Role of Cal, the colicin A lysis protein, in two steps of colicin A release and in the interaction with colicin A–porin complexes

Danièle Cavard

Laboratoire d’Ingénierie des Systèmes Macromoléculaires, Centre National de la Recherche Scientifique, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

Release of colicin A was studied in Escherichia coli cells that differed in expressing the colicin A lysis protein (Cal). Pools of released and unreleased colicin A were harvested throughout colicin A induction. The amount of colicin A in each pool varied with the time of induction, allowing the definition of two sequential steps in colicin A release, one of which was dependent on Cal. Each step of colicin A release was differently affected in cells containing Cal mutants in which the N-terminal cysteine residue was substituted by either proline or threonine, preventing them from being acylated and matured. These Cal mutants were only observed in degP cells, indicating that the DegP protease cleaved the unacylated precursor of Cal. Cal was found in the insoluble fraction of the pools of released and unreleased colicin A together with the hetero-oligomers of colicin A and porins (colicins Au). The biogenesis of colicins Au was studied in temperature-sensitive secA and secY strains and found to be Sec-independent, indicating that they are formed by newly synthesized colicin A binding to mature porins already incorporated in the outer membrane. Cal is a lipoprotein similar to VirB7, a constituent of the type IV secretion system. It would interact with colicins Au to constitute the colicin A export machinery.

INTRODUCTION

Colicins are bactericidal proteins produced by strains of Escherichia coli that harbour a colicinogenic plasmid. The colicin operon is under the control of an SOS promoter and colicins are synthesized in large amounts upon induction by mutagenic agents. Colicins of group A are expressed with a colicin lysis protein and are released into the medium late after synthesis, in contrast to colicins of group B. Colicin lysis proteins are lipopeptides of 28 to 41 residues with highly homologous sequences. They are the unique components needed for colicin secretion. Colicin release is thought not to be mediated by a specific machinery, but rather by loss of envelope integrity as leakage of cellular proteins occurs during colicin A release. The leakage follows activation of the outer membrane phospholipase A (OMPLA), which provokes quasi-lysis of the culture (reviewed by van der Wal et al., 1995).

The colicin A lysis protein (Cal) is responsible for colicin A release. It is synthesized like every lipoprotein as a precursor form (pCal) which is modified (pCalm) before being processed. The mature form of Cal polymerizes and is found in the outer membrane (Cavard, 1991). Colicin A is found in every cell compartment, except in the inner membrane. In the outer membrane, colicin A is complexed with an outer-membrane protein, either a porin or OMPLA. These hetero-oligomers of colicin A, called colicins Au, are stable in urea and in SDS, and dissociate after boiling in SDS, as do the Cal polymers. In most colicins Au, colicin A interacts through its C-terminal domain with either OmpC or OmpF porins (Cavard, 2002). Such an interaction has previously been demonstrated in vitro (Dover et al., 2000). A role for colicins Au in colicin A release has been suggested, but is not obvious since cells devoid of porins and, consequently, of most colicins Au, secrete colicin A. Furthermore, cells that do not express Cal produce colicins Au, although they do not release colicin A (Cavard, 2002).

Whether colicins Au play a role in colicin A secretion, they must interact with Cal. Release of colicin A was studied in strains differing in expressing Cal. Two steps of colicin A release were described, one of which at least was dependent on Cal. Cal was found in extracellular fractions together with colicins Au. It is suggested that colicins Au and Cal interact and are part of a specific machinery for colicin A secretion.

Abbreviations: Cal, mature form of the colicin A lysis protein; colicins Au, heat-labile oligomers of colicin A and outer-membrane proteins; KU, killing unit; LE, lytic extract; pCal, unacylated Cal precursor; pCalm, modified Cal precursor; SE, salt extract; SP, ammonium sulfate precipitation; ts, temperature-sensitive.
METHODS

Bacterial strains and plasmids. E. coli K-12 W3110, W3110 degP, MC4100, UT5600 ompT and UT5600 ompT degP were from the laboratory collection. Plasmid pCoIA9 contains the wild-type colicin A operon. Plasmids pJMM1 contains a colicin A operon in which Tn10 has been inserted upstream of the cal gene. Plasmids pAL16 and pAK31 have been constructed from pCoIA9 to substitute threonine or proline, respectively, for the cysteine residue in position 1 in the mature Cal sequence (Cavard et al., 1987). Plasmid pPC978 contains the virB7 gene of Agrobacterium tumefaciens under control of the lac promoter on the pBluescript plasmid pBCSK + (Berger & Christie, 1994).

Growth conditions. Strains were grown at 37°C in Luria–Bertani (LB) medium with ampicillin (100 µg ml⁻¹) as required. To induce colicin synthesis, mitomycin C, which is a mutagenic agent, was added at 300 ng ml⁻¹ to a culture whose optical density value at 600 nm had been adjusted to 1. Sodium azide (2 mM) was added when necessary at the time of induction.

Colicin A assays. These were performed as described previously (Cavard, 1976). A 100 µl aliquot of a dilution in LB medium of the sample to be assayed was added to 1-3 ml of MC4100 cells grown in LB medium to an OD₆₀₀ value of 0.25. After incubation for 20 min at 37°C with shaking, 100 µl of an SDS solution at 7.5 mg ml⁻¹ was added. The OD₆₀₀ value of the samples was measured 10 min later. The percentage ratio was determined from the OD ratio of colicin-treated and control samples and was converted to killing units (KUs). The number of KUs obtained was multiplied by the dilution factor of the assayed sample and by 3.25 × 10⁸, which corresponds to the number of MC4100 cells added to the assayed sample.

Extraction of released and unreleased colicin A. After mitomycin C addition, the culture in LB medium (50 ml) was incubated for various times, before being centrifuged at 12,500 g. The supernatant was added to 60 % ammonium sulfate (390 g l⁻¹). The precipitate was harvested by centrifugation and re-dissolved in 5 ml of 20 mM Tris/HCl (pH 6-8), 10 % (v/v) glycerol (SP). The cell pellet was suspended in 4 ml of 10 mM Tris/HCl (pH 6-8), 500 mM NaCl. After 30 min at room temperature, cells were re-centrifuged and incubated in 3 ml of water for 30 min before being centrifuged again. The NaCl and water supernatants were combined and designated the salt extract (SE). The final cell pellet was suspended in 8 M urea in 10 mM Tris/HCl (pH 6-8), 1 mM EDTA, 100 µg lysozyme. After 1 h at room temperature, 2 ml of 10 mM Tris/HCl (pH 8) was added. Cells were incubated for 2 h at 4°C before centrifugation, then washed once with 1 ml of 10 mM Tris (pH 6-8). Both supernatants were mixed and corresponded to the lytic extract (LE).

Urea and trypsin treatment. SP, SE and LE were dialysed in 10 mM Tris/HCl (pH 6-8) before centrifugation at 75 000 g (TLA 100.2 rotor; Beckman) for 30 min at 4°C. The pellets were resuspended in 8 M urea in 10 mM Tris/HCl (pH 6-8), vortexed, incubated at room temperature for 30 min and washed once. The pellets were resuspended in 100 mM Tris/HCl (pH 8) to which 100 µg bovine trypsin ml⁻¹ (Sigma) was added. The suspension was incubated at 37°C for 30 min. Trypsin was then inactivated by the addition of 1 mM PMSF.

Protein analysis. Proteins were resolved by SDS-PAGE, as described previously (Cavard, 2002). The SDS-polyacrylamide gels contained either 11 or 6 % acrylamide. Urea/SDS-polyacrylamide gel contained 7 % acrylamide and 7 M urea. To detect Cal, analyses were performed in Tricine/SDS gels (Schägger & von Jagow, 1987), with a continuous running gel of 13-5 % acrylamide. Proteins were transferred onto nitrocellulose filters (200 nm pore size; Schleicher & Schuell). The membranes were incubated first with monoclonal antibody (mAb) 1C11 to colicin A at 1:5000, or with polyclonal antibodies (pAbs) to OmpF (Cavard, 2002) or to Cal (Howard & Lindsay, 1992) at 1:1000, and, then, with phosphatase-conjugated anti-mouse or anti-rabbit antibodies as appropriate. Protein bands were visualized by incubation with Nitro Blue Tetrazolium in the presence of MgCl₂ and 5-bromo-4-chloro-3-indolyl phosphate di-sodium salt.

RESULTS

Pools of released and unreleased colicin A

To study colicin A secretion, colicin A synthesis was induced in cells expressing or not the cal gene, i.e. in W3110 cells carrying either pCoIA9 or pJMM1. Released and unreleased colicin A were collected and their amounts evaluated throughout induction. Released colicin A was obtained by centrifugation of the culture. The culture supernatant was analysed before (medium) or after SP.

To obtain unreleased colicin A, the pellet of induced cells was suspended in buffer containing 1 M NaCl and centrifuged to collect the SE, as described by Herschman & Helsinki (1967) and Schwartz & Helsinki (1971). However, the amount of colicin A recovered increased when the NaCl concentration of the buffer was decreased in such a way that it was higher in the absence than in the presence of NaCl (not shown). The centrifuged cells were then incubated in 1/10 volume of 10 mM Tris/HCl (pH 6-8) containing 0·5 M NaCl, centrifuged and washed in water before re-centrifugation. The NaCl and water washes were combined and represented the SE. The cells in the pellet were then treated with EDTA/lysozyme and the resulting sphaero-plasts were lysed at low osmotic pressure. The lysed cells were centrifuged and washed once. The pooled supernatants corresponded to the LE. The final pellets containing mainly DNA and cell envelopes were discarded.

Each pool obtained from induced W3110(pCoIA9) cells was analysed on SDS-polyacrylamide gels stained with Coomassie brilliant blue (Fig. 1). The amount of colicin A varied significantly in each pool with the time of induction, as expected. At zero time of induction, there was no colicin A in any of the pools. After 90 min induction, there was a significant amount of colicin A in the LE and a low amount in the SE. After 180 min induction, most colicin A was found in the SP extract, low amounts in the SE and traces in the LE. In W3110(pJMM1) cells devoid of Cal, colicin A was only found in the LE at all times after induction. Thus, with the help of Cal, colicin A synthesized in the cell cytoplasm moved first to a cell location where it could be extracted by washing with buffer, before being released into the medium.

The amount of colicin A collected throughout the induction period in the SP, SE and LE pools was assessed by performing colicin A assays (Table 1). At zero time of induction, the number of colicin A KUs was around the limit of detection. After 90 min induction, in W3110(pCoIA9)
minor ones constitute a group of oligomeric species with apparent molecular masses of 120 to 135 kDa, called colicins Ao. To determine whether the various forms of colicin A were present in the colicin A extracts of the pools collected after 3 h induction from W3110(pColA9) and W3110(pJMM1) cells, analysis of each pool was performed on an SDS-polyacrylamide gel containing 6% acrylamide. On the blot of the gel probed with mAb to colicin A, colicins As, Af and colicins Ao were detected regardless of whether the cells had been boiled in sample buffer before loading. In contrast, the heat-labile colicins Au were only detected in the unheated samples. All forms of colicin A were present in all pools, except in the SP and SE from W3110(pJMM1) cells. The level of colicins Au compared to that of monomeric colicins A was higher in the SE and LE of both W3110(pColA9) and W3110(pJMM1) cells than in SP (Fig. 2a).

The presence of Cal was checked after trichloroacetic acid precipitation on Tricine gels. Cal was detected in each pool on the blot of the gel probed with pAbs to Cal (Fig. 2b). Its amount was low in the SP, high in the SE and more significant in the LE. In the LE, a band with an apparent molecular mass of about 10 kDa which seemed to correspond to a Cal dimer was detected. Cal was absent in the pools from W3110(pJMM1) cells, as expected.

**Cal and colicins Au are present in the insoluble fraction of each pool of released and unreleased colicin A**

The presence of Cal and colicins Au in the three pools of colicin A was not expected since they are membrane-associated and should not be present in aqueous solutions. To know whether they were associated with either membrane fragments or organelles such as pili or vesicles, the SP, SE and LE pools obtained from W3110 _degP_(pColA9) cells were dialysed and centrifuged at high speed. The pellets were resuspended in 8 M urea, centrifuged, washed and resuspended in 100 mM Tris/HCl (pH 8) before being treated or not with trypsin and analysed on urea/SDS-polyacrylamide gel in order to detect colicins Au. Colicins Au were indeed best detected in SDS-polyacrylamide gels

### Table 1. Number of KUs of colicin A present in 1 ml of induced culture

The number of KUs present in each fraction obtained from 1 ml of induced culture at various times after induction is indicated. The percentage (%) of KUs present in each fraction was calculated from the total number of KUs.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (min)</th>
<th>LB medium</th>
<th>SE KUs</th>
<th>LE KUs</th>
<th>Total no. of KUs</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>KUs</td>
<td>%</td>
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<tr>
<td>W3110(pColA9)</td>
<td>0</td>
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<td>86</td>
<td>0·5×10⁹</td>
<td>5·8×10⁹</td>
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<tr>
<td></td>
<td>180</td>
<td>1·4×10¹²</td>
<td>78</td>
<td>3·4×10¹¹</td>
<td>1·8×10¹²</td>
</tr>
<tr>
<td>W3110(pJMM1)</td>
<td>90</td>
<td>1·9×10⁸</td>
<td>0·2</td>
<td>3·8×10⁸</td>
<td>9·8×10¹⁰</td>
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<td></td>
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containing 7 M urea, confirming they are not formed on the SDS-polyacrylamide gels and suggesting they were associated with complexes of high molecular masses. Urea is known to suppress non-specific aggregation of membrane proteins in SDS-polyacrylamide gels (Soulie et al., 1996). On the blot of the gel probed with mAb to colicin A, colicins Au were detected without monomeric colicin A in the unheated samples of the insoluble fractions treated with urea. They dissociated into monomeric colicin A after boiling. Colicins Ao were present in the heated as in the unheated samples with a band with an apparent molecular mass of about 260 kDa. All colicin A species were destroyed by trypsin. On the blot probed with pAbs to OmpF, colicins Au were detected in the unheated samples, but not in the trypsin-treated ones. In the heated samples, OmpC and OmpF were detected in both trypsin-treated and untreated fractions. The same results were obtained from each pool of colicin A (shown for SE in Fig. 3a). Thus, colicins Au could be isolated from monomeric colicin A by centrifugation, but not from colicins Ao. The colicin part of colicins Au was sensitive to trypsin, but not their porin part, indicating that colicin A was associated with porins integrated into the outer membrane.

The presence of Cal in the soluble and insoluble fractions was checked on immunoblots of the Tricine gel (Fig. 3b). Cal was only detected in the SE and the LE insoluble fractions treated with urea, with traces of a dimeric form in the LE. It was not hydrolysed by trypsin.

Thus, both Cal and colicins Au were found extracellularly in the insoluble fractions of each pool of released and unreleased colicin A.

**Steps of colicin A release are impaired in cells with an unacylated Cal precursor**

Whether one or both steps of colicin A release were dependent on Cal, they should be modified in cells with a non-functional Cal. The above experiments were repeated in cells carrying a plasmid with a mutated cal gene. Amino acid substitutions of the cysteine residue present at the N-terminal end of mature Cal inhibit Cal acylation and processing and make the cal gene product unstable (Cavard...
et al., 1987). Plasmids pAL16 and pAK31, which contain, respectively, the C1T and C1P substitutions in the cal gene product, were introduced into W3110 degP cells since a Cal form has been shown to be a substrate of the DegP protease (Cavard et al., 1989). After induction, the C1T (see below) and C1P (not shown) Cal precursors were expressed and remained stable, indicating that the DegP protease hydrolysed the unacylated precursor of Cal (pCal).

The production and release of colicin A were analysed after induction of W3110 degP cells carrying pColA9 or pAL16 (Fig. 4a) or pJMM1 (not shown). In W3110 degP(pColA9) cells, the distribution of colicin A in the three pools SP, SE and LE was similar to that obtained in W3110(pColA9) cells, but the amount of colicin A released was greater (83% of the KUs were found in the medium) due to the increased Cal stability, as reported previously (Cavard et al., 1989). In contrast, in W3110 degP(pAL16) cells, the colicin A content of each pool was drastically modified, as 2 × 10^{11} KUs were found in the medium (8% of the total KUs), 6 × 10^{11} KUs in the SE (26% of the KUs) and 1.5 × 10^{12} in the LE (65% of the KUs). Colicin A release was inhibited as expected, but some colicin might be extracted in the SE in contrast to what was observed in the absence of Cal, as the harvest of colicin A in W3110 degP(pJMM1) cells was similar to that observed in W3110(pJMM1) cells. Both steps of colicin A release were impaired, the second step more drastically than the first, indicating that pCal was not as efficient as Cal.

**Colicins Au are unstable in the presence of an unacylated Cal precursor**

The three pools of colicin A obtained from W3110 degP cells carrying either pColA9 or pAL16 were dialysed, centrifuged and the pellets were analysed as described above. The composition of the insoluble fractions obtained from the pools of W3110 degP(pColA9) cells was similar to that obtained from W3110(pColA9) cells. In contrast, the composition of the pools from W3110 degP(pAL16) cells was different. The amount of pCal detected by pAbs to Cal on blots of Tricine gels (Fig. 4b) was significant in the insoluble fractions of the SE and LE. It was present mainly in a dimeric form that was resistant to urea and trypsin treatment. The colicins Au were present with monomeric colicin A in the unheated samples on the blot probed with mAb to colicin A (shown for SE in Fig. 4c) in contrast to what was observed in cells harbouring pColA9. Furthermore, a band below monomeric colicin A was observed in the heated samples, suggesting proteolysis. On the blot probed with pAbs to OmpF, colicins Au, OmpC and OmpF were observed as in cells harbouring pColA9. Thus, colicins Au were less stable in the presence of pCal than in that of Cal.

**Loss of OmpF in the absence of Cal in degP cells**

Colicins Au were detected similarly in the unheated samples of W3110 degP cells carrying pColA9 or pAL16 or pJMM1 when analysed on a blot probed with mAb to colicin A. However, on a blot probed with pAbs to OmpF, they were not detected in the unheated samples of the W3110 degP(pJMM1) cells, while they were in the cells carrying either pColA9 or pAL16 (Fig. 5). In the heated samples, both OmpC and OmpF were present in the W3110 degP cells carrying either pColA9 or pAL16, while only OmpC was detected in the cells harbouring pJMM1 cells. W3110 degP(pJMM1) cells behave like an ompF mutant in which colicins Au are detected by mAb to colicin A, but not by pAbs to OmpF (Cavard, 2002). W3110 degP cells lost OmpF after transformation with pJMM1, even when not induced for colicin A production (Fig. 5b), suggesting that
the colicin A–OmpF complex made during spontaneous colicin A production was toxic in the absence of Cal. Either wild-type Cal or non-functional C1T Cal protected the cells, suggesting an interaction between Cal and/or pCal with the colicin A–OmpF complex.

The loss of OmpF was also observed in UT5600 \textit{ompT} (pJMM1) cells in a few generations after transformation (Fig. 5b). In UT5600 \textit{ompT} \textit{degP} (pJMM1) cells, OmpF was absent and cells were mucoid, indicating that an additional mutation was necessary for cell survival. The \textit{cal}, \textit{degP} and \textit{ompT} gene products seemed to play a protective role against the toxic colicin A–OmpF complex.

The export of colicins Au to the outer membrane is Sec-independent

The formation of colicins Au has been shown to be a rapid process as monitored by radiolabelling of their heat-labile forms. Kinetics indicated that they rapidly reached the outer membrane (Cavard, 2002). To determine whether colicins Au were using the Sec secretion pathway as porins do (Danese & Silhavy, 1998), induction of colicin A was performed in temperature-sensitive (ts) \textit{sec} mutants. Isogenic strains HJM114, CJ105 \textit{secA} \textit{ts} and CJ107 \textit{secY} \textit{ts} carrying pColA9 were grown at 30°C and induced for colicin A production at 42°C. In these conditions, the export of Sec-exported proteins such as \textit{b}-lactamase was inhibited as described previously (Cavard, 1992). After 3 h induction, samples of the cultures were run on an urea/SDS-polyacrylamide gel (Fig. 6a). On the blot probed with mAb to colicin A, colicins Au were similarly observed in the unheated samples of wild-type and \textit{sec} \textit{ts} mutant cells.

Monomeric colicin A and colicins Ao were detected regardless of whether the cells had been boiled in sample buffer before loading. On the blot probed with \textit{pAbs} to OmpF, colicins Au were detected in the unheated samples of each strain tested, whereas the two porins OmpC and OmpF were observed in the heated samples. Thus, colicins Au seemed to require neither SecA nor SecY to exhibit heat-modifiability.

To verify whether colicins Au were exported with Cal, which uses a Sec-independent pathway (Cavard, 1992), production of colicins Au was studied in W3110 cells carrying either pColA9 or pJMM1, i.e. in cells containing or not the \textit{cal} gene. Sodium azide, which is a potent inhibitor of the SecA translocase (Oliver \textit{et al}.., 1990), was added at the time of induction. Samples were analysed as above. Colicins Au and the various forms of colicin A were observed in both cultures in the presence and in the absence of sodium azide on the blots probed with either \textit{mAb} to colicin A or \textit{pAbs} to OmpF (Fig. 6b). Their amount was reduced in the presence of azide, as expected. Thus, Cal was not needed for the Sec-independent export of colicins Au.

\textbf{Fig. 5.} Loss of OmpF by W3110 \textit{degP} cells carrying pJMM1. (a) Samples of induced W3110 \textit{degP} cells carrying pColA9 (A) or pAL16 (AL) or pJMM1 (JM) as indicated were heated (+) or not (−) in sample buffer before loading onto a urea/SDS-polyacrylamide gel. The Western blots of the gel probed with either \textit{mAb} 1C11 to colicin A or \textit{pAbs} to OmpF are presented. (b) Samples of uninduced W3110 (wt) or W3110 \textit{degP} or UT5600 \textit{ompT} cells carrying either pColA9 (A) or pJMM1 (JM) were boiled in sample buffer before loading onto a urea/SDS-polyacrylamide gel. The Western blot of the gel probed with \textit{pAbs} to OmpF is presented. The positions of the various proteins are indicated by arrowheads.

\textbf{DISCUSSION}

In this report, colicin A release was studied and the role played by both Cal and colicins Au during secretion was investigated. Two steps of release were proposed. In the first step, colicin A produced in the cytoplasm of induced cells moved to a cell localization where it can be extracted by washing. In the second step, colicin A was released in the medium. In both steps, numerous proteins were found

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with colicin A. Cal was required at least for the first step of colicin A export since neither step occurred in cells devoid of Cal. In cells with a mutation in the lipobox of the cal gene product, both steps were inhibited, the first one less strongly than the second one, confirming that both acylation and processing of Cal were needed for its functioning.

Many lines of evidence for interactions between Cal and colicins Au are reported. First, Cal and colicins Au were both found in the pools of released and unreleased colicin A, demonstrating they were loosely associated with the outer membrane and might become extracellular. Both Cal and colicins Au co-localized after centrifugation of the pools and were insoluble in 8 M urea, suggesting they were associated with the same organelle. Second, colicins Au produced either in the presence of pCal or in the absence of Cal were unstable and some of them dissociated into colicin A monomers. Third, in the absence of Cal and/or pCal, degP cells lost OmpF, suggesting that colicins Au made of colicin A bound to OmpF were toxic and interacted with Cal and/or pCal which detoxified them.

The extracellular release of colicins Au might be due to their interaction with Cal since it did not occur in the absence of Cal. Porins, which are part of colicins Au complexes, are integral outer-membrane proteins and, consequently, refractory to removal into aqueous solutions. In contrast, Cal binds to the membrane by its diglyceride moiety as do all lipoproteins and this hydrophobic association might be removed (Jakubowski et al., 2003; Sagulenko et al., 2001). Both Cal and colicins Au might be present in a structure that would constitute either a barrier or a conduit for colicin A. Cal would allow association of the structure with the outer membrane. The Cal sequence is homologous to that of VirB7, the lipoprotein of the type IV secretion system of A. tumefaciens (Fig. 7), as pointed out previously (Shirasu et al., 1990). VirB7 forms homodimers and heteromultimeric complexes at the outer face of the outer membrane (Anderson et al., 1996; Spudich et al., 1996; Baron et al., 1997; Jakubowski et al., 2003; Sagulenko et al., 2001). In the same way, homodimers of Cal and/or pCal were observed and Cal seemed to interact with hetero-oligomeric complexes such as colicins Au. Preliminary results indicated that expression of the virB7 gene was as deleterious for cells as that of the cal gene, but did not complement the cal gene deletion as observed by transformation of W3110(pJMM1) cells with pPC978 (not shown).

Alternatively, Cal and colicins Au might be released as vesicles. Outer-membrane vesicles (OMVs) are produced during bacterial growth (reviewed by Beveridge, 1999) and

Fig. 6. Sec-independent export of colicins Au. (a) HJM114 (wt), CJ105 secA ts (A) and CJ107 secY ts (Y) cells carrying pColA9 were induced or not induced (ni) with mitomycin C. (b) W3110 cells carrying either pColA9 (A9) or pJMM1 (JM) were induced with mitomycin C in the presence (az) or absence (c) of azide for 3 h. Samples were boiled (+) or not (−) in sample buffer before loading onto a urea/SDS-polyacrylamide gel. The Western blots of the gel probed with either mAb 1C11 to colicin A or pAbs to OmpF are presented. The position of the porins and of the various colicin A forms is indicated by an arrowhead, and that of colicins Ao by a vertical line.

Fig. 7. Comparison of the amino acid sequences of Cal and VirB7. The sequences of Cal and VirB7 deduced from the nucleotide sequences of the cal gene of plasmid pColA (SWISS-PROT P06962), and of the virB7 gene of plasmid pTiC58 (SWISS-PROT P17792), respectively, are presented. Identical amino acids are in bold, related ones in italic letters. The number of residues in the signal peptide and in the mature form is indicated.
are used for toxin export in *E. coli* (Wai *et al.*, 2003). Colicin-producing cells are likely to be similar to *tol* mutants which produce more OMVs than wild-type cells (Bernadac *et al.*, 1998). The Tol proteins they contain should bind the produced colicin A and become non-functional. Such inhibition of the Tol machinery has been described in cells producing the N-terminal domain of colicins (Bouveret *et al.*, 1998, 2002). Cal and colicins Au would be present in the membrane of such vesicles.

Until now, colicin A secretion was thought to be caused by the loss of integrity of the bacterial envelope since a drop in the absorbance of the cultures, called quasi-lysis, occurs at the time of colicin A release (Pugsley & Schwartz, 1984; Baty *et al.*, 1987). Quasi-lysis is provoked by outer membrane phospholipase A (OMPLA) activation following the induction of the colicin lysis protein (Dekker *et al.*, 1999). However, it is not known whether the cells lysed during release are the induced cells or the uninduced ones in which either the uptake or the action of mitomycin C does not take place. Numerous uninduced cells that do not contain colicin have been observed by electron microscopy in colicin A-induced cultures (Cavard *et al.*, 1984) and by immunofluorescence microscopy in colicin K-induced cultures (Mulec *et al.*, 2003). Lysis of the uninduced cells would explain the presence of cytoplasmic and periplasmic proteins in the medium together with colicin A, but colicin A would be released by a specific machinery present in induced cells.

Colicins Au appeared to be formed by binding of colicin A to mature porins already incorporated in the outer membrane. Their assembly did not need the Sec translocase in contrast to that of porins. Porins are produced as precursors that are processed after Sec translocation by the leader peptidase before integration into the outer membrane. Colicin A lacks a signal peptide and is thus exported by an unknown pathway first to the periplasm where it has been observed in cells expressing Cal (Cavard, 2002), suggesting an interaction between Cal and colicin A for crossing the inner membrane. From the periplasm, colicin A would associate with integral outer-membrane proteins such as OmpC and OmpF, OMPLA and maybe LamB and OmpT, to form the heterogeneous group of colicins Au. Colicins Au are produced in an *ompT* null mutant (Cavard, 2002), but proteolysis occurred during colicins Au purification which was inhibited by benzamidine (not shown), an OmpT-specific inhibitor (White *et al.*, 1995), suggesting the presence of OmpT, or homologues, in some colicins Au complexes.

The two gene products of the colicin A operon might be involved in the biogenesis of a machinery for colicin A transport. By making complexes with membrane proteins, both colicin A and Cal might be able to assemble a machinery as efficient as the one encoded by the 10 to 14 genes required for other protein secretion processes described in Gram-negative bacteria (Pugsley, 1993).

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