. IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma) was used at 1 mM.

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2.3. *Radioactive labelling*

Cultures in M9 medium were grown at 30°C to an OD600 of 0.8. They were then shifted to 42°C for 60 min before being induced for lysis protein expression. After 30 min of induction, cells were labelled with [^{35}S]methionine (1000 Ci/mmol) at 20 μCi/ml (1 μCi=37 kBq) for a given time and chased with unlabelled methionine (50 μg/ml). Labelling with [^{35}S]cysteine (600 Ci/mmol) at 30 μCi/ml (1 μCi=37 kBq) was done in the presence of unlabelled methionine (50 μg/ml) and chased with unlabelled cysteine (1 μg/ml) in the presence of threonine (10 μg/ml) to diminish the transient amino-acid starvation caused by cysteine [20]. Radioactive products were purchased from Amersham Corp. In routine experiments, 10 μl samples of the cultures were taken at various times after the chase, mixed with 10 μl of sample buffer, heated at 96°C and analysed on urea-SDS-polyacrylamide gels. Gels were electrophoresed and treated as described previously [19].

2.4. *Immunoprecipitation*

Immunoprecipitations were performed as described [21].

3. RESULTS

3.1. *Different effects of SecA and SecY inactivation on the export of colicin A and of colicin E1*

The role of the SecA and SecY proteins on colicin release was first checked. *E. coli* wild-type HJM114 and its thermosensitive *secA* and *secY* mutants carrying either pColA9 or pColE1 were grown in LB at 30°C and shifted to non-permissive temperature before being induced with mitomycin C for colicin and lysis protein expression. After 3 h of induction, samples were removed and centrifuged; pellets and supernatants were analysed on SDS gels to control the effect of the mutations on colicin export (Fig. 1). Release of colicin A was observed in the three strains. The amount of colicin present in the cells was, however, higher in the mutants than in the wild type. In contrast, export of colicin E1 was strikingly altered in both mutants: little colicin was released by the *secA* cells and none by the *secY* mutant.

Quasi-lysis of the cultures that occurs with release of colicin was obtained with the *secA* and the *secY* mutants carrying pColA9 and not with the same strains carrying pColE1 (not shown).

3.2. *Processing of Cal is unmodified after either SecA or SecY inactivation*

The effect of the *secA* and *secY* mutations on Cal synthesis was next studied. It could not be done in cells carrying pColA9 as induction in synthetic medium at the non-permissive temperature was rapidly lethal for the *secY* mutant and, more slowly, for the *secA* cells. Then cells carrying either pAT1 which encodes Cal and a truncated colicin A called protein AT1, or pCK4 which encodes Cal under the control of the *lac* promoter were used.

Cells were induced at the restrictive temperature. They were pulse-labelled for 1 min with [^{35}S]methionine and analysed on urea-SDS gels during the chase. The slow processing of Cal previously reported [18,19] was observed in every strain (Fig. 2A). After 1 min of chase, the unmodified (pCal) and the modified (pCal^m) precursor forms of Cal were preferentially labelled. After 30 min, pCal^m was chased into the Cal mature form and the signal peptide, which both accumulated. The kinetics of Cal synthesis and processing were similar in the wild type as in the two thermosensitive mutants, indicating that neither the SecA nor the SecY protein seemed to be required for Cal biogenesis and membrane insertion.

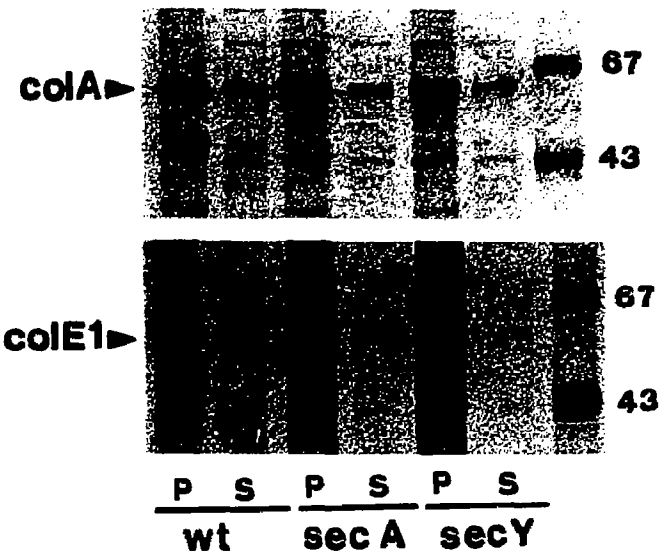

Fig. 1. Export of colicin A and E1 in the *secA* and the *secY* mutants. Cells HJM114 (wt), CJ105 (secA) and CJ107 (secY) carrying either pColA9 (top) or pColE1 (bottom) were grown in LB at 30°C. They were shifted at 42°C 30 min before induction with mitomycin C. After 3 h of induction, samples were withdrawn and centrifuged. Pellets (P) and supernatants (S) were analysed on SDS gels. The relevant part of the Coomassie-stained gel is shown. The molecular weights of protein markers are indicated on the right.

The protein AT1 induced with Cal is truncated at the time of release by 2 kDa by the OmpT protease of the producing cells [22]. Cleavage of protein AT1 was observed with the same timing in wild-type and mutant cells, indicating that the release of proteins provoked by Cal occurred simultaneously in every strain (Fig. 2A).

Labelling of cells carrying pCK4 after induction by IPTG gave the same results (Fig. 2B). No alteration of the rate of Cal maturation was observed in either mutant except for the amount of pCal^m synthesized that appears to be less in mutant cells than in wild type.

3.3. *Synthesis of CelA is altered by inactivation of both SecA and SecY*

Strains carrying pColE1 induced with mitomycin C at 42°C were labelled with [^{35}S]cysteine during 1 min and analysed after 1 min of chase. Incorporation of radioactive cysteine was similar in the 3 isogenic strains, but colicin E1 was less stable in M9 medium in mutant strains than in wild-type (not shown). The mature form of CelA was well detected in wild-type cells but less in the *secA* conditional mutant and almost not in the *secY* cells (Fig. 3A).

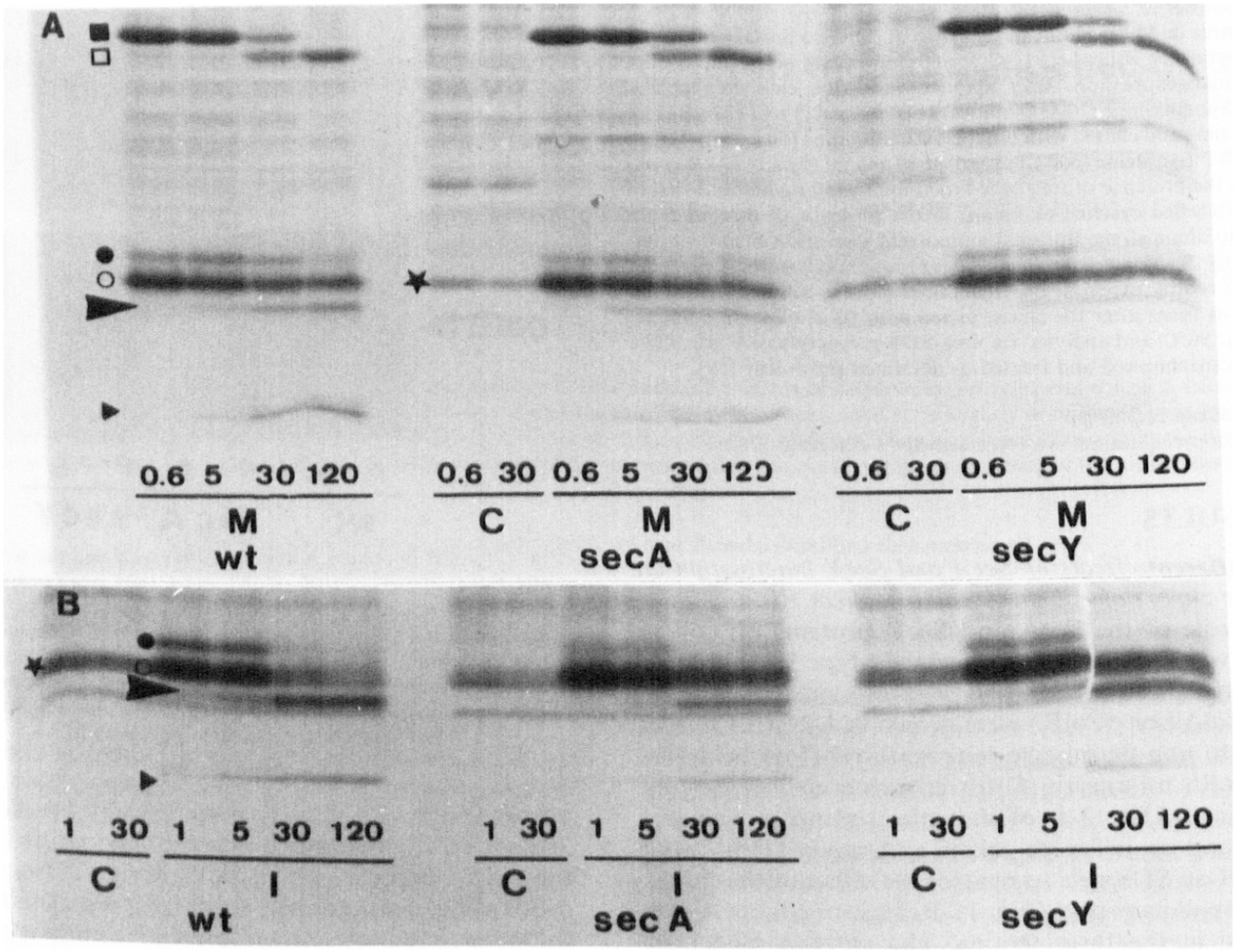

Fig. 2. Synthesis of Cal in the *secA* and *secY* mutants. (A) Cells of HJM114 (pAT1) (wt), CJ105 (pAT1) (secA) and CJ107 (pAT1) (secY) were grown in M9 medium at 30°C and brought to 42°C before being induced with mitomycin C (M) or not induced (C). After 30 min of induction, they were pulse-labelled with [^{35}S]methionine for 1 min. Aliquots were withdrawn at various times of the chase as indicated in minutes and analysed on urea-SDS gels. (B) Same experiment as above with strains carrying pCK4. Labelling was performed 10 min after induction with IPTG (I). Part of the fluorograms are presented. The various forms of Cal are indicated: precursor form, ○; modified precursor form, ●; mature form, ▶; signal sequence, ➤. The murein-lipoprotein is indicated by a star. The two forms of protein A71 are indicated by squares: intact protein, ■, cleaved protein, □.

The same experiment was performed on cells carrying pCTB113, which contains a synthetic *celA* gene under the control of a *lac* promoter [13]. After induction by IPTG, CelA was weakly detected in the *secA* mutant and badly in the *secY* mutant (Fig. 3B). Despite the strong inhibition of the synthesis of CelA mature form, neither the precursor form nor the modified precursor form of CelA was observed in the induced thermosensitive mutants carrying either pColE1 or pCTB113.

Processing of some precursor proteins which requires the SecA and SecY proteins, was checked as a control of the mutants. Processing of the preOmpA and of the pre-β-lactamase was indeed affected in the two thermosensitive mutants: the precursor form was detected after 1 min of labelling with [^{35}S]methionine in the mutants and not in wild-type cells (Fig. 4) as reported [14,18].

3.4. *Assembly of CelA and Cal after SecA and SecY inactivation*

Protein release induced by lysis proteins is a delayed event, occurring late after synthesis. At this time, the mature form of lysis protein is detected in SDS gels only in samples heated to 96°C before loading [18]. This property, common to outer-membrane proteins, was chosen as a control for the correct assembly and functioning of Cal and CelA. Labelled cells were analysed on gels after 4 h of induction at the non-permissive temperature (Fig. 5). The mature form of Cal was observed in heated samples and was absent from unheated samples of the *sec* mutants as in wild-type. Other intermediate forms of Cal present in induced cells: pCal, pCal^{m} and the signal sequence were unmodified by the heating conditions. In contrast, CelA mature form was detected in both heated and unheated samples of the *sec*

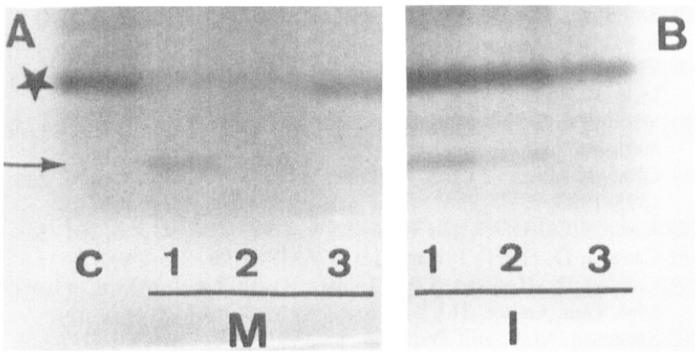

Fig. 3. Synthesis of CelA in the *secA* and *secY* mutants. (A) Cells of HJM114 (pColE1), CJ105 (pColE1) and CJ107 (pColE1) were grown in M9 medium at 30°C and shifted to 42°C before being induced with mitomycin C (M) or not induced (C). After 45 min of induction, they were pulse-labelled with [^{35}S]cysteine for 1 min. Aliquots were withdrawn after 1 min of the chase and analysed on urea-SDS gels. (B) Same experiment as above with strains carrying pCTB113. Labelling was performed 10 min after induction with IPTG (I). Part of the fluorograms is presented. The CelA mature form is indicated with a small arrow. The murein-lipoprotein is indicated by a star. 1: HJM114; 2: CJ105; 3: CJ107.

mutants indicating an incorrect assembly of CelA in the cell envelope.

The same observations were made in cells expressing the lysis proteins after induction by IPTG (not shown).

4. DISCUSSION

The present work demonstrates that dependence upon SecA and SecY proteins for export varies for colicin lysis proteins despite their high level of genetic and sequence homologies. For Cal, the colicin A lysis protein, the *secA* and *secY* gene products do not seem to be important for processing and assembly. In contrast, for CelA, the colicin E1 lysis protein, both gene products are essential for synthesis and functioning.

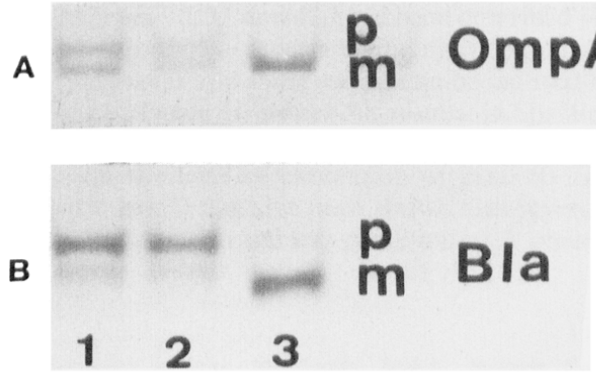

Fig. 4. Synthesis of OmpA and β-lactamase in the *secA* and the *secY* mutants. Cells of HJM114 (pAT1), CJ105 (pAT1) and CJ107 (pAT1) were treated as reported in Fig. 1. After 1 min of labelling with [^{35}S]methionine, samples were removed, immunoprecipitated with specific antisera against (A) OmpA, (B) β-lactamase, and analysed on gels. The relevant part of the fluorograms is shown. Indications are: p, precursor; m, mature form; 1, CJ105; 2, CJ107; 3, HJM114.

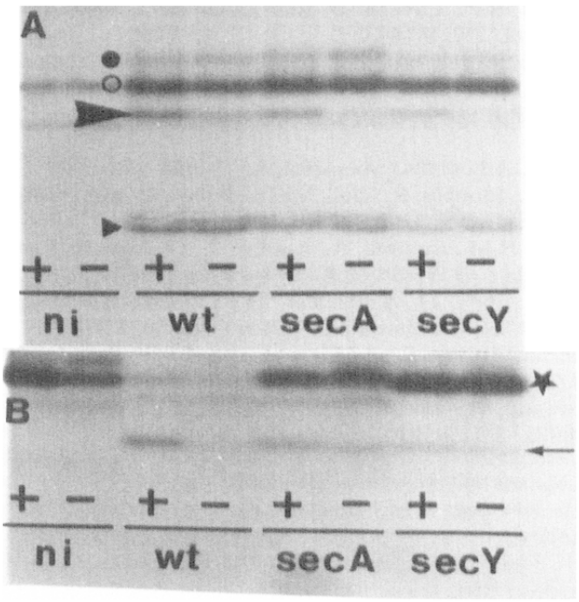

Fig. 5. Assembly of Cal and CelA in the *secA* and *secY* mutants. (A) Cells of HJM114 (pAT1) (wt), CJ105 (pAT1) (secA) and CJ107 (pAT1) (secY) were grown in M9 medium at 30°C and shifted to 42°C before being induced with mitomycin C (M) or not induced (ni). After 30 min of induction, they were pulse-labelled with [^{35}S]methionine for 1 min. Aliquots were withdrawn after 4 h of chase, mixed with sample buffer and heated (+) or unheated (−) before being analysed on urea-SDS gels. (B) Same experiment as above with strains carrying pColE1 and labeling with [^{35}S]cysteine. Part of the fluorograms is presented. Indications are as in Figs. 2 and 3.

The opposite dependence on the SecA and SecY proteins of these two lysis proteins should be related to the differences observed in their rate of synthesis. CelA is rapidly matured as the murein-lipoprotein and, like Lpp, requires both SecA and SecY proteins to be correctly exported and assembled. Cal presents a particular slow processing with accumulation of every intermediate and does not seem to depend strongly on the general export pathway either for its own assembly or to promote secretion of proteins. The dependence on the *secA* and *secY* gene products of the two colicin lysis proteins would be either the cause or, more probably, the consequence of their different mode of processing.

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