Functioning of the Colicin A Lysis Protein is Affected by Triton X-100, Divalent Cations and EDTA

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The colicin A lysis protein, Cal, is synthesized at the same time as colicin A by *Escherichia coli* harbouring plasmid pColA after induction by mitomycin C. Its function in the induced bacteria involves the release of colicin A, quasi-lysis, the death of the producing cells and the activation of the outer membrane phospholipase A. We have found that these various functions are affected differently by treatment of the induced cells with Triton X-100, divalent cations or EDTA. Triton X-100 and EDTA caused increased quasi-lysis and a higher level of mortality of the producing cells, but while Triton X-100 enhanced the release of colicin A, EDTA reduced it. Divalent cations protected the cells against both killing and quasi-lysis without greatly affecting colicin release. The effects of these agents were similar for both wild-type and phospholipase A mutants and depended only on the presence of a functional *cal* gene.

INTRODUCTION

It has been demonstrated that the release of colicin A from producing *Escherichia coli* cells is entirely dependent on the synthesis of the colicin A lysis protein (Baty *et al.*, 1987b). This protein, called Cal, is a small lipoprotein of 33 amino acids that is induced under SOS control at the same time as colicin A. It is synthesized in precursor form and undergoes modification and processing reactions similar to those of the major lipoprotein of the outer membrane. A small amount of Cal is released to the medium together with large amounts of colicin A (Cavard *et al.*, 1985, 1987).

The Cal protein is highly homologous to lysis proteins encoded by other colicin or cloacin plasmids (De Graaf & Oudega, 1986; Pugsley, 1988) with the exception of that one encoded by pColE9-J (James *et al.*, 1987). The mode of action of these lysis proteins is unknown. They promote the release of colicins or cloacin by a lethal mechanism that involves a so-called 'quasi-lysis' of the producing cells and a necessary activation of the phospholipase A of the outer membrane at the time of colicin release (Pugsley & Schwartz, 1984; Luirink *et al.*, 1986; Cavard *et al.*, 1987). Pugsley & Schwartz (1983, 1984) have reported that Triton X-100 promotes colicin E2 release from induced cells carrying a functional lysis gene, even if the cells are devoid of phospholipase A. The detergent thus appears to bypass the requirement for phospholipase A activation. On the other hand, the divalent cations Mg^{2+} and Ca^{2+} have been shown to inhibit the quasi-lysis of induced cells provoked by the lysis proteins of colicins E1 and E2 and of cloacin DF13 (Altieri *et al.*, 1986; Pugsley & Schwartz, 1984; Luirink *et al.*, 1986, 1987).

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Abbreviation: Cal, colicin A lysis protein.

We report in this paper that Triton X-100 causes early release of both colicin A and its lysis protein, and that it is possible to selectively alter the functions of the Cal lysis protein, and furthermore, that Cal causes severe damage to induced cells even in the absence of phospholipase activation.

METHODS

Bacterial strains and plasmids. The strains *Citrobacter freundii* CA31 (pColA) and *E. coli* W3110 (pColA9) were used in most experiments. The plasmids pColA9, pJMM1, pX345, pAE11 and pAK31 have been described (Llobès *et al.*, 1986; Cavard *et al.*, 1985, 1986, 1987).

Growth conditions. Strains were grown in LB medium or in M9 medium supplemented with thiamin (1 µg ml⁻¹), glycerol (0.4%, v/v) and Casamino acids (0.01%, w/v). Plates of minimal or LB medium contained 1.5% (w/v) agar and 40 µg ampicillin ml⁻¹. Mitomycin C was used in cultures having an OD₆₀₀ of 1, using 300 ng ml⁻¹ for *E. coli* strains and 100 ng ml⁻¹ for *Citrobacter* CA31. All cultures were incubated at 37 °C with good aeration. Optical density of the cultures was measured in a Kontron spectrophotometer.

Analysis of colicin A release and immunoblotting. Samples of a culture in LB medium treated with mitomycin C, or of a supernatant of the culture, were analysed by 12.5% (w/v) SDS-PAGE (Cavard *et al.*, 1985). Immunoblotting was performed as described by Baty *et al.* (1987b) with a rabbit anti-colicin A serum.

Labelling with radioactive methionine. Labelling conditions with [³⁵S]methionine [10 µCi ml⁻¹, 1200 Ci mmol⁻¹ (44.4 TBq mmol⁻¹)] were as previously described (Cavard *et al.*, 1987). Labelling was performed after 60 or 90 min of mitomycin C treatment for *Citrobacter* and *E. coli* strains, respectively. For pulse-chase experiments, [³⁵S]methionine [30 µCi ml⁻¹ (1.11 MBq ml⁻¹)] was added and after 45 s unlabelled methionine was added to 100 µg ml⁻¹. Samples of 10 µl were taken at various times after the chase and mixed with 10 µl of loading buffer and heated to 96 °C before being analysed. Urea-SDS-polyacrylamide gels were electrophoresed and treated as described previously (Cavard *et al.*, 1987).

Strain construction. An *E. coli* W3110 *pldA* strain was constructed by P1 transduction. First, a W3110 *metE::Tn10* was constructed by P1 transduction from *E. coli* 403 *metE::Tn10* and selection on tetracycline plates. This strain was then transduced with P1 grown on strain PC1602 *pldA* (a generous gift of Dr J. Tommassen, Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Utrecht, Holland). Transductants were selected on minimal medium plates and screened for the loss of tetracycline resistance. The *pldA* maker was tested as follows. Cells were transformed with pColA9, grown in LB medium and treated or not treated with mitomycin C for 4 h, after which their phospholipids were extracted as described by Ames (1968) and analysed by thin-layer chromatography on silica gel plates. Clones in which the level of lysophosphatidylethanolamine was not increased by mitomycin C were considered as *pldA* and were called CBM.

Chemicals. Mitomycin C was purchased from Sigma. All other chemicals used were reagent grade.

RESULTS

Effects of divalent cations, EDTA and Triton X-100 on Cal protein function

In a culture of *E. coli* W3110(pColA9), addition of mitomycin C induces the synthesis of both Cal and colicin A (Llobès *et al.*, 1986). In LB medium, the optical density of the culture increases during the first hour of induction and then decreases with time until a value lower than that at the time of mitomycin C addition is reached. This 'quasi-lysis' indicates a functioning lysis protein and is correlated with the release of colicin A (Cavard *et al.*, 1987).

In the presence of Triton X-100 (0.1%) or of EDTA (1 mM), the quasi-lysis of the induced cells was more significant than that of the control cells (Fig. 1a). In contrast, in the presence of 20 mM-MgSO₄, no decline in OD₆₀₀ was observed but the OD₆₀₀ of the induced cells reached a plateau after 2 h of induction. Elimination of Mg²⁺ ions by the addition of EDTA provoked an immediate decrease of 40% in the OD₆₀₀ of the culture (Fig. 1c). The addition of CaCl₂ had the same effect on quasi-lysis as that of MgSO₄ (data not shown). [It should be noted that the growth of control (uninduced) cells was slower in the presence of EDTA but normal in the presence of Triton X-100 or MgSO₄ at the concentrations used.]

We have found that quasi-lysis and colicin A release are greatly reduced in strain PC1602 *pldA* (Cavard *et al.*, 1987). We then tested the effect of Mg²⁺ ions, EDTA or Triton X-100 addition on strain CBM(pColA9), a *pldA* mutant isogenic to W3110. Quasi-lysis was much reduced for *pldA* cells but was partially restored in the presence of Triton X-100 and was almost normal in the

Fig. 1. Effect of Triton X-100, Mg^{2+} ions and EDTA on the quasi-lysis of colicin A producing cells. (a); A culture of *E. coli* W3110(pColA9) in LB medium was split into two parts, one of which was treated with mitomycin C (M, filled symbols) and the other untreated (C, open symbols) at zero time. Each one was distributed into four flasks: in one, Triton X-100 was added to 0.1% concentration ($\blacktriangledown$, MTx; or ∇ , CTx), in the second, EDTA was added to 1 mM ($\blacktriangle$, ME; or $\triangle$, CE), in the third, $MgSO_4$ was added to 20 mM ($\blacksquare$, MMg; or $\square$, CMg), and in the fourth, nothing was added ($\bullet$, M; or $\circ$, C). The OD₆₀₀ of the cultures was measured at various times of induction. (b) The same experimental conditions were used as in (a) but with cells of *E. coli* CBM(pColA9). The OD₆₀₀ of the control cultures in the presence of either Triton X-100 (CTx) or $MgSO_4$ (CMg) was similar to that of the control cultures (C) and is not plotted on the figure. (c) After 3 h of induction, samples of control and mitomycin C cultures treated with $MgSO_4$ were taken and added to various concentrations of EDTA immediately before their OD₆₀₀ was measured. $\square$, Control cells of W3110(pColA9); $\blacksquare$, induced cells of W3110(pColA9); $\bullet$, induced cells of CBM(pColA9).

Fig. 2. Effect of Triton X-100, Mg^{2+} ions and EDTA on colicin A release. In the same experiment as shown in Fig. 1, samples of cultures were taken after 2 and 4 h of induction and centrifuged. Equal amounts (10 μ l) of the cultures (C) and supernatants (S) were resuspended in loading buffer and analysed by SDS-PAGE. Part of the gel stained with Coomassie blue is presented. The position of colicin A is indicated by an arrow. Numbers on the right are the molecular masses of the standard proteins.

presence of EDTA (Fig. 1*b*). In the presence of $MgSO_4$, induced *pldA* cells behaved like control cells, with no decrease or early plateau in the OD_{600} of the culture.

The release of colicin A under these conditions was examined by analysis of the cultures and culture supernatants by SDS-PAGE (Fig. 2). Triton X-100 slightly increased the amount and the rate at which colicin A was released while both Mg^{2+} and EDTA decreased the rate and extent of release. Less colicin was synthesized in the presence of EDTA or Triton X-100 than in control cells. The early drop of OD_{600} provoked by EDTA or Triton X-100 thus seemed to be correlated with a decrease in the rate of synthesis of the colicin. In contrast, more colicin and more cell proteins were synthesized in presence of Mg^{2+} than in control cells, indicating that the continuous increase of OD_{600} was correlated to cell growth. In *pldA* cells, the release of colicin A was greatly reduced, as previously reported (Cavard *et al.*, 1987). It was further decreased by EDTA or Mg^{2+} and slightly increased by Triton X-100 (Fig. 2).

The mortality of induced cells was checked in the presence of these various effectors by measuring the loss of colony-forming ability. As shown in Table 1, it was altered by the various treatments in the same manner as quasi-lysis. EDTA and Triton X-100 increased the killing significantly, while $MgSO_4$ protected the cells, for both wild-type and *pldA* cells. In addition, the number of CBM(pColA9) cells surviving after induction was 50 times higher than for the wild-type, suggesting that the activation of phospholipase A by Cal is an important part of the lethal process.

Fig. 3 shows the analysis on urea-SDS gels of pellets and supernatants of a culture in M9 medium of *E. coli* W3110(pColA9) induced with mitomycin C in the presence of Triton X-100 or

Fig. 3. Effect of Triton X-100 and EDTA on the release of colicin A and Cal. A culture of *E. coli* W3110(pColA9) in M9 medium was induced with mitomycin C in the absence (C) or presence of 0.1% Triton X-100 (Tx) or 2 mM-EDTA (E), or both Triton X-100 and EDTA (ETx). They were labelled for 5 min with [35 S]methionine after 90 min of induction. At various times after labelling samples were collected and centrifuged for 1 min at 13000 g. Pellets (P) were resuspended in loading buffer and heated before loaded on the gel. Samples (10 μ l) of supernatants were mixed with loading buffer, heated and loaded on the gel. The fluorogram of the urea-SDS gel is presented. Migrations of the colicin (■), colicin A fragments (▪), modified precursor Cal (●), precursor Cal (○), mature Cal (►) and Cal signal peptide (▶) are indicated.

Table 1. *Effects of various treatments on colony-forming ability of induced cells*

Treatment*	C.f.u. ml ⁻¹		
	0	Time: 1 h	4 h
W3110(pColA9)			
Non-induced	1 $\times$ 10 ⁹	ND	6.2 $\times$ 10 ⁹
Non-induced + EDTA	1 $\times$ 10 ⁹	ND	2.5 $\times$ 10 ⁹
Induced	1 $\times$ 10 ⁹	1.7 $\times$ 10 ⁷	1 $\times$ 10 ⁶
Induced + MgSO ₄	1 $\times$ 10 ⁹	2.2 $\times$ 10 ⁸	5.5 $\times$ 10 ⁷
Induced + EDTA	1 $\times$ 10 ⁹	1.3 $\times$ 10 ⁶	3 $\times$ 10 ⁴
Induced + Triton X-100	1 $\times$ 10 ⁹	1.3 $\times$ 10 ⁷	8 $\times$ 10 ⁵
CBM(pColA9)			
Non-induced	2 $\times$ 10 ⁹	ND	7 $\times$ 10 ⁹
Induced	2 $\times$ 10 ⁹	2 $\times$ 10 ⁸	1 $\times$ 10 ⁸
Induced + MgSO ₄	2 $\times$ 10 ⁹	ND	5.2 $\times$ 10 ⁸
Induced + EDTA	2 $\times$ 10 ⁹	ND	6 $\times$ 10 ⁴
Induced + Triton X-100	2 $\times$ 10 ⁹	ND	5 $\times$ 10 ⁶

ND, Not determined.

* Bacteria in LB medium were treated as reported in Fig. 1.

EDTA or both and labelled with [35 S]methionine. As can be seen in the cell pellets (P), differences in the accumulation of Cal were observed according to the effector added. In Triton X-100 treated cells, the level of mature Cal decreased after 30 min of labelling and then stayed constant with time. In contrast, in EDTA-treated cells, with or without added Triton X-100,

Fig. 4. Effect of Triton X-100 or EDTA on the release of colicin A and Cal in *pldA*⁺ or *pldA* strains. Cultures of *E. coli* W3110(pColA9) (+) and of CBM pColA9 (–) were induced, treated and labelled as indicated in Fig. 2. After 5 h of induction, samples were centrifuged and 10 µl of the supernatants analysed on a urea-SDS gel. On the fluorogram, the migration of colicin A (■), fragments of colicin A (●) and mature Cal (▴) are indicated.

mature Cal was as stable as in control cells. In the supernatants (S), the release of both colicin A and mature Cal was observed. The release of Cal was significantly increased by treatment with Triton X-100, which explained the low level found in the cells. The release of Cal was unmodified by EDTA while the release of colicin A was inhibited. The addition of both Triton X-100 and EDTA prevented the release of Cal caused by Triton X-100 alone, which was consistent with what was observed in the pellets. The addition of divalent cations had no effect on either Cal synthesis or release (data not shown). Colicin A in the supernatant was partly hydrolysed by proteases into fragments of about 30 kDa, indicated by dots in Fig. 3 and identified with anti-colicin A antibodies in an immunoblot (data not shown).

The addition of Triton X-100 or EDTA had the same effects on strain CBM(pColA9) as on strain W3110(pColA9). The supernatants of both *pldA* and *pldA*⁺ cells after 5 h of induction and labelling with [³⁵S]methionine are compared in Fig. 4. The release of colicin A and of Cal was greatly reduced in CBM(pColA9) compared to the wild-type strain but was greatly increased in the presence of Triton X-100. Under these conditions, the release of Cal approached the level observed for Triton X-100-treated wild-type cells. Treatment with EDTA also increased the release of Cal in CBM(pColA9) cells, although to a much lesser extent, as was observed for W3110(pColA9) cells. Again large amounts of colicin A fragments (shown by dots in Fig. 4) were observed; but these fragments were formed in larger amounts when CBM(pColA9) cells were treated with either Triton X-100 or EDTA than when untreated (Fig. 4, lane C). This

Fig. 5. Maturation and processing of Cal are unaffected by either Triton X-100 or EDTA. A culture of *Citrobacter* CA31 was induced with mitomycin C in the absence (M) or presence of 0.1% Triton X-100 (M+Tx) or of 2 mM-EDTA (M+E). After 1 h of induction, cells were pulse-labelled for 40 s with [35 S]methionine (50 μ Ci ml $^{-1}$). Samples (10 μ l) of the culture were taken at various times after the chase (indicated in the figure in min), mixed with 10 μ l of loading buffer and loaded on a urea-SDS gel. A fluorogram of the gel is presented. Migrations of the various forms of Cal are indicated as in Fig. 3.

suggests that either some *E. coli* proteases are activated by detergent or that the susceptibility of colicin A to protease action is increased in the presence of either detergent or EDTA.

The effects described above were also observed with strains carrying plasmids encoding the *cal* gene but without an entire *caa* gene (Baty *et al.*, 1987*b*), both in *E. coli* or *Citrobacter* strains, and in either LB or M9 medium. The same effects were also obtained with cells carrying plasmid pColE1 (data not shown).

The modification and processing of Cal are not affected by either Triton X-100 or EDTA

The lipid modification and processing of Cal during colicin induction were examined in *Citrobacter* CA31 cells by pulse-labelling for 40 s with [35 S]methionine followed by a chase with unlabelled methionine. As seen in Fig. 5, addition of either Triton X-100 or EDTA at the time of induction did not affect the various steps of modification and processing of Cal. In each case, the Cal protein was synthesized under precursor form, pCal, which was slowly chased into a higher M_r modified precursor form (pCal^m) by acylation. This form was then chased to the mature form plus its signal peptide, both of which accumulated in the cell with time. In each case, the entire process of Cal modification and processing was very slow, requiring at least 30 min. The same kinetics were obtained in *E. coli*, as previously reported for wild-type cells (Cavard *et al.*, 1987).

Effective concentrations of Triton X-100, divalent cations and EDTA

The effect of various concentrations of Triton X-100 on the release of Cal from the induced cells to the culture supernatant is shown in Fig. 6. The extraction of Cal from the cells was maximum with the lowest concentration used (0.04%) and did not increase with higher concentrations. It was also apparent that while increasing concentrations of Triton X-100 caused a progressive loss in the amount of the signal peptide in the cells, this material was not recovered in the supernatant. Increasing concentrations of Triton X-100 also caused increased

Fig. 6. Effect of various concentrations of Triton X-100 on Cal release. A culture of *E. coli* W3110 (pColA9) was treated with mitomycin C in the absence or presence of 0, 0.04, 0.1 or 1% Triton X-100. They were labelled with [35 S]methionine as indicated in Fig. 3. After 5 h of induction, samples were collected and centrifuged and pellets (P) and supernatants (S) were analysed on urea-SDS gel. On the fluorogram, the migrations of colicin A (Col), fragments of colicin A (■) and mature Cal (►) are indicated.

break-down of colicin A released in the medium with formation of fragments around 30 kDa (shown by dots in Fig. 6). This breakdown was not obtained with cells of *Citrobacter* CA31, indicating that it was due to *E. coli* protease(s).

The concentration range of Mg^{2+} ions effective in decreasing the quasi-lysis of induced cells was similar to that described for cloacin DF13 producing cells, between 5 and 20 mM (Luirink *et al.*, 1986). The optimal concentration for W3110(pColA9) cells was 20 mM. The effective concentration of EDTA was also tested. In M9 medium, which contains 1 mM- Mg^{2+} and 0.1 mM- Ca^{+2} , a concentration of 1.5 mM-EDTA was effective in reducing the release of colicin A without causing the release of Cal. Further increases in the concentration of EDTA had no further effect, but inhibited growth.

Effect of Triton X-100 on strains carrying plasmids with caa or cal mutations

We have previously shown that strains carrying plasmids containing mutations in the *cal* gene (at the modification and cleavage site of the Cal precursor form) are unable to release colicin A (Cavard *et al.*, 1987). In contrast, strains carrying a mutation in the *caa* gene are able to export the mutated colicin as long as the *cal* gene is functional and the mutant colicin is soluble (Baty *et al.*, 1987a, b). In strain *E. coli* W3110(pAK31), the *cal* gene carries a mutation at the processing site such that the Cal protein is rapidly hydrolysed rather than being correctly modified. In strain W3110(pJMM1), transposon TnI is inserted just upstream of the *cal* gene. In strain W3110(pX345), the *caa* gene has been modified by mutagenesis such that the mutated colicin A

Fig. 7. Effect of Triton X-100 on Cal accumulation in the cells of various strains. Cultures of *E. coli* W3110 containing various plasmids derived from pColA were treated with mitomycin C in the absence (M) or presence of 0.1% Triton X-100 (MTx). They were labelled with [35 S]methionine as indicated in Fig. 3. At various times after the chase (indicated in min on the figure), samples were centrifuged and the pellets mixed with loading buffer before being loaded onto the gel. Part of a fluorogram of the gel is presented. Migrations of the various forms of Cal are indicated as in Fig. 3.

aggregates and cannot be released to the medium despite the presence of a functional *cal* gene. These strains were induced with mitomycin C in the presence of Triton X-100, labelled with [35 S]methionine, and analysed after various times of chase on urea-SDS gels. Triton X-100 had no effect on strain W3110(pAK31), in which no functional Cal protein is present (Fig. 7). The same results were obtained with strain W3110(pJMM1) (data not shown). The effect of Triton X-100 on strain W3110(pX345) was similar to that observed for the wild-type, resulting in a loss of Cal from the cells. Fig. 8 shows the effect of Triton X-100 on the culture supernatants of these various strains and on *Citrobacter* CA31 after 5 h of induction. The detergent enhanced the release of colicin A and of Cal in each *cal*⁺ strain. In particular, release of colicin A-X345 was obtained under these conditions and became similar to the release of wild-type colicin A.

DISCUSSION

The experiments described in this paper show that the various functions of the Cal protein can be individually modified by various effectors. These functions are the release of colicin A, quasi-lysis, the activation of the outer membrane phospholipase A and the killing of the producing cells.

Divalent cations abolished quasi-lysis and reduced killing but allowed a delayed release of colicin A for both wild-type and *pldA* cells. EDTA, in contrast, caused a severe drop of OD₆₀₀ and a high level of killing but weak release of colicin A and a normal release of Cal in both types of cells. Triton X-100 increased the quasi-lysis, lethality and release of colicin, again with similar effects for *pldA*⁺ or *pldA* cells. It also caused a significant release of Cal to the culture supernatants.

Evidence that the colicin lysis proteins might exert their various functions by activating the outer membrane phospholipase A was first presented by Pugsley & Schwartz (1984). This activation was proposed to trigger a cascade of events first involving an alteration of the membrane permeability due to the formation of lysophospholipids with detergent-like properties. This would result in the release of colicin and various other proteins, with quasi-lysis reflecting the leakage of these various cell components to the medium. It has been shown that mutants defective in phospholipase A do not undergo a pronounced quasi-lysis or release significant amounts of colicin E2 (Pugsley & Schwartz, 1984), cloacin DF13 (Luirink *et al.*, 1986) or colicin A (Cavard *et al.*, 1987). The fact that these mutants could release colicin E2 (Pugsley & Schwartz, 1984) in the presence of Triton X-100 has been explained as a bypass of the phospholipase A activation by the detergent.

Fig. 8. Effect on Triton X-100 on Cal and colicin A release in various strains. Cultures of *E. coli* W3110 containing various plasmids as indicated on the figure were treated as described in Fig. 7. After 5 h of induction, samples were centrifuged and the supernatants (10 μ l) were mixed with loading buffer and analysed on a gel. On the fluorogram presented, the positions of colicin A ($\rightarrow$), fragments of colicin A ($\blacksquare$) and mature Cal ($\blacktriangleright$) are indicated.

Here we have shown that, in both *pldA*⁺ and *pldA* cells, Triton X-100 causes the early release of not only colicin A but also the lysis protein, Cal, itself. This probably reflects the solubilization of the Cal protein from the envelope by the detergent. Fractionation of induced cells has indicated that Cal is localized mainly in the inner membrane of the cell (unpublished). Thus this result suggests that after a certain period of induction, the inner membrane becomes accessible to the detergent present in the medium whether or not the cells contain the PldA phospholipase. The detergent also results in quasi-lysis and the death of the cell without phospholipase activation, but all of these effects still depend on the presence of a functioning lysis protein, since none of them are observed in cells containing a mutant Cal (Fig. 7 and data not shown).

The effect of divalent cations in preventing quasi-lysis without markedly interfering with the release of colicin has been interpreted as reflecting either an interference with phospholipase A activation (Pugsley & Schwartz, 1984) or an interference in an autolytic process which follows phospholipase A activation (Luirink *et al.*, 1986). It has been shown that Mg²⁺ ions inhibit the autolysis of *E. coli* cells while EDTA provokes it (Leduc *et al.*, 1982, 1984). However, experiments using autolytic-deficient mutants suggest that the autolytic system is not required for the function of the Cal protein and thus is not responsible for either the release of colicin A or quasi-lysis (Howard *et al.*, 1987).

Furthermore, as for Triton X-100, the effects of varying the concentration of divalent cations were similar for both *pldA*⁺ and *pldA* cells. The addition of Mg²⁺ ions protected both W3110 and CBM cells from the lethal effects of induction, while EDTA increased these effects for both types of cells. Similarly, Mg²⁺ ions decreased the severity of quasi-lysis while EDTA increased it, again for both *pldA*⁺ and *pldA* cells, and for both strains the protection by Mg²⁺ was reversible, since the addition of EDTA caused an immediate loss of OD₆₀₀. The effects of

addition or removal of divalent cations on colicin release were more complex. For both *pldA*⁺ and *pldA* cells the level of colicin release was reduced in the presence of EDTA, while it was slightly decreased in the presence of Mg²⁺. In fact, site-directed mutagenesis studies suggest that a positive charge of Cal protein may be important for its function (Howard *et al.*, 1989). Therefore, Cal may disturb the membrane surface potential in such a way that addition of divalent cations could attenuate the resulting cytolysis and maintain the envelope structure.

The effects of the various agents tested on the functions of Cal suggest that it causes extensive damage to the *E. coli* cell envelope irrespective of the presence of phospholipase in the outer membrane of the producing cells. Although *pldA* cells are less likely to die, release less colicin and display less quasi-lysis than wild-type cells, all of these effects are still pronounced during colicin induction. We propose that the presence of the Cal lipoprotein accumulating throughout induction disorganizes the structure of the envelope of the cells. Thus the outer membrane becomes permeable and sensitive to the action of detergents and control over the activity of the phospholipase is lost. Further damage is caused by the activity of this enzyme or by added detergent, resulting in the actual loss of membrane material (including Cal) by solubilization or blebbing, as previously described for membrane structure mutants (Chakraborti *et al.*, 1986). Divalent cations would presumably protect the envelope against these events by forming salt bridges between the negatively charged phospholipid and LPS constituents of the envelope.

Further experiments are required in order to determine both the molecular nature of the changes in the envelope caused by Cal and the mechanism by which they are brought about.

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