JOURNAL OF BACTERIOLOGY, Jan. 1989, p. 410–418
0021-9193/89/010410-09$02.00/0

Vol. 171, No. 1

Amino Acid Sequence and Length Requirements for Assembly and Function of the Colicin A Lysis Protein

S. PETER HOWARD, DANIÈLE CAVARD, AND CLAUDE LAZDUNSKI*

Centre de Biochimie et de Biologie Moléculaire du Centre National de la Recherche Scientifique, 31 Chemin Joseph Aiguier, B.P. 71, 13402 Marseille Cedex 9, France

Received 15 July 1988/Accepted 8 October 1988

The roles of the various parts of the mature colicin A lysis protein (Cal) in its assembly into the envelope and its function in causing "quasi-lysis," the release of colicin A, and the activation of phospholipase A were investigated. By using cassette mutagenesis, many missense mutations were introduced into the highly conserved portion of the lysis protein. In vitro mutagenesis was also used to introduce stop codons after amino acids 16 and 18 and a frameshift mutation at amino acid 17 of the mature Cal sequence. The processing and modification of the mutants were identical to those of the wild type, except for the truncated Cal proteins, which were neither acylated nor processed. Thus, the carboxy-terminal half of Cal must be present (or replaced by another peptide) for the proper processing and assembly of the protein. However, the specific sequence of this region is not required for the membrane-damaging function of the protein. Furthermore, the sequence specificity for even the conserved amino acids of the amino-terminal half of the protein is apparently exceedingly relaxed, since only those mutant Cal proteins in which a highly conserved amino acid has been replaced by a glutamate were impaired in their function.

Most of the plasmids which encode colicins also encode a small protein, called lysis protein (or bacteriocin release protein), which is required for the release of colicins from producing cells to the extracellular medium (6, 11, 12, 24, 26, 28, 30, 31). The genes for these small, 28- to 33-amino-acid proteins form an operon with the colicin structural gene and are coregulated under SOS control (8, 11, 16, 27, 29). However, lysis proteins are produced in lower amounts than the colicins due to the presence of a transcription terminator after the colicin gene (8, 10, 11, 17).

The primary structures of many colicin lysis proteins have been determined from the nucleotide sequences of the corresponding colicin operons (9, 25). They all contain a signal peptide with a cysteine residue at the cleavage site, and in all cases so far examined they have been found to be modified by the addition of lipid, very likely through the same pathway as the major lipoprotein of *Escherichia coli* (7).

The CloDF13 lysis protein has been localized to both inner and outer membranes of *E. coli* (11, 24), while the colicin N lysis protein was found only in the outer membrane (25). In addition, in *Citrobacter freundii*, the mature form of the colicin A lysis protein (Cal) was found to be partly released into the extracellular medium (6).

The induction of lysis proteins has been shown to activate the normally dormant phospholipase A located in the outer membrane (7, 18, 27), thereby increasing the permeability of this membrane. However, it is not yet known whether lysis proteins cause direct or indirect activation of the phospholipase A or whether this is the primary effect of these proteins. Furthermore, as yet the mechanism by which lysis proteins allow the transfer of colicins across both the inner and outer membranes remains poorly understood. However, it has been demonstrated that the release process is nonspecific with respect to the colicin itself, which was shown not to contain any topogenic export signal in the case of colicin A (2).

The colicin A lysis protein (Cal) is a 33-amino-acid lipoprotein which is responsible for "quasi-lysis," which involves the release of colicin A and many other proteins from induced colicinogenic cells (6). It is produced as a precursor of 51 amino acids which undergoes remarkably slow processing, during which the unmodified precursor, modified precursor, mature Cal, and the signal sequence are visible for at least 30 min following pulse-labeling (7).

The amino acid sequences of almost all lysis proteins are very homologous and even identical for certain of the E colicins (9, 25, 30). The sequence of the mature (lipid-modified) form of Cal, with the "consensus" lysis protein amino acids underlined, is Cys-Gln-Val-Asn-Asn-Val-Arg-Asp-Thr-Gly-Gly-Gly-Ser-Val-Ser-Pro-Ser-Ser-Ile-Val-Thr-Gly-Val-Ser-Met-Gly-Ser-Asp-Gly-Val-Gly-Asn-Pro. Cal displays marked homology with other lysis proteins in its amino-terminal 18 amino acids but appears unrelated to the others in the carboxy-terminal half of the peptide.

Despite the small size of lysis proteins, their structure-function relationships have not yet been extensively studied. It has been shown for both Cal and CelB (the ColE2 lysis protein) that if the amino-terminal cysteine residue is replaced or prevented from being modified, the lytic properties of the proteins are severely reduced or abolished (7, 26). However, the functional significance of the rest of the consensus amino-terminal region of the mature protein is not known.

In an attempt to address this question, we have used in vitro mutagenesis to alter selected amino acids or regions of Cal. The mutants were then examined with respect to the function of the lysis protein in causing the quasi-lysis phenomenon and the release of colicin and other proteins following induction of the colicin A operon with mitomycin (MTC). The results indicate that the carboxy-terminal portion of Cal is not required for its function but is required for the proper assembly and processing of the protein in the envelope of the producing cells. In addition, when any one of three of the most highly conserved amino acids in the amino-terminal portion of the protein was replaced by an amino acid which was completely unrelated in physical

* Corresponding author.

properties, quasi-lysis and the release of colicin A were found to be unaffected except when the introduced amino acid carried a negative charge.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strains W3110 and JE5505 *lpp pps his proA argE thi gal xyl mtl tsx* were used as the host strains in all experiments involving the synthesis and release of colicin A. JM105 *thi rpsL endA sbc-15 hsdR4* Δ(*lac-pro*) (F' *traD36 proAB lacI*^{q}ZΔM15) (32), JM105 made *mutL* by P1 transduction from BMH 71-18 *mutL* (14), M13mp18 *amIV* (4), and C600 *thr leu thi lacY1 supE44* (1) were used for mutagenesis and recombinant DNA procedures.

The plasmid pAE11 (7), derived from pColA9 (16), was used as the source of the *cal* gene for in vitro mutagenesis.

Growth conditions. Strains were routinely grown in LB medium. For radiolabeling of proteins with [^{35}S]methionine or [^{35}S]cysteine, they were grown in M9 medium supplemented with thiamine (1 μg/ml), glycerol (0.4%), required amino acids (50 μg/ml), and methionine assay medium (0.5%) or cysteine assay medium (0.5%) (Difco Laboratories, Detroit, Mich.). The same medium was used for [2-^{3}H]glycerol labeling, except that it contained 0.4% lactate as the carbon source.

MTC was used as the inducer of the colicin A operon at 300 ng/ml, in cultures having an OD_{600} of 1. All cultures were incubated at 37°C with good aeration.

Labeling experiments. All labeling was performed after 45 min of MTC treatment. Cells were labeled with [^{35}S]methionine (45 μCi/ml, 1,350 Ci/mmol) for 1 min and chased with 500 μg of unlabeled methionine per ml. [^{35}S]cysteine was added at a concentration of 25 μCi/ml (1,200 Ci/mmol) for 2 min in the presence of methionine (25 μg/ml) and chased with unlabeled cysteine (250 μg/ml). Lipoproteins were labeled with [2-^{3}H]glycerol (133 μCi/ml, 1 Ci/mmol) for 90 min without a chase. After various periods of chase as noted in the figure legends, 125-μl samples were precipitated with an equal volume of 25% ice-cold trichloroacetic acid and washed with 90% acetone before being solubilized in sample buffer in preparation for electrophoresis. For analysis of phospholipids, cells were prelabeled by overnight growth in LB containing 10 μCi of [U-^{14}C]acetate (57 mCi/mmol) per ml. The cells were then diluted to an OD_{600} of 0.2 in fresh medium, grown to an OD_{600} of approximately 1.0, and induced with 300 ng of MTC per ml. After a further 4 h of incubation, the cultures were sampled and their lipids were extracted as described previously (7). After thin-layer chromatography, the labeled lipids were located by autoradiography, scraped from the plates, and counted in scintillation fluid.

Site-directed mutagenesis and recombinant DNA procedures. Oligonucleotides (Table 1) were synthesized by phosphoramidite chemistry on an Applied Biosystems model 381A DNA synthesizer. For in vitro M13 mutagenesis (used to construct pKA with oligomers a and b and pS18 with oligomer c), the *Sph*I-*Hin*dIII fragment of pAE11, which contains the coding sequence for the mature Cal protein in which the *Sph*I site had been previously created (7), was inserted into M13mp18 *amIV* (4, 21). Single-stranded DNA (1 μg) was prepared and incubated at 85°C with 10 pmol of unphosphorylated mutagenic primer and 10 pmol of phosphorylated amber correction oligonucleotide in 10 μl of 10 mM Tris hydrochloride (pH 8.0)–10 mM $MgCl_2$. After being cooled to room temperature, the primers were extended for 8 h at 10°C with 1 U of DNA polymerase I (Klenow fragment) and 5 U of T4 DNA ligase in the presence of 0.25 mM deoxyribonucleoside triphosphates and 0.5 mM ATP. Samples were used to transfect $CaCl_2$-treated JM105 *mutL* cells, and mutants were identified either by direct analysis of mini-RF preparations or by hybridization screening with the mutagenic oligonucleotide as described previously (4). After dideoxy sequencing of a region encompassing the entire *cal* gene, the *Sph*I-*Hin*dIII fragment which contained the mutation was exchanged with the same fragment of pAE11 or pKA.

Cassette mutagenesis was performed as outlined in Fig. 1.

TABLE 1. Deoxyribonucleotides used for the construction of *cal* mutants

Oligomer	Sequence[a]
a	5'-GTTTCACCCTCATCTATCG-3'
b	5'-CACCCTCGAGTATCGTTACC-3'
c	5'-CCCTCGAGTTAATAGTTACCGG-3'
d	5'-CCAAGTAAACAATGTCGNAGATACTG-3'
e	5'-TCTNCGACATTGTTTACTTGGCATG-3'
f	5'-GAGGTGGTTCTGTTTCACCC-3'
g	5'-TCGAGGGTGAAACAGAACCACCTCCAGTA-3'
h	5'-CCAAGTAAACAATGTCAGGGATACTG-3'
i	5'-TCCCTGAGATTGTTTACTTGGCATG-3'
j	5'-GAGNAGGTTCTGTTTCACCA-3'
k	5'-TCGATGGTGAAACAGAACCTNCTCCAGTA-3'
l	5'-GAGGTGGTTCTCNATCACCA-3'
m	5'-TCGATGGTGATNGAGAACCACCTCCAGTA-3'
n	5'-GAGGTGGTTCTGTTTCACCCTAG-3'
o	5'-TCGACTAGGGTGAAACAGAACCACCTCCAGTA-3'

[a] N, Any nucleotide.

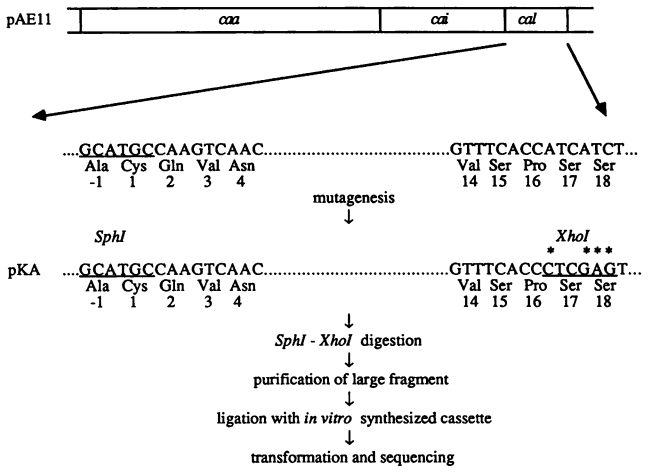


FIG. 1. Strategy for the mutagenesis of *cal*. Part of the sequence of the *cal* gene of plasmid pAE11 is shown, with the nucleotides of the *Sph*I site underlined. By using in vitro mutagenesis, four bases (starred) of *cal* were changed, creating an *Xho*I site (underlined) without changing the amino acid composition of the gene product. After *Sph*I-*Xho*I digestion of pKA, the large fragment was ligated with a cassette containing a degenerate base, allowing the isolation of multiple mutations from the same cassette. Thus, codon 7 of the Arg-7 mutagenesis cassette contained the sequence GNA (where N is any nucleotide) instead of AGG in the wild type, codon 11 of the Gly cassette contained GNA instead of GGT, and codon 14 of the Val cassette contained CNA instead of GTT. The numbering of the Cal amino acids is that of the mature form.

The cassettes used to create the various mutations were composed of the oligonucleotides listed in Table 1 as follows: oligomers d, e, f, and g for the Arg-7 substitutions; oligomers h, i, j, and k for the Gly-11 substitutions; oligomers h, i, l, and m for the Val-14 substitutions; oligomers h, i, n, and o for S16. In each case, the 4-oligonucleotide cassette (the internal 5'-deoxynucleotides of which had been phosphorylated with polynucleotide kinase) was allowed to prehybridize in 10 mM $MgCl_2$–10 mM Tris (pH 7.5) after being heated to 80°C. This was then ligated at a 100:1 molar ratio to the large *Sph*I-*Xho*I fragment of pKA, which had been purified from low-melting-point agarose. To create the FS2 mutation, pKA was digested with *Xho*I, treated with T4 DNA polymerase in the presence of deoxyribonucleoside triphosphates, and religated. This resulted in the duplication of the T of the *Xho*I site in pKA, causing a +2 frameshift. For all of the mutants constructed by using plasmids, minipreparations of the plasmids obtained after transformation of C600 cells were sequenced to identify the mutations before being transformed into W3110 and resequenced.

General methods. For analysis of proteins released into the culture medium upon induction of the colicin A operon, samples were taken from induced or uninduced cultures and centrifuged for 1 min at 15,000 × *g*. Cell pellets and supernatants were dissolved in electrophoresis sample buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and SDS-PAGE gels with urea were electrophoresed and treated for fluorography as previously described (7).

Materials. All enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used according to their recommendations. MTC was purchased from Sigma Chemical Co. (St. Louis, Mo.). Globomycin was a gift from Mamoru Arai (Sankyo, Tokyo). [^{35}S]methionine, [^{35}S]cysteine, [U-^{14}C]acetate, and [2-^{3}H]glycerol were purchased from the Radiochemical Centre, Amersham, United Kingdom. All other chemicals used were reagent grade.

RESULTS

Construction of *cal* gene mutants. An *Sph*I restriction site had been previously introduced into the *cal* gene of pColA9 to facilitate site-directed mutagenesis at the processing and modification site of pro-Cal (7). This led to plasmid pAE11. Here, the construction strategy was to create a cassette containing the DNA region encoding the 18-amino-acid consensus part of Cal to investigate the functional significance of this region and of the carboxy-terminal sequence of the protein.

To this end, an *Xho*I site was first introduced by oligonucleotide-directed mutagenesis of pAE11 (Fig. 1). The restriction site introduced was chosen so that the base changes resulting in the creation of the site did not change the amino acid sequence of Cal. The resulting plasmid (pKA) was then cut with *Sph*I-*Xho*I, and various mutations were introduced into the *cal* gene by insertion of synthetic oligonucleotides (Fig. 1). These oligonucleotides were designed to substitute conserved amino acids by others having widely different physical properties, to determine their importance to the function of Cal. This series of Cal mutants consisted of 10 point mutants (Fig. 2) in which the highly conserved amino acids Arg-7, Gly-11, and Val-14 were substituted by using the procedure described in the legend to Fig. 1.

A second series of mutants were designed to test the importance of the C-terminal region of Cal to its function (Fig. 2). Two mutant plasmids (pS16 and pS18) coded for truncated Cal proteins which contained only the very homologous first 16 or 18 amino acids of the normal 33-amino-acid lysis protein. Cleavage of pKA with *Xho*I followed by incubation with T4 DNA polymerase and religation resulted in a frameshift mutation. This plasmid (pFS2) coded for a Cal protein in which the amino acid sequence (amino acids 17 to 33) following the conserved amino-terminal portion of Cal was completely altered by the frameshift, as well as being shortened by 6 amino acids (Fig. 2).

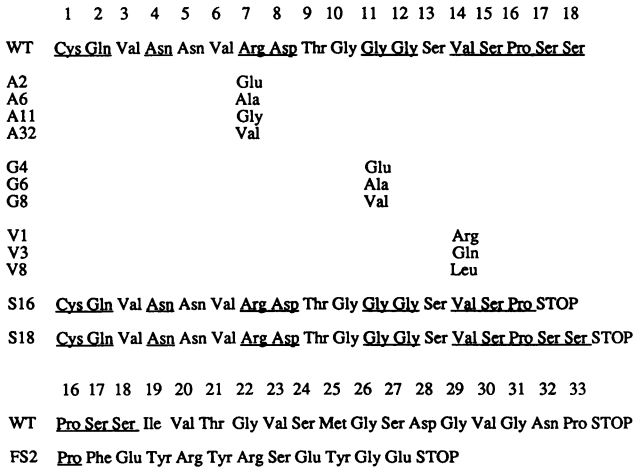
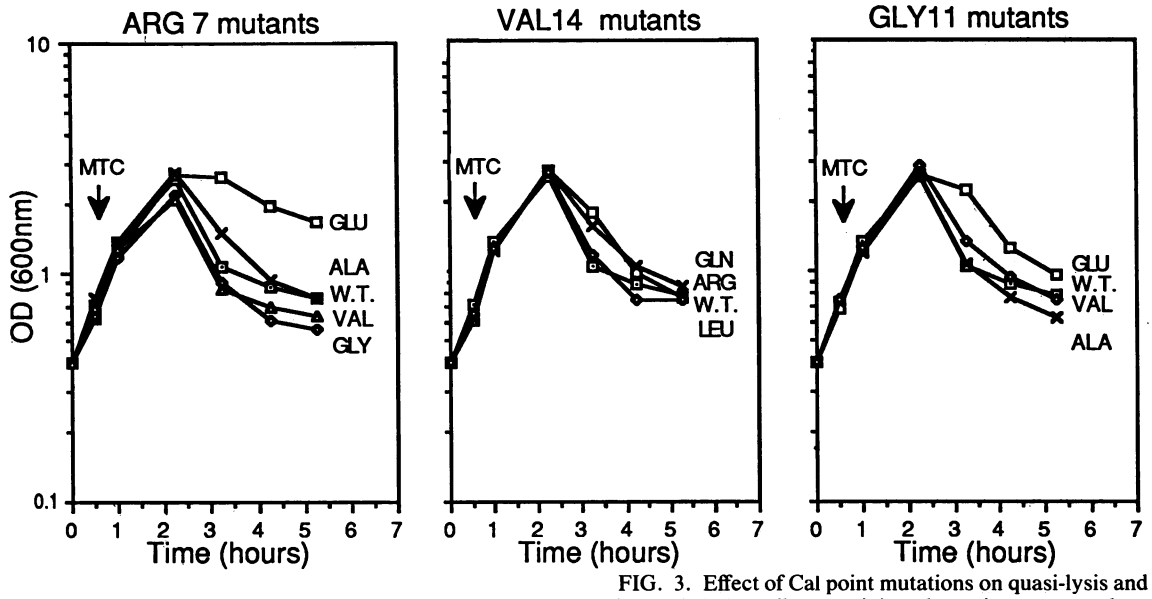


FIG. 2. Primary structure of the Cal point mutants. The primary structure of the wild-type (WT) Cal protein is indicated, with the consensus amino acids underlined. The numbering of the amino acids is that of the mature form of Cal. For the point mutations only the changed amino acid is shown, while the complete primary sequences of the S16 and S18 mutants are shown. These were constructed by the insertion of a TAA codon after residues 16 and 18 of Cal, respectively. At the bottom of the figure, the sequence of the carboxy-terminal part of the FS2 mutant is presented and compared with that of the wild type.

Effect of the *cal* point mutations on quasi-lysis, colicin release, and phospholipase A activation. The three major phenotypic effects of colicin operon induction which have been shown to be strictly dependent on lysis gene expression are a large but incomplete drop in the optical density of the colicinogenic culture (quasi-lysis), the concurrent release of large quantities of colicin and other soluble cell proteins, and a pronounced degradation of cellular lipids, caused by the activation of the phospholipase A of the outer membrane. Cells containing the mutant plasmids were thus induced with MTC and examined for these effects as a measure of the function of the mutant Cal proteins.

The mutants for which the Arg at position 7 had been changed to Ala, Val, or Gly were found to be unaffected for either quasi-lysis or the release of colicin A (Fig. 3). The wild-type and mutant cultures began to lyse at the same time, and both the spectrum and quantity of proteins released from the cells were very similar. Among the four mutants constructed involving this amino acid, only the replacement by Glu was found to impair the function of the protein. In this case, essentially none of the colicin was released over the course of the experiment, and the culture suffered only a slight decline in optical density long after induction. Similar results were obtained for the mutants involving Gly-11, which could be changed to Val or Ala without significantly affecting the function of the protein. Again, among those amino acids tested, only the replacement by Glu was found to affect quasi-lysis or the release of



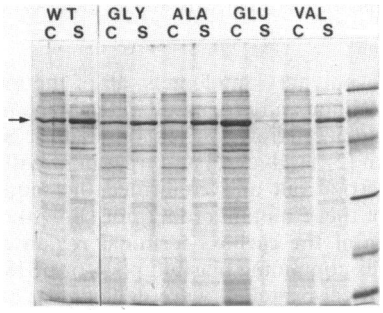


FIG. 3. Effect of Cal point mutations on quasi-lysis and colicin A release. W3110 cells containing the point mutant plasmids were grown in LB medium and induced with MTC at the time indicated with the arrows (top). The OD_{600} of the cultures was measured at intervals and plotted against the time of incubation at 37°C. After 3 h of induction, samples of the four Arg-7 mutants were centrifuged, and quantities of the cells (C) and culture supernatants (S) representing equal amounts of the original culture were analyzed by SDS-PAGE. The Coomassie blue-stained gel is presented (bottom). The standards in the right lane are (from top to bottom) phosphorylase A (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). The position of colicin A is indicated by an arrow. WT, Wild type.

the colicin, and in this case, the effect was less pronounced than for the replacement of Arg-7 by Glu (Fig. 3). The amino acid Val-14 could be changed to Gln, Arg, or Leu without affecting the function of Cal in causing either quasi-lysis or the release of colicin A (Fig. 3).

The lipid composition of cells containing the various plasmids was evaluated after [^{14}C]acetate labeling followed by induction with MTC. As shown in Table 2, induction of the wild-type *cal* gene in W3110(pColA9) cells resulted in a dramatic decrease in the levels of phosphatidylethanolamine and phosphatidylglycerol and concurrent large increases in the amount of lysophospholipids and of free fatty acids. Induction of W3110 cells carrying *cal* point mutant plasmids such as pV1 or pA11 provoked the same effects as observed for the wild type, whereas those containing pA2 and pG4, in which a glutamate residue had been introduced, did not (Table 2). In these cells there were only slight increases in the quantities of lysophospholipids and free fatty acids. The results for these strains resembled those previously obtained for *cal* mutants containing alterations at the lipid modification site, which also display a reduction in the extent of quasi-lysis and release of colicin A (7).

TABLE 2. Effect of Cal and mutant Cal induction on lipid composition

Strain	% of radioactivity recovered after thin-layer chromatography[a]					
	Lyso	PG	PE	DPG	FFA	NL
W3110(pColA9),N.I.[b]	0.4	7.1	90.7	0.4	0.4	1.1
W3110(pColA9)	8.1	1.1	35.8	0.8	51.6	2.6
W3110(pA11)	8.5	2.9	47.4	0.9	37.8	2.5
W3110(pV1)	7.7	2.9	46.4	1.2	38.8	3.0
W3110(pA2)	1.4	5.8	83.4	0.7	5.7	3.0
W3110(pG4)	1.7	7.7	79.6	0.7	6.2	4.2
W3110(pS16)	1.0	6.5	86.8	0.6	2.7	2.4
W3110(pS18)	1.2	7.0	87.3	0.5	2.8	1.3
W3110(pFS2)	8.8	1.9	45.7	1.0	39.7	2.9

[a] Lyso, Lysophosopholipids; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; FFA, free fatty acids; NL, neutral lipids.

[b] N.I., Not induced. All other strains were induced with MTC (300 ng/ml) for 4 h before harvesting and lipid extraction.

Modification and processing of the *cal* point mutants. We have previously observed that Cal is a slowly processed lipoprotein for which the unmodified precursor, lipid-modified precursor, mature form, and signal sequence can be identified on fluorograms after electrophoresis of [^{35}S]methionine-pulse-labeled cells (7). The various forms of Cal migrate very close or similarly to the various forms of the major lipoprotein Lpp.

To clearly demonstrate the migration of the various Cal forms in our gel system, we compared the synthesis of Cal in both *lpp*$^+$ and *lpp* strains carrying pColA9. As shown in Fig. 4a and c, after pulse-labeling with [^{35}S]methionine and 30 min of chase, the Cal mature form and its signal peptide were clearly detected in MTC-treated cells. The Cal mature form migrated underneath the major lipoprotein (missing in the

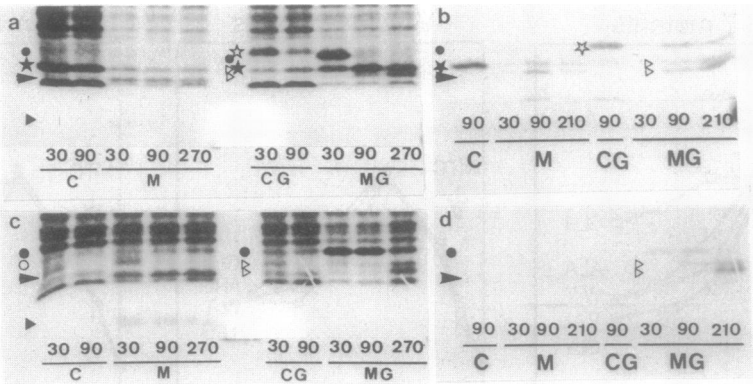


FIG. 4. Modification and processing of the Cal protein in *lpp*$^+$ and *lpp* strains. Cells of W3110(pColA9) and JE5505(pColA9) were grown in LB medium, centrifuged, and incubated in M9 medium before being induced with MTC (M) or not induced (C). Globomycin was added to control cells (CG) or induced cells (MG) after 10 min of induction. (a) After 45 min of induction, W3110(pColA9) cells were pulsed for 2 min with [^{35}S]methionine. After 30, 90, and 270 min of chase, samples (10 μl) were taken and analyzed by urea-SDS-PAGE. Part of the fluorogram is presented. The various forms of Cal are indicated: precursor (○), modified precursor (●), mature (►), signal sequence (▶), and fragment of modified precursor (▷). The two forms of the major lipoprotein are indicated: mature form (★) and modified precursor form (☆). (b) After 40 min of induction, W3110(pColA9) cells were labeled with [2-^{3}H]glycerol. After 30, 90, and 210 min of labeling, samples (10 μl) were taken and analyzed by urea-SDS-PAGE. Part of the fluorogram is presented. The various forms of Cal and of Lpp are indicated as in panel a. (c) Same experiment as in a but with JE5505(pColA9) cells. (d) Same experiment as in b but with JE5505(pColA9) cells.

lpp strain), and its signal peptide migrated much lower than the mature form due to its low molecular mass (1.5 kilodaltons [kDa]). The unmodified Cal precursor migrated with the major lipoprotein and therefore could be distinguished only in the induced *lpp* strain. The modified Cal precursor migrated above the precursor form, just underneath the Lpp-modified form, as demonstrated by globomycin treatment of labeled cells. In the globomycin-treated *lpp*$^+$ strain, it accumulated for 30 min after labeling and was then degraded (Fig. 4a), as reported previously (7). It should be noted that the modified Lpp precursor form was also unstable, as seen in control cells. In the globomycin-treated *lpp* strain, the modified Cal precursor was more stable than in the *lpp*$^+$ strain, since a substantial amount was still present after 4 h of chase (Fig. 4c).

The presence of the two acylated forms of Cal (mature form and modified precursor) was also demonstrated by [2-^{3}H]glycerol labeling (Fig. 4b and d). In the *lpp*$^+$ strain, both Cal forms were observed after 30 min of labeling in induced cells together with a small amount of Lpp mature form. The modified Cal precursor form was chased into mature form after 90 min. Lpp was absent in *lpp* cells, in which only the Cal mature form was present after MTC induction. After globomycin treatment, the Cal precursor form accumulated until 90 min after labeling and then was split into two fragments of lower molecular weight. The same pattern was obtained in induced cells of both strains. A decrease in Lpp synthesis by MTC treatment (7) was more marked in the presence of globomycin than in its absence in the *lpp*$^+$ strain. The hydrolysis of modified precursor Cal observed with [^{35}S]methionine labeling (7) (Fig. 4a and c) must be due to a proteolytic cleavage giving rise to acylated fragments containing either one or both methionine residues in positions −5 and +25.

In order to investigate whether the processing and modification of the mutant Cal proteins (especially those in which its function was affected) remained normal, the mutants Arg-7→Glu (A2), Gly-11→Glu (G4), and Val-14→Arg (V1) were pulse-labeled with [^{35}S]methionine, chased, and subjected to SDS-PAGE (Fig. 5). Although the presence of the point mutations altered the mobility of the Cal derivatives on the urea-SDS-polyacrylamide gels, all of the mutants examined were modified and processed in the same manner as the wild type, as demonstrated by labeling with [^{35}S]methionine. Labeling with [2-^{3}H]glycerol and treatment with globomycin were also carried out and confirmed the modification and processing of these mutants (data not shown).

Importance of the carboxy-terminal region of Cal for its function. Cells containing plasmid pS16 or pS18, which code for truncated Cal proteins, were deficient in causing quasilysis (Fig. 6) and the release of colicin A (Fig. 6) despite the fact that they decreased cell viability (data not shown). This result suggested that the sequence of the carboxy-terminal portion of Cal is crucial for its function. To test this

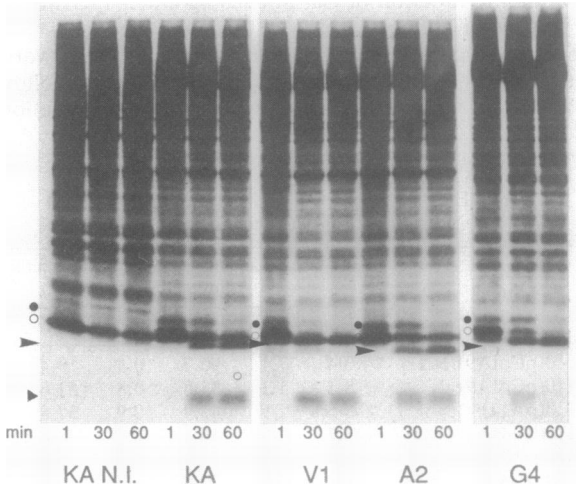


FIG. 5. Modification and processing of the Cal point mutants. W3110 cells containing plasmid pKA, pV1, pA2, or pG4 were grown in M9 medium and induced with MTC or not induced (N.I.), and after a further 45 min were pulsed for 1 min with [^{35}S]methionine. After 1, 30, and 60 min of chase, samples were taken and analyzed by urea-SDS-PAGE. The fluorogram is presented. The various forms of Cal are indicated: precursor (○), modified precursor (●), mature (►), and signal sequence (▶).

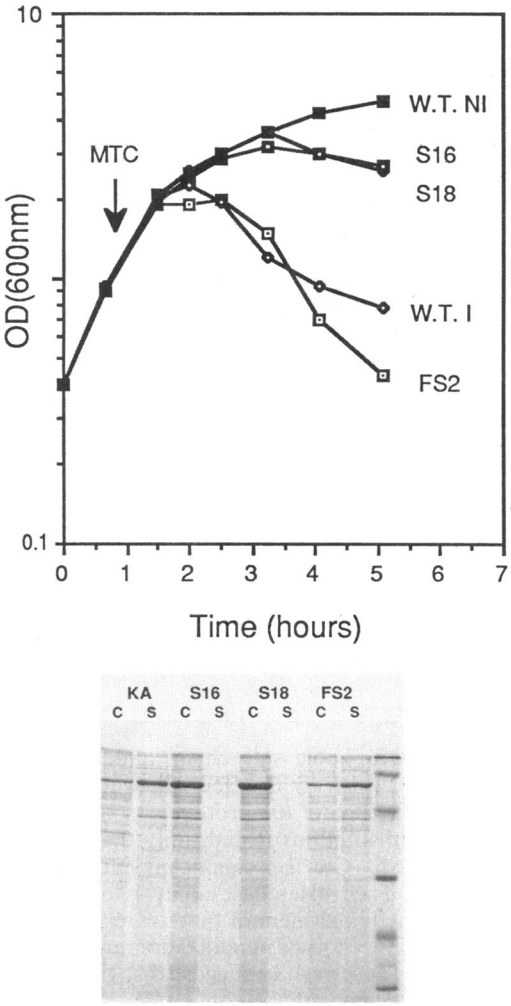


FIG. 6. Effect of nonsense and frameshift mutations in Cal on quasi-lysis and colicin A release. W3110 cells containing plasmid pKA (wild type [W.T.]), pS16, pS18, or pFS2 were grown in LB medium and induced (I) with MTC at the time indicated by the arrow or not induced (NI). The OD_{600} of the cultures was measured at intervals and plotted against the time of incubation (top). After 3 h of induction, samples of the cultures were centrifuged, and cells (C) and supernatants (S) were analyzed by SDS-PAGE. The Coomassie blue-stained gel is presented (bottom). M_r markers (rightmost lane) are as in Fig. 3.

hypothesis, the frameshift mutant plasmid pFS2 was constructed. The mutant lysis protein encoded by pFS2 had a carboxy-terminal sequence totally different from that of the wild-type protein (Fig. 2). However, when cells containing this plasmid were induced with MTC, they lysed and released colicin and other cellular proteins as efficiently as cells producing wild-type Cal (Fig. 6). Again, phospholipid analysis gave results which mirrored those obtained for quasi-lysis and the release of colicin A. The S16 and S18 mutant Cal proteins did not cause significant activation of phospholipase A, while induction of FS2 expression resulted in degradation of cellular phospholipids that was very similar to that observed for cells producing wild-type Cal (Table 2).

Truncated Cal proteins are neither modified nor processed. The results described above indicated that the Cal proteins which were truncated after amino acid 16 or 18 were completely inactive while a mutant protein which was only 6 amino acids shorter than Cal but completely different in sequence following amino acid 17 was fully functional. This suggested that the specific carboxy-terminal amino acid sequence of Cal is not required for its function but that a minimum length of the protein is required for it to function at all. We examined the processing of these mutants in order to determine whether this length requirement acts at the level of Cal assembly.

Wild-type Cal contains methionine and cysteine residues in both the signal sequence and the mature portion of the protein. In contrast, the three mutant proteins S16, S18, and FS2, in addition to being smaller than the wild type, contain a cysteine residue in both the signal sequence and the mature protein but only methionine residues in the signal sequence. Cells containing the mutant plasmids were induced and pulse-chased with both [^{35}S]methionine and [^{35}S]cysteine in separate experiments, allowing us to confidently identify precursor and processed forms of these radically altered Cal proteins.

For both S16 and S18, only a single labeled band running well below the major lipoprotein was observed upon labeling with [^{35}S]methionine (Fig. 7), which appeared to be very slowly degraded. No forms of lower apparent molecular weight were observed, even after long periods of chase. Exactly the same pattern resulted after [^{35}S]cysteine labeling of these mutants (not shown). W3110(pFS2) cells labeled with [^{35}S]methionine contained a band of slightly lower apparent molecular weight than wild-type precursor Cal, which decreased in intensity with increasing times of chase, and the signal sequence, which increased in intensity with increasing time of chase. When these cells were labeled with [^{35}S]cysteine, an additional band, corresponding to the mature form of the mutant Cal, also appeared with increasing time of chase (Fig. 7). For both types of labeling, a faint band running above the major lipoprotein was also observed, which may correspond to the lipid-modified precursor form of this mutant Cal.

In order to assess the extent of lipid modification and processing of truncated and frameshift Cal mutants, induced W3110 cells carrying the mutated plasmids were labeled with [2-^{3}H]glycerol in the presence or absence of globomycin. A band corresponding to Cal was obtained in wild-type and in induced W3110(pFS2) cells but was absent in control cells and in induced W3110(pS16) and W3110(pS18) cells (Fig. 8). In the presence of globomycin, two bands were obtained for wild-type induced cells that corresponded to the modified precursor Cal and to its fragment(s), while only one band was obtained for induced W3110(pFS2) cells. This band corresponded to the acylated FS2 precursor form, which was more stable than the wild-type precursor, as also indicated by [^{35}S]methionine labeling of globomycin-treated cells (data not shown). No band was seen for S16 or S18, indicating either that no modification and processing took place or that rapid hydrolysis occurred. Shutoff of Lpp synthesis was observed in all induced cells, as demonstrated by comparing the labeling with that of control cells. In control cells, the Lpp mature form was strongly labeled in the absence of globomycin and weakly labeled in its presence, together with the modified Lpp precursor form.

These results indicated that FS2 is both processed and modified just like the wild-type Cal protein, whereas S16 and S18, despite having the same sequence for at least 16 amino acids on either side of the modification and processing site, are not.

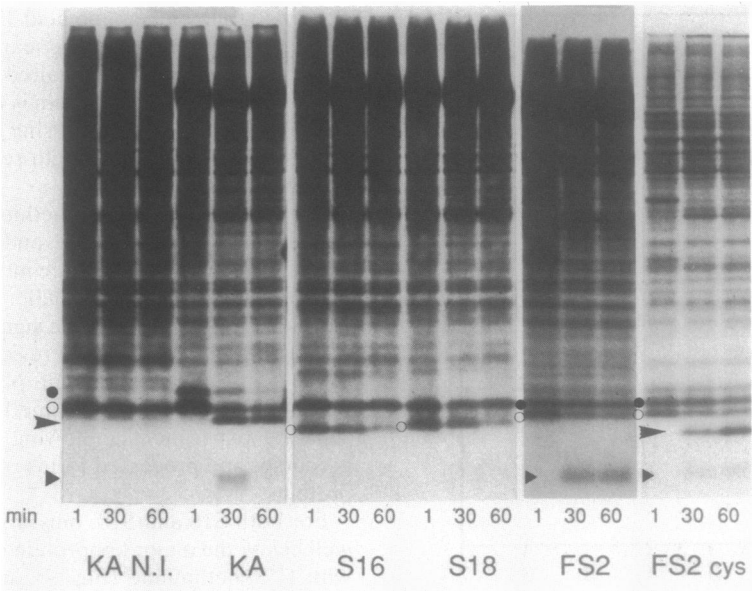


FIG. 7. Truncated Cal proteins are neither modified nor processed. W3110 cells containing plasmid pKA, pS16, pS18, or pFS2 grown in M9 medium were induced with MTC or not induced (N.I.), and after a further 45 min were pulsed for 1 min with [^{35}S]methionine or for 2 min with [^{35}S]cysteine (cys). After 1, 30, and 60 min of chase, samples were taken and analyzed by urea-SDS-PAGE. The fluorogram is presented. The various forms of the Cal protein are indicated as in Fig. 4.

DISCUSSION

The high degree of conservation of the first 18 amino acids of the mature form of lysis proteins has been pointed out by various authors (6, 9, 11, 25). This conservation suggested some structural requirement related to the function. This idea has even been extended to the leader sequences of the various lysis proteins, which have significant local similarities with portions of phage lysis proteins, suggesting some functional and evolutionary relationship between lysis proteins encoded by colicinogenic plasmids and coliphages (15).

The results described above with the amino-terminal point mutants indicate that, despite a very high conservation of the amino-terminal amino acid sequence among the colicin lysis proteins, the sequence specificity in this region is very relaxed. Not only could each of the conserved amino acids tested be replaced by another amino acid, they could also be replaced by amino acids of entirely different chemical properties. Thus, Arg-7 could be replaced by Val, an amino acid with a large hydrophobic side chain, and Val-14 could be replaced by the positively charged Arg without detrimental effects on the function of the protein.

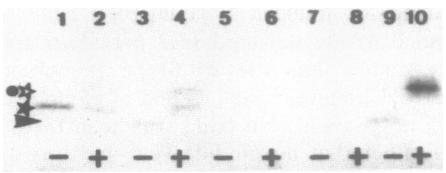


FIG. 8. Acylation and processing of truncated and frameshift Cal mutants. W3110 cells containing plasmid pKA, pS16, pS18, or pFS2 grown in M9 medium were induced with MTC or not induced for 10 min, and then globomycin was added (+) or not added (−). After 40 min of induction, cells were labeled with [2-^{3}H]glycerol and incubated for 90 min before being analyzed by urea-SDS-PAGE. Part of the fluorogram is presented. The mature and modified precursor forms of Cal and Lpp are indicated as in Fig. 4. Lanes 1 and 2, W3110(pKA) control cells; lanes 3 and 4, W3110(pKA) induced cells; lanes 5 and 6, W3110(pS16) induced cells; lanes 7 and 8, W3110(pS18) induced cells; lanes 9 and 10, W3110(pSF2) induced cells.

Among the substitutions tested, only the replacement of Arg-7 or Gly-11 by Glu had significant effects on the lytic activity of Cal. The pulse-chase analysis of these mutants indicated that the impairment of function was not caused by an inhibition of the normal modification and processing of these mutants. These results suggest that the charge of the amino-terminal region of Cal may play some role in its function. Although there is already an aspartate in this region, the addition of further negative charge appears to have an adverse effect. In contrast, the addition of a positive charge in this region in the V1 mutant and the presence of many positive and negative charges in the carboxy-terminal region of FS2 did not impair the function of Cal. It should also be mentioned that the lysis protein of colicin N contains an additional positive charge in the amino-terminal region relative to the other lysis proteins and five additional positive charges in its carboxy-terminal region (25).

A possible role for the leader sequence of lysis proteins in the lytic function has been proposed on the basis of loose homology with portions of phage lysis proteins (15). Indeed, the likelihood of this possibility was strengthened on the one hand by the observation that the signal sequences of Cal (6) and the ColE2 lysis protein (26) are apparently very stable, and on the other by the observation that when Cal or the ColE2 lysis protein was modified so that they could not be processed (preventing the release of the stable signal sequence), the lytic events following their induction were sharply attenuated or delayed (7, 26). However, the results presented here argue strongly against this hypothesis, since the Cal mutants A2 and G4, although impaired in the lytic function, were not impaired in processing and thus accumulated the signal sequence just as the wild-type did (Fig. 5). The question remains why the signal sequences of lysis proteins are so stable. For Cal, the observation that the band representing the signal sequence is only observed upon

processing of the modified Cal precursor (i.e., is absent during globomycin treatment of induced cells) (7) as well as the results of selective labeling experiments (6) indicate that it is indeed the signal sequence of Cal that is observed and not the signal sequences of the cell in general that are accumulated during colicinogenic induction. It may be that for structural reasons the lysis protein signal sequences are not susceptible to cleavage by signal peptide peptidase, and another possibility is that for some reason they are not able to contact the peptidase in the envelope. A study on the properties of various lipoprotein signal sequences has indicated that the Cal signal sequence is more hydrophobic than those of other lipoproteins (13), a factor which could be important in determining its stability.

The fact that the truncated proteins S16 and S18 were neither modified nor processed is very interesting. Like the mutant Cal proteins which were altered at the processing and cleavage site that we constructed previously (7), the S16 and S18 proteins were completely inactive. The ability to be modified and processed was entirely restored by the addition of an 11-amino-acid carboxy-terminal sequence in the frameshift mutant FS2. This addition also restored activity to the mutant protein. This result again supports the hypothesis that the modification is the key requirement for the activity of the lysis proteins. Moreover, this suggests that the carboxy-terminal sequence, not conserved among lysis proteins, functions only in the assembly of the nascent lysis protein polypeptide chains.

We do not yet know why the truncated Cal proteins cannot be posttranslationally processed while the full-length protein can be. It is possible that the explanation lies in a coupling between synthesis and export of the lysis protein. About 35 amino acids are masked within the large subunit of the ribosome during translation (3). When the polypeptide chain is truncated, as in S16 and S18, with the signal sequence of 18 amino acids, the nascent chains (totaling 34 or 36 amino acids) would not emerge from the ribosome and thus would not be accessible to the cellular machinery involved in export and modification. As a consequence, these nascent chains may be released into the cytoplasm. In contrast, the addition of a "spacer sequence" of 12 amino acids in FS2 may allow the nascent chain to emerge from the ribosome and be recognized and inserted into the pathway of export and modification. It must be noted, however, that Toba et al. found that the ColE2 and ColE3 lysis proteins could be truncated to 20 amino acids (39 for the precursor) without loss of activity (30). This would also seem to preclude recognition of anything more than the few positively charged amino-terminal amino acids of the signal sequence before translation was completed. Since we have shown that acylation and processing are essential for lysis protein function, we presume that these truncated lysis proteins were correctly modified and processed. It is possible that a conformation specific to the truncated Cal proteins renders them unable to enter the pathway of modification and processing posttranslationally, while those of the truncated colicin E2 and E3 lysis proteins can. Experiments are in progress to determine more precisely both the minimum length of the Cal precursor for its assembly and its dependence on the *E. coli* secretion loci which have been identified to date.

All of the results so far obtained indicate that aside from the structural features required for correct assembly, the modification of Cal by the addition of lipid to the amino-terminal cysteine seems to be the most important, if not the only, requirement for quasi-lysis and the release of colicin A. In this context, it is interesting that a family of lipopeptides extracted from the culture medium of various microorganisms have been shown to exhibit antibiotic properties. For example, iturin A and bacillomycin L and D, produced by *Bacillus subtilis*, and peptidolipin NA, produced by *Nocardia asteroides*, all have antifungal activity. These lipopeptides have been shown to dramatically increase the electrical conductance across planar lipid membranes (19, 20). Taking into account the large range of the conductance values, it has been proposed that local modifications in the structure of the bilayer may be induced by interaction with lipopeptide micelles (19). We have recently observed that the induction of Cal causes depolarization of the inner membrane in *E. coli* cells (manuscript in preparation). Disorganization of the lipid structure of the envelope may be the primary effect of Cal, with the activation of phospholipase A being a consequence of this perturbation. If this is the case, it is not surprising that the modification of Cal by lipid, rendering it amphiphilic, is required for its function.

Finally, our results leave unanswered the question of why the N-terminal region of mature lysis proteins is conserved. At present, we cannot present any simple explanation for this conservation. However, we did observe a high level of DNA sequence homology between regions involved in the replication control of the pColA, pColE1, and pCloDF13 plasmids, and this region borders the region encoding the lysis proteins of these three plasmids (22, 23). Some structural feature in this region may be required for a purpose related to the regulation of DNA replication and copy number control. This could explain a conservation of sequence which is not related to the function of the gene products. A second and perhaps more likely hypothesis is that the selective pressure on a functioning lysis protein has been insignificant in comparison to that acting on the colicin and immunity protein structural genes. Any cells producing a new colicin protein which could kill neighboring cells which had previously been immune would presumably quickly outgrow those neighbors. A similarly strong advantage would be expected to accrue to cells producing a variant immunity protein which had become resistant to a colicin which was scourging the local population. Such pressures are not as easy to envisage for the lysis proteins, which may thus be highly homologous only because they are separated by a short period of evolution, as is suggested by the high level of homology among the three colicin plasmids sequenced to date (22).

ACKNOWLEDGMENTS

We thank P. Sauve and J. P. Bourdineaud for providing the oligonucleotides, M. Chartier for technical assistance, and M. Payan for careful preparation of the manuscript.

This work was supported by the Centre National de la Recherche Scientifique, the Commission of the European Communities, the Fondation pour la Recherche Médicale, and the Ministère de la Recherche et de la Technologie. S.P.H. is the recipient of a Natural Sciences and Engineering Research Council of Canada postdoctoral fellowship.

LITERATURE CITED

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525.
2. **Baty, D., R. Lloubès, V. Geli, C. Lazdunski, and S. P. Howard.** 1987. Extracellular release of colicin A is non-specific. EMBO J. **6**:2463–2468.
3. **Bernabeu, C., and J. A. Lake.** 1982. Nascent polypeptide chains emerge from the exit domain of the large ribosomal subunit. Proc. Natl. Acad. Sci. USA **79**:3111–3115.

4. **Carter, P., H. Bedouelle, and G. Winter.** 1985. Improved oligonucleotide site-directed mutagenesis using M13 vectors. Nucleic Acids Res. **13**:4431–4443.
5. **Cavard, D., V. Crozel, J. P. Gorvel, F. Pattus, D. Baty, and C. Lazdunski.** 1986. A molecular, genetic and immunological approach to the functioning of colicin A, a pore-forming protein. J. Mol. Biol. **187**:449–459.
6. **Cavard, D., R. Lloubès, J. Morlon, M. Chartier, and C. Lazdunski.** 1985. Lysis protein encoded by plasmid ColA-CA31. Gene sequence and export. Mol. Gen. Genet. **199**:95–100.
7. **Cavard, D., D. Baty, S. P. Howard, H. M. Verheij, and C. Lazdunski.** 1987. Lipoprotein nature of the colicin A lysis protein: effect of amino acid substitutions at the site of modification and processing. J. Bacteriol. **169**:2187–2194.
8. **Cole, S. T., B. Saint-Joanes, and A. P. Pugsley.** 1985. Molecular characterisation of the colicin E2 operon and identification of its products. Mol. Gen. Genet. **198**:465–472.
9. **de Graaf, F. K., and B. Oudega.** 1986. Production and release of cloacin DF13 and related colicins. Curr. Top. Microbiol. Immunol. **125**:183–205.
10. **Ebina, Y., and A. Nakazawa.** 1983. Cyclic AMP-dependent initiation and ρ-dependent termination of colicin E1 gene transcription. J. Biol. Chem. **258**:7072–7078.
11. **Hakkaart, M. J. J., E. Veltkamp, and H. J. J. Nijkamp.** 1981. Protein H encoded by plasmid CloDF13 involved in lysis of the bacterial host. I. Localisation of the gene and identification and subcellular localisation of the gene H product. Mol. Gen. Genet. **183**:318–325.
12. **Jakes, K., and N. D. Zinder.** 1984. Plasmid ColE3 specifies a lysis protein. J. Bacteriol. **157**:582–590.
13. **Klein, P., R. I. Somorjai, and P. Lau.** 1988. Distinctive properties of signal sequences from bacterial lipoproteins. Protein Eng. **2**:15–20.
14. **Kramer, B., W. Kramer, and H. J. Fritz.** 1984. Different base-base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of *E. coli*. Cell **38**:879–887.
15. **Lau, P. C., M. A. Hefford, and P. Klein.** 1987. Structural relatedness of lysis proteins from colicinogenic plasmids and icosahedral coliphages. Mol. Biol. Evol. **4**:544–556.
16. **Lloubès, R., D. Baty, and C. Lazdunski.** 1986. The promoters of the genes for colicin production, release and immunity in the ColA plasmid: effects of convergent transcription and LexA protein. Nucleic Acids Res. **14**:2621–2636.
17. **Lloubès, R., D. Baty, and C. Lazdunski.** 1988. Transcriptional terminators in the *caa-cal* operon and *cai* gene. Nucleic Acids Res. **16**:3739–3749.
18. **Luirink, J., C. van der Sande, J. Tommassen, E. Veltkamp, F. K. de Graaf, and B. Oudega.** 1986. Effects of divalent cations and of phospholipase A activity on excretion of cloacin DF13 and lysis of host cells. J. Gen. Microbiol. **132**:825–834.
19. **Maget-Dana, R., F. Heitz, M. Ptak, F. Peypoux, and M. Guinand.** 1985. Bacteria lipopeptides induce ion-conducting pores in planar bilayers. Biochem. Biophys. Res. Commun. **129**:965–971.
20. **Maget-Dana, R., M. Ptak, F. Peypoux, and G. Michel.** 1985. Pore-forming properties of iturin A, a lipopeptide antibiotic. Biochim. Biophys. Acta **815**:405–409.
21. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–78.
22. **Morlon, J., M. Chartier, M. Bidaud, and C. Lazdunski.** 1988. The complete nucleotide sequence of the colicinogenic plasmid ColA. High extent of homology with ColE1. Mol. Gen. Genet. **211**:231–243.
23. **Morlon, J., R. Lloubès, S. Varenne, M. Chartier, and C. Lazdunski.** 1983. Complete nucleotide sequence of the structural gene for colicin A, a gene translated at non-uniform rate. J. Mol. Biol. **170**:271–285.
24. **Oudega, B., A. Ykema, F. Stegehuis, and F. K. de Graaf.** 1984. Detection and subcellular localization of mature protein H involved in excretion of cloacin DF13. FEMS Microbiol. Lett. **22**:101–108.
25. **Pugsley, A.** 1988. The immunity and lysis genes of ColN plasmid pCHAP4. Mol. Gen. Genet. **211**:335–341.
26. **Pugsley, A. P., and S. T. Cole.** 1987. An unmodified form of the ColE2 lysis protein, an envelope lipoprotein, retains reduced ability to promote colicin E2 release and lysis of producing cells. J. Gen. Microbiol. **133**:2411–2420.
27. **Pugsley, A. P., and M. Schwartz.** 1983. Expression of a gene in a 400-base-pair fragment of colicin plasmid ColE2-P9 is sufficient to cause host cell lysis. J. Bacteriol. **156**:109–114.
28. **Pugsley, A. P., and M. Schwartz.** 1984. Colicin E2 release: lysis, leakage or secretion? Possible role of a phospholipase. EMBO J. **3**:2393–2397.
29. **Sabik, J. F., J. L. Suit, and S. E. Luria.** 1983. *cea-kil* operon of the ColE1 plasmid. J. Bacteriol. **153**:1479–1485.
30. **Toba, M., H. Masaki, and T. Ohta.** 1986. Primary structures of the ColE2-P9 and ColE3-CA38 lysis genes. J. Biochem. **99**:591–596.
31. **Watson, R. J., P. C. K. Lau, T. Vernet, and L. P. Visentin.** 1986. Characterization and nucleotide sequence of a colicin-release gene in the *hic* region of plasmid ColE3-CA38. Gene **42**:351–355.
32. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33**:103–119.