Colicin E2 Production and Release by *Escherichia coli* K12 and Other *Enterobacteriaceae*

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Previous work has shown that *Escherichia coli* K12 ColE2⁺ cells undergo a form of partial lysis and exhibit increases in lysophosphatidylethanolamine (lysoPE) and free fatty acid content due to activation of phospholipase A when induced to produce and release colicin E2. The increase in lysoPE content was assumed to be essential for efficient colicin release. These same characteristics are also presented by some natural ColE2⁺ isolates, and by other representatives of the *Enterobacteriaceae* after transformation with derivatives of a ColE2 plasmid. However, *Salmonella typhimurium* strains carrying ColE2 plasmids released colicin without partial lysis and without increasing their lysoPE content. A previously undetected minor phospholipid, which appeared in these and other strains only when they were induced to produce colicin, may be an important factor in colicin release. In ColE2⁺ *E. coli* K12, production of this new lipid was dependent on phospholipase A activation following expression of the ColE2 lysis gene. Some other ColE2⁺ strains did not respond to induction of colicin production in the same way as ColE2⁺ *E. coli* K12. These strains were less sensitive to inducer (mitomycin C) or unable to produce increased amounts of colicin in response to induction, or unable to degrade colicin once it was released. In general, the results suggest that colicin release occurs by the same or similar processes in the various strains tested, and support the continued use of *E. coli* K12 as the model strain for studying the mechanisms of colicin release.

INTRODUCTION

The processes of colicin E2 production and release have been extensively characterized in the laboratory strain *Escherichia coli* K12 (reviewed by Pugsley, 1984a). The eventual release of colicin E2 into the medium is the culmination of a sequence of events which starts with the induction of the plasmid-borne colicin E2 operon by inactivation of the chromosome-encoded repressor, LexA protein. In the laboratory, this is usually achieved by activating the host cell SOS regulatory circuit by treating the cells with normally sub-lethal doses of mitomycin C. The cells then produce and accumulate large amounts of the colicin E2-immunity protein complex, the product of the first two genes (*ceaB* and *ceiB*) of the colicin E2 operon (Cole *et al.*, 1985). The induced cells also produce lower amounts of a small, envelope lipoprotein called the lysis protein (Cole *et al.*, 1985). After 2–3 h, the endogenous PldA phospholipase is activated, provoking significant changes in envelope phospholipid composition which are apparently essential for efficient colicin release (Pugsley & Schwartz, 1984). The cells then start to release colicin, and simultaneously become permeable to sucrose and other normally impermeant solutes (Pugsley & Rosenbusch, 1981, and unpublished data), become sensitive to lysozyme and to SDS (Pugsley & Schwartz, 1984), cease all metabolic activity, and lose viability (Pugsley & Rosenbusch, 1981;

Abbreviations: FFA, free fatty acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; ONPG, *o*-nitrophenyl β -D-galactoside.

Pugsley & Schwartz, 1983*a*). The cells also release some other, mainly periplasmic proteins along with the colicin (Pugsley & Rosenbusch, 1981). The culture turbidity begins to decline at this time, but this can be prevented, without affecting colicin release, by including 20 mM-Mg²⁺ in the growth medium (Pugsley & Schwartz, 1984). A similar sequence of events is involved in the production and release of other colicins encoded by small, high copy number plasmids (A, D, E1, E3, E8, N and DF13; reviewed by Pugsley, 1984*a*).

E. coli K12 is not a natural colicin-producing strain. It has been stored, manipulated and intentionally or unintentionally mutagenized during its prolonged existence as the standard laboratory *E. coli* strain. Furthermore, its envelope structure differs from that of most other *Enterobacteriaceae* in that it lacks the long polysaccharide side chains which are normally attached to the lipopolysaccharide core sugars on the cell surface (Lugtenberg & van Alphen, 1983). All of these features could conceivably affect the way in which ColE2⁺ derivatives of *E. coli* K12 respond to induction of colicin production, so that some of the events which we previously considered to be important for efficient colicin release may in fact only occur in *E. coli* K12. In order to test this possibility, we selected a variety of natural and laboratory-constructed ColE2⁺ derivatives of different representatives of the family *Enterobacteriaceae* and examined their responses to induction of colicin production.

METHODS

Strains, plasmids and bacteriophages. The strains of *E. coli* K12 used are listed in Table 1. Strain PAP542 was constructed by subcloning the entire colicin E2 operon (*ceaB-ceiB-celB*; Cole *et al.*, 1985) into plasmid pOM40 (Raibaud *et al.*, 1984) and then integrating it into the *malA* region of the chromosome of strain PAP488 by homologous recombination followed by spontaneous loss of the pOM40 derivative (Raibaud *et al.*, 1984). The *btuB* mutation resulting in the loss of colicin E2 receptor activity was introduced into this and other strains by simultaneous selection for resistance to colicin E5 and to bacteriophage BF23 (Pugsley, 1985). ColE2⁺ *Shigella sonnei* P9 and *E. coli* CA42 are standard colicin E2-producing strains. Other ColE2⁺ strains (Table 3) were selected from a collection of Col⁺ strains by the fact that they appeared to produce only colicin E2 (Pugsley, 1985). Other strains (Table 3) were selected for study because they were sensitive to the antibiotics kanamycin, ampicillin and tetracycline, did not produce colicin or substantial amounts of any other protein (potential bacteriocin), lyse, or undergo significant changes in lipid composition when grown in L broth containing mitomycin C (see below), did not flocculate when grown in liquid culture, and were transformable by pAPIP219 or pCHAP16 (see below).

ColE2-related plasmids are listed in Table 2. Plasmid pCHAP16 contains approximately 4000 bp of pColE2-P9 DNA cloned in pBR322. pCHAP23 is similar to pAPIP692 (Pugsley & Schwartz, 1983*b*) except that the vector DNA is pEMBL9. The Φ (*ceaB-lacZ*)101 operon fusion was obtained following the infection of strain PAP542 with bacteriophage Mu dX (Baker *et al.*, 1984). The infected cells were plated on MacConkey lactose agar containing 0.1 µg mitomycin C ml⁻¹ with selection for resistance to ampicillin and chloramphenicol (see below). Red (Lac⁺) clones were screened for colicin production in the patch test (Pugsley, 1985), and for their Lac phenotype in the absence of mitomycin C. A single clone out of over 5000 tested fulfilled the requirements for having Mu dX inserted into *ceaB* to give a *ceaB-lacZ* operon fusion (i.e. Col⁻ Imm⁺, and Lac⁺ only in the presence of mitomycin C). Chromosomal DNA from this strain was digested with *Eco*RI, and the fragments ligated into *Eco*RI-digested pMBL524, a pBR322 derivative (Silhavy *et al.*, 1984), according to Maniatis *et al.* (1982). Strain PAP488 was transformed with the ligated mixture with selection for ampicillin resistance on L agar plates containing 0.1 µg mitomycin C ml⁻¹ and 1 µg 5-bromo-4-chloro-3-indolyl β-D-galactoside ml⁻¹ (X-gal). Plasmid pCHAP28 was obtained from one of the blue (LacZ⁺) clones which appeared after overnight incubation at 37 °C. Restriction endonuclease mapping of pCHAP28 indicated the structure expected for pMBL524 Φ (*ceaB-lacZ*), with the insertion site of the Mu dX phage being 50–100 bp downstream from the *Clal* site in *ceaB*. Expression of Φ (*ceaB-lacZ*)101 in *E. coli* K12 derivatives carrying pCHAP28 (as measured by specific β-galactosidase activity; Miller, 1972) was shown to parallel that of colicin E2 production in strains carrying pColE2-P9 or its derivatives, i.e. it was induced by mitomycin C and nalidixic acid, and dependent upon the presence of wild-type *recA*, *lexA* and *recB* (for nalidixic acid only) alleles (data not shown).

Plasmid pUH51 [*ori* pBR322 Φ (*lacZp-E*) *lacI*^{q1}] carries the bacteriophage ϕ X174 lysis gene *E* under the control of the *lacZ* promoter (Henrich *et al.*, 1983), and was supplied by W. Lubitz (Munich, FRG) and R. Plapp (Kaiserslautern, FRG).

The bacteriophages used were λ , λ c i21, Mu cts *bla*⁺ (W. Schumann, Darmstadt, FRG) and P1::Tn921 (Cm^r) cts.

Media and growth conditions. L broth (Miller, 1972) was used for most experiments, and was supplemented with 15 µg kanamycin ml⁻¹, 20 or 200 µg ampicillin ml⁻¹, 15 µg tetracycline ml⁻¹ or 25 µg chloramphenicol ml⁻¹ as

Table 1. *E. coli* K12 strains

Strain	Genotype, other characteristics, use and reference
BZB1011	<i>gyrA</i> . Host for most Col plasmids
PAP488	<i>rpsL</i> Δ (<i>pro-lac</i>). Strain for testing Φ (<i>ceaB-lacZ</i>) expression
BZB1019	Met ⁻ <i>hsdR</i> Gal ⁻ <i>rpsL</i> . Recipient for Col plasmids from strains other than <i>E. coli</i> K12
PC1602	<i>metE</i> Bio ⁻ <i>thyA</i> <i>endA</i> (de Geus <i>et al.</i> , 1983)
PAP274	Met ⁺ <i>pldA</i> * derivative of PC1602 (de Geus <i>et al.</i> , 1983)
PAP572	<i>thr-1 leuB6 lacY</i> Thi ⁻ <i>supE44</i> (λ^- derivative of strain W1; Ohki <i>et al.</i> , 1972)†
PAP573	As PAP572 but SDS [†] (λ^- derivative of strain 15; Ohki <i>et al.</i> , 1972)
PAP576	As PAP573 but <i>pldA</i> (λ^- derivative of strain 23; Ohki <i>et al.</i> , 1972)
PAP575	As PAP573 but lacking detergent-sensitive phospholipase (λ^- derivative of strain 21; Ohki <i>et al.</i> , 1972)
PAP574	As PAP575 but <i>pldA</i> (λ^- derivative of strain 17; Ohki <i>et al.</i> , 1972)
PAP542	As PAP488 but <i>btuB malA::</i> (<i>ceaB-celB-ceiB</i>)

* *pldA* is the structural gene for detergent-resistant phospholipase (Raetz, 1978).

† The original λ lysogens described by Ohki *et al.* (1972) were cured by super-infection with λ i²¹ and screening colonies for absence of immunity to λ and to λ i²¹. These strains were originally described as carrying a *malA* mutation, but they are in fact Mal⁺ λ vir⁸.

‡ This mutation renders the strains more sensitive to mitomycin C. The increased sensitivity to mitomycin C and to SDS is only overcome in our derivatives by the absence of the detergent-sensitive phospholipase (unpublished data).

Table 2. *Col* plasmids

Plasmid	Structure, origin and reference
pColE2-P9	Wild-type ColE2 from <i>Shigella sonnei</i> P9; <i>ceaB</i> ⁺ <i>ceiB</i> ⁺ <i>celB</i> ⁺ (Pugsley & Schwartz, 1983a; Watson <i>et al.</i> , 1983)
pColE2-CA42	Wild-type ColE2 from <i>E. coli</i> CA42; <i>ceaB</i> ⁺ <i>ceiB</i> ⁺ <i>celB</i> ⁺ (Watson <i>et al.</i> , 1983)
pAPIP219	pColE2-P9::Tn5; <i>ceaB</i> ⁺ <i>ceiB</i> ⁺ <i>celB</i> ⁺ (Pugsley & Schwartz, 1983a)
pCHAP16	pBR322-pColE2-P9 recombinant; <i>ter</i> ⁺ <i>bla</i> ⁺ <i>ceaB</i> ⁺ <i>ceiB</i> ⁺ <i>celB</i> ⁺ (Cole <i>et al.</i> , 1985)
pCHAP23	pEMBL9; Φ (<i>lacZp-ceiB⁺-celB⁺</i>) <i>bla</i> ⁺ (Pugsley & Schwartz, 1983b, 1984)*
pCHAP28	<i>ori</i> pBR322; Φ (<i>ceaB-lacZ</i>)101 <i>bla</i> ⁺ †
pAPIP421	pBR322-pColN-284 recombinant; <i>cna</i> ⁺ <i>cni</i> ⁺ <i>cnl</i> ⁺ <i>bla</i> ⁺ ::Tn5 (<i>neo</i> ⁺) (Pugsley, 1984b)
pAPIP408	pColE1-K53::Tn5; <i>ceaA</i> ⁺ <i>ceiA</i> ⁺ <i>celA</i> ⁺

* *E. coli* K12 strains carrying this plasmid also carried pAPIP502 (*lacI^q*). The presence of *lacI^q* was apparently unnecessary for the maintenance of pCHAP23 in other strains, presumably because their own *lacI⁺* alleles were more able to repress Φ (*lacZ-celB*) than the *E. coli* K12 *lacI⁺* allele (see Pugsley & Schwartz, 1983b).

† Details of the construction and analysis of this plasmid are given in Methods.

appropriate for a given plasmid or bacteriophage (see above). Minimal medium was M63 (Miller, 1972) containing 1% (v/v) glycerol. Media were solidified with 1.6% (w/v) agar where appropriate. Broth cultures were grown at 37 °C with good aeration, and mitomycin C (usually 0.5 μ g ml⁻¹) was added when the OD₆₀₀ reached 0.15–0.2. Changes in OD were then recorded at 20–30 min intervals. A decline in OD is referred to here as lysis or partial lysis, although this term is not strictly correct (see Pugsley, 1984a). IPTG (1 mM) was used to induce Φ (*lacZp-E*) or Φ (*lacZp-celB*) expression.

Assays. Colicin was assayed by the spot dilution method (Pugsley & Rosenbusch, 1981). In most cases, comparisons of colicin production by ColE2⁺ *E. coli* K12 and other strains were based on the analyses of total TCA-precipitated proteins in the entire culture or in cell-free medium by SDS-PAGE (Pugsley & Schwartz, 1984). For the direct comparison of colicin production and stability in ColE2⁺ *E. coli* K12 and *E. coli* O2, cells were grown in I. broth or M63 glycerol medium containing mitomycin C. After 70 min, samples were withdrawn, washed twice with M63 medium and resuspended in the same medium containing ¹⁴C-labelled amino acids (50 μ Ci ml⁻¹; 1.85 MBq ml⁻¹) (CEA, France) and incubated at 37 °C for 15 min. These cells were then returned to the main culture, and samples were withdrawn immediately and at subsequent intervals for the analysis of total culture proteins by SDS-PAGE and autoradiography (Pugsley & Schwartz, 1983a). β -Galactosidase activity was assayed according to Miller (1972). Extracellular β -galactosidase activity was assayed as described previously (Pugsley & Rosenbusch, 1981), and the release of β and β' subunits of RNA polymerase was detected by SDS-PAGE. *o*-Nitrophenyl β -D-galactoside (ONPG) permeation was determined by comparing the rates of ONPG hydrolysis (Miller, 1972) by intact and chloroform/SDS-lysed, IPTG-induced cells taken from I. broth cultures growing in the presence or absence of mitomycin C. The background permeability coefficient (rate in intact/lysed cells) in wild-type *E. coli* K12 was 5–8%. A similar coefficient was also obtained with uninduced ColE2⁺ strains. Lysozyme and

SDS sensitivity were indicated by lysis of cultures growing in the presence of mitomycin C plus 25 mM-MgSO₄ (Mg²⁺ prevents 'lysis' resulting from mitomycin C induction of colicin operon expression). SDS (1%) or lysozyme (50 µg ml⁻¹) were added after 3 h incubation. Changes in viability were computed by plating out dilutions of cultures on L agar plates and counting colonies formed by surviving cells after overnight incubation at 37 °C. Results are presented as the reduction in viability relative to the highest viable count obtained in the first 2–3 h of mitomycin C induction. Lipid analyses were done essentially as described previously (Pugsley & Schwartz, 1984). Cultures were prelabelled with [¹⁴C]acetic acid (20–50 µCi ml⁻¹; 0.74–1.85 MBq ml⁻¹) (CEA, France) before addition of mitomycin C. The solvent used for most TLC analyses was the chloroform/methanol/acetic acid/water (65:25:2:6, by vol.) system used previously (Pugsley & Schwartz, 1984), but other systems described by Ames (1968) were used in attempts to identify minor lipids. Lipid standards, phosphatidylethanolamine (PE), lysoPE, phosphatidylserine (PS), lysoPS, phosphatidylglycerol (PG), lysoPG and phosphatidylcholine, were from Serva (FRG) or Sigma. TLC plates were developed with iodine, molybdenum blue or ninhydrin (Skipski & Barclay, 1969) before being exposed to X-ray film.

Plasmids. Plasmids were extracted by the method of Holmes & Quigley (1981), and analysed by restriction endonuclease digestion and agarose gel electrophoresis as described by Maniatis *et al.* (1982). Transformation, with selection for antibiotic resistance or for immunity to a partially purified preparation of colicin E2-P9, was generally as described by Maniatis *et al.* (1982), except that solutions containing 50 mM-Tris/HCl (pH 7.4), 5 mM-MgCl₂ and 10 or 100 mM-CaCl₂ were used for strains which were exceptionally difficult to transform. Colicin production by Col⁺ transformants were tested by the patch method (Pugsley, 1985), and plasmids were extracted from recipients and analysed as above to confirm that major rearrangements had not occurred. Plasmid copy numbers were estimated by comparing plasmid yields (as determined by agarose gel electrophoresis) from cultures grown under identical conditions.

RESULTS

Further studies with ColE2⁺ E. coli K12

The basic characteristics associated with colicin E2 production and release by *E. coli* K12 have already been documented (Pugsley, 1984*a*; Pugsley & Schwartz, 1984; Cole *et al.*, 1985). Our earlier results implicated phospholipase A1-A2 (the *pldA* gene product) as the cause of the most prominent changes in envelope phospholipid composition which occurred immediately prior to colicin release, namely a reduction in PE content, and increases in lysoPE and free fatty acid (FFA) content (Pugsley & Schwartz, 1984; Pugsley, 1984*a*). As a result of more recent studies with other ColE2⁺ strains (see below), and subsequently with ColE2⁺ *E. coli* K12, we have detected increases in the amounts of a second lipid accompanying colicin release from mitomycin C-induced cells (Fig. 1). This as-yet unidentified lipid migrated close to lysoPE and PS on thin-layer chromatographs. It gave positive reactions with molybdenum blue and with ninhydrin, indicating the presence of phosphate and amine groups (Skipski & Barclay, 1969). Its appearance, like that of lysoPE and FFA, was dependent on the presence of a functional *pldA* gene in the chromosome, and of a functional *celB* (lysis) gene on the ColE2 plasmid (data not shown). This lipid (hereafter referred to as lipid Y) was also present in increased amounts after induction of $\Phi(lacZ-celB)$ in *E. coli* K12(pCHAP23) (data not shown).

Increases in lysoPE, FFA and lipid Y content were also observed when *E. coli* K12 λ^+ was treated with mitomycin C (see also Sakakibara *et al.*, 1972), or when *E. coli* K12 Mu *cts* Ap^r or P1 *cts* Cm^r lysogens were induced by raising the incubation temperature from 30 °C to 42 °C. Similarly, induced expression of the bacteriophage ϕ X174 gene *E* in *E. coli* K12(pUH51) (*lacZ-E*) also resulted in increases in lysoPE, FFA and lipid Y content (W. Lubitz and co-workers, unpublished observations), as did a 5 min 56 °C heat-shock in cultures of wild-type *E. coli* K12 (data not shown). In every case, these changes did not occur in derivatives carrying the *pldA1* mutation. The presence of a second mutation affecting the detergent-sensitive phospholipase (Table 1) did not affect the appearance of increased amounts of these products (data not shown).

The effect of copy number on the expression of $\Phi(ceaB-lacZ)101$ was determined using derivatives of PAP488 with the operon fusion in the chromosome or on pCHAP28 (see Methods). In mitomycin C-induced cultures, the level of β -galactosidase activity was approximately 25-fold higher in the strain carrying pCHAP28. This figure agrees reasonably well with estimates of pCHAP28 copy number per chromosome (data not shown). A strain devoid of the colicin E2 receptor protein (BtuB) and carrying the colicin E2 operon recombined

Fig. 1. TLC of chloroform/methanol extracts from [^{14}C]acetate-labelled cells of *E. coli* K12(pColE2-P9) (lanes A, B), *Erwinia carotovora* 3937(pAPIP219) (lanes C, D) or *Salmonella typhimurium* S/756 (natural Col $^+$ strain) (lanes E, F) grown for 4 h in L broth in the absence (lanes A, C, E) or presence (lanes B, D, F) of 0.5 μg mitomycin C ml^{-1} . Note the relatively high amounts of lysoPE, lipid Y and FFA in uninduced *E. coli* K12(pColE2-P9), which is due to spontaneous induction of colicin production and release as the cells near the end of exponential growth. CL, cardiolipin.

into the chromosomal *malA* locus also produced less colicin than strains carrying pColE2-P9 or pCHAP16, but in this case the amounts of colicin recovered after 3 h of mitomycin C induction were 50- to 150-fold higher in cultures of cells carrying the plasmid; this is considerably greater than the difference predicted by the higher operon copy number in these strains. Indeed, mitomycin C-induced cultures of strain PAP542 (*btuB malA::ceaB-ceiB-celB*) did not produce

sufficient colicin E2 to be detected by SDS-PAGE of whole cell extracts, did not show any changes in phospholipid composition and, significantly, released less than 5% of their colicin into the growth medium. Reductions in ColE2 plasmid copy number will therefore affect colicin release in a similar way.

Analysis of other ColE2⁺ strains

Various ColE2⁺ strains representing different members of the family *Enterobacteriaceae* were compared with ColE2⁺ *E. coli* K12 with respect to colicin production and release, and associated characteristics. The cultures were grown in L broth with or without 0.5 µg mitomycin C ml⁻¹ as inducer. Two sets of strains were tested. The first comprised ten natural ColE2⁺ strains which, with the exception of *Shigella sonnei* P9 (ColE2-P9, ColIb-P9), produced only one colicin and carried a single species of small, high-copy-number potential Col plasmid (see below). As shown in Table 3, all of these strains, like *E. coli* K12(pAPIP219), produced and released large amounts of colicin E2, and became sensitive to SDS. Only *Shigella sonnei* strains P9 and 1291, and *E. coli* CA42 showed any signs of a decline in culture turbidity, extensive changes in lysoPE content or substantial reductions in viability up to 6 h after addition of mitomycin C. Indeed, increases in lysoPE content could not be detected at any time in any of the *Salmonella typhimurium* ColE2⁺ strains, and increases in lipid Y and FFA content were far less prominent than in *E. coli* K12 ColE2⁺ (Fig. 1).

Plasmids extracted from the ColE2⁺ *Salmonella typhimurium* strains were transferred to *E. coli* K12 strain BZB1019 by transformation with selection for colicin E2 immunity (Methods). The recipients produced and released colicin E2 in response to mitomycin C induction in the same way as *E. coli* K12(pColE2-P9), i.e. including production of lysoPE and partial lysis. Restriction endonuclease analysis of these plasmids indicated that they were either pColE2-P9-like or pColE2-CA42-like (Pugsley & Schwartz, 1983*a*; Watson *et al.*, 1983) (Table 3), with differences in restriction endonuclease cleavage patterns being noted only with enzymes with several cleavage sites in the respective plasmids (data not shown). In particular, the colicin, immunity and lysis gene regions of all plasmids (Cole *et al.*, 1985) were apparently highly conserved. Thus, it would appear that the differences between *E. coli* K12 ColE2⁺ and *Salmonella typhimurium* ColE2⁺ strains were due to host cell factors rather than to differences in the Col plasmids they carried.

The use of higher concentrations of mitomycin C to induce colicin production did not cause partial lysis of the *Salmonella typhimurium* ColE2⁺ strains (data not shown), nor did it result in an increase in the amount of lysoPE appearing before or during colicin release (data not shown). However, these cells were shown to have a phospholipase similar to that of *E. coli* K12 by the fact that the appearance of lysoPE, lipid Y and FFA was induced to the same extent as in *E. coli* K12 by heat-shock treatment (Fig. 2). We were unable to verify that lysis protein was being produced by these cells, due to technical difficulties in detecting low molecular weight proteins in these cells.

These studies indicated that some natural ColE2⁺ strains did not behave in the same way as ColE2⁺ *E. coli* K12 after mitomycin C induction. We therefore tested other strains of *Enterobacteriaceae* which were converted to ColE2⁺ by transformation with the pColE2-P9 derivatives pAPIP219(ColE2::Tn5) or pCHAP16(pBR322-pColE2). Most of these strains responded to mitomycin C treatment in the same way as *E. coli* K12 ColE2⁺, except for the fact that the *E. coli* K12 strains were the only ones which became sensitive to lysozyme (Table 4). However, lysis and increases in lysoPE, lipid Y and FFA content were sometimes less pronounced than in *E. coli* K12 ColE2⁺. Some ColE2⁺ strains produced an additional lipid (lipid Z) after mitomycin C induction (Fig. 3). This lipid usually co-migrated with lysoPS when analysed by TLC. There were also some variations in the apparent specificity of colicin release, with some strains such as *Enterobacter cloacae* 1028(pAPIP219) releasing only colicin E2 and a protein tentatively identified as elongation factor EFTu in amounts detectable by SDS-PAGE [compare results in Fig. 4 and results with *E. coli* K12 strains in Pugsley & Rosenbusch (1981) and Pugsley & Schwartz (1984)].

Table 3. Colicin production and release, and other characteristics associated with growth of natural ColE2⁺ strains and *E. coli* K12(pColE2-P9) in *L* broth containing 0.5 µg mitomycin C ml⁻¹

Strain	Source/ origin*	Biotype/ phage type	ColE2 plasmid†	Induced colicin production‡	Lysis inhibited by 25 mM- § Partial lysis§	Loss of viability§	Protein release¶			Increased content of¶		
							Colicin	RNA polymerase	Lyso-	Lipid	PE	Y FFA
<i>E. coli</i> K12												
<i>Shigella sonnei</i> P9			pColE2-P9	100	+	10 ⁴	+	-	+	+	+	+
<i>Shigella sonnei</i> 1291	P. Fredericq Scotland		pColE2-P9	120	+	10 ³	+	-	+	+	+	+
<i>E. coli</i> CA42			pColE2-P9-like	140	+	10 ⁴	+	-	+	+	+	+
<i>Salmonella typhimurium</i> S/1297	P. Fredericq Scotland		pColE2-CA42	150	+	10 ³	+	-	+	+	+	+
<i>S. typhimurium</i> S/756		26a/204	pColE2-CA42-like	80	-	0	+	-	-	-	+	+
<i>S. typhimurium</i> S/726	Scotland	9f/141	pColE2-P9-like	80	-	10	+	-	-	-	+	+
<i>S. typhimurium</i> S/726	Denmark	17a/85	pColE2-CA42-like	60	-	0	+	-	-	-	+	+
<i>S. typhimurium</i> S/152		26a/204	pColE2-CA42-like	100	-	0	+	-	-	-	+	+
<i>S. typhimurium</i> S/1884	Scotland	26a/49	pColE2-CA42-like	120	-	<10	+	-	-	-	+	+
<i>S. typhimurium</i> S/1274	Scotland	26a/204	pColE2-CA42-like	100	-	0	+	-	-	-	+	+
<i>S. typhimurium</i> S/3305	England	26a/85	pColE2-CA42-like	90	-	0	+	-	-	-	+	+

NA, Not applicable; (+), positive, but considerably less positive than the *E. coli* K12(pColE2-P9) cultures.

* *Shigella sonnei* 1291 and all *Salmonella typhimurium* strains were from the Dundee collection. All *Salmonella typhimurium* strains were from infected humans except S/1884 and S/1297, which were from infected cattle. Strains with the same biotype/phage type may be identical, although they were isolated at different times from different patients. Address for P. Fredericq: University de Liege, Belgium.

† Based on analysis with restriction enzymes *Pvu*II, *Bgl*II, *Eco*RI, *Cla*I, *Eco*RV, *Hinc*II and *Hae*III and comparison of the restriction digest patterns as revealed by agarose gel electrophoresis with those of authentic pColE2-P9 or pColE2-CA42 plasmids.

‡ Determined by SDS-PAGE of total culture proteins after 3 h induction. Cultures which lysed ceased colicin production at this time, but non-lysing cultures were shown by labelling experiments to continue colicin synthesis for considerably longer periods. The results are expressed as percentages of the colicin produced by *E. coli* K12(pColE2-P9).

§ During the period up to 8 h after addition of mitomycin C. Lysis started 2.5–3 h after induction.

¶ All strains were shown to undergo lysis when these cultures were supplemented with 1% SDS. SDS-induced lysis was far less dramatic in all the *S. typhimurium* cultures, which showed outgrowth of unaffected cells within 30–45 min of the addition of SDS. Only *E. coli* K12(pColE2-P9) cultures were sensitive to lysozyme.

¶ Analysis of proteins released was done 3–8 h after induction. The extent and timing of colicin release was similar in all cultures. Changes in lipid content were determined 2–6 h after induction.

Fig. 2

Fig. 3

Fig. 2. TLC of chloroform/methanol extracts from [¹⁴C]acetate-labelled cells from L broth cultures of *Salmonella typhimurium* S/756 (lane A) or the same cells after a 5 min heat-shock and continued incubation (lane B), and of *Salmonella typhimurium* LT2(pUH51) grown in the absence (lane C) or presence (lane D) of 1 mM-IPTG to induce $\Phi(lacZ-E)$ expression and total lysis. CL, cardiolipin.

Fig. 3. TLC of chloroform/methanol extracts from [¹⁴C]acetate-labelled cells from L broth cultures of *Klebsiella aerogenes* strain 10B(pAPIP219) grown for 4 h in the absence (lane A) or presence (lane B) of 0.5 µg mitomycin C ml⁻¹. Note the presence of lipid Z, which was not detected in the other strains. CL, cardiolipin.

Table 4. Characteristics associated with induction of the wild-type colicin E2 operon in derivatives of various strains of *Enterobacteriaceae* carrying pAPIP219 or pCHAP16, and growing in L broth + 0.5 µg mitomycin C ml⁻¹ at 37 °C

Strain	Plasmid	Colicin production*	Onset of 'lysis' by 20 mM-Mg ²⁺ (h)	Lysis inhibited by 20 mM-Mg ²⁺	ONPG permeation (% of maximum)	Proteins released†			Sensitivity tot‡	Increased content of‡				
						Colicin	β-Galactosidase‡	RNA polymerase		Lysozyme	Lysozyme PE	Lipid Y	Lipid Z	FFA
<i>Escherichia coli</i> K12	pAPIP219 or pCHAP16	100	2.5-3.5	+	70	+	-	-	+	+	+	+	-	+
<i>E. coli</i> O2	pAPIP219	2	ND	NA	14	-	-	-	NA	-	-	-	-	-
<i>E. coli</i> O6	pAPIP219	80	2	+	NT	+	-	-	-	+	+	+	-	+
<i>E. coli</i> O10	pAPIP219	70	2.5	+	NT	+	§	-	+	+	+	+	-	+
<i>E. coli</i> C	pAPIP219	100	(2.5)	+	85	+	-	-	NT	-	+	+	-	+
<i>Enterobacter cloacae</i> 438	pAPIP219	30	(2.5)	+	60	+	NT	-	+	+	+	+	-	+
<i>E. cloacae</i> 1028	pCHAP16	50	3	+	NT	+	NT	-	+	+	+	+	-	+
<i>Klebsiella aerogenes</i> 10B	pAPIP219	80	3	+	70	+	-	-	+	+	+	+	+	+
<i>K. aerogenes</i> M7	pAPIP219	80	3	+	NT	+	-	-	+	+	+	+	+	+
<i>Citrobacter freundii</i> 8581	pAPIP219	15	(4)	+	35	+	-	-	(+)	-	+	+	+	+
<i>Salmonella typhimurium</i> LT2	pCHAP16	80	ND	NA	55	+	-	-	(+)	-	-	+	-	(+)
<i>S. panama</i>	pAPIP219	100	2.5	+	NT	+	NT	-	+	+	+	+	-	+
<i>S. brandenburg</i>	pAPIP219	150	2.5	+	NT	+	NT	-	+	+	+	+	-	+
<i>S. java</i>	pAPIP219	200	3	+	NT	+	NT	-	+	+	+	+	-	+
<i>S. paratyphi</i> B	pAPIP219	50	3	+	NT	+	NT	-	+	+	+	+	-	+
<i>Shigella sonnei</i> 493	pAPIP219	20	ND	NA	15	-	-	-	-	-	-	-	-	-
<i>Erwinia carotovora</i> 3937	pAPIP219	20	ND	NA	NT	Trace	NT	+¶	-	-	-	-	-	-
<i>E. herbicola</i> H	pAPIP219	2	ND	NA	NT	-	-	+¶	-	-	-	-	-	-

ND, Not detected; NA, not applicable; NT, not tested; (+), positive, but less so than in cultures of *E. coli* K12(pAPIP219, pCHAP16).

* The amount of colicin produced is expressed relative to the amount produced by *E. coli* K12(pAPIP219) after 3 h incubation in the presence of inducer; it was determined by assaying the colicin, or by comparing the amounts of protein in the band corresponding to colicin after SDS-PAGE of standard amounts of protein precipitated from the cultures with TCA.

† Increases in sensitivity to SDS and to lysozyme were determined after 3 h incubation, changes in lipid content were determined after 3.5 h incubation, and the analysis of proteins released was done after 4 h incubation.

‡ Where tested, β-galactosidase was <5% of total protein recovered from the medium, as determined by enzyme assays (β-galactosidase) or by comparing protein recovery in total culture or culture media.

§ Released colicin was apparently rapidly degraded.

|| Tested in a Lac⁺ derivative carrying F' lac pro from *E. coli* K12.

¶ These strains seemed to release small amounts of all soluble proteins when grown in L broth at 37 °C.

Fig. 4. Production and release of colicin E2 by *Enterobacter cloacae* 498(pAPIP219). Cells were grown in L broth (lane A), in L broth + 25 mM-MgSO₄ + 0.5 µg mitomycin C ml⁻¹ (lanes B, D), or in L broth + 0.5 µg mitomycin C ml⁻¹ (lanes C, E) for 4 h. Samples A–C are total culture proteins after precipitation by TCA; samples D and E are the proteins in the culture medium. All samples were equivalent to 200 µl of culture. Note that Mg²⁺ prevented partial lysis (Table 4) but did not affect colicin production or release.

The response of some of the ColE2⁺ strains was apparently different from that of *E. coli* K12 ColE2⁺ (Table 4). *Salmonella typhimurium* LT2(pCHAP16) resembled the natural ColE2⁺ *Salmonella typhimurium* strains (Table 2) in all respects, although ColE2⁺ derivatives of other *Salmonella* species responded to mitomycin C induction in the same way as ColE2⁺ *E. coli* K12 (Table 4). *Salmonella typhimurium* LT2 produced substantially larger amounts of lysoPE, lipid Y and FFA when subjected to heat-shock or after induction of the ϕ X174 *E* gene in derivatives carrying pUH51 (Fig. 2 and not shown). Increasing the amount of mitomycin C used to induce colicin production in *Salmonella typhimurium* LT2(pCHAP16) did not elicit partial lysis or the appearance of substantially increased amounts of lysoPE (data not shown). However, *Salmonella typhimurium* LT2 was slightly more resistant to the growth-reducing effects of mitomycin C than was *E. coli* K12, and expression of Φ (*ceaB-lacZ*)101 in derivatives carrying pCHAP28 at a given concentration of mitomycin C was slightly lower in *Salmonella typhimurium* LT2 than in *E. coli* K12 (Fig. 5).

ColE2⁺ *Shigella sonnei* 493 released only trace amounts of colicin even after prolonged incubation in the presence of mitomycin C (Table 4). An unusually high level of resistance was indicated by the absence of growth inhibition by 0.5 µg mitomycin C ml⁻¹, and by the relatively low level of expression of Φ (*ceaB-lacZ*)101 at this dose of mitomycin C (Fig. 5 and data not shown). Cultures of *Shigella sonnei* 493(pAPIP219) grown in the presence of 1.5 µg mitomycin C ml⁻¹ produced more colicin E2, and released it at the normal time with concomitant partial lysis and increases in lysoPE, lipid Y and FFA content.

Fig. 5. Expression of $\Phi(\text{ceaB-lacZ})101$, encoded by plasmid pCHAP28, in *E. coli* K12 (a), *Shigella sonnei* 493 (b), *Salmonella typhimurium* LT2 (c) or *E. coli* O2 (d) growing in L broth containing various concentrations of mitomycin C: 0.5 $\mu\text{g ml}^{-1}$ ($\square$), 1 $\mu\text{g ml}^{-1}$ ($\bullet$), 1.5 $\mu\text{g ml}^{-1}$ ($\blacksquare$), 5 $\mu\text{g ml}^{-1}$ ($\circ$). β -Galactosidase units (Miller, 1972) were corrected in (b) and (d) by deducting the activity measured in parallel cultures of the same strain without pCHAP28 (these strains carry a chromosomal *lacZ*⁺ allele).

E. coli O2(pAPIP219) was unusual among all of the strains tested in that the amount of colicin produced after mitomycin C induction was extremely low [$<2\%$ of that in *E. coli* K12(pAPIP219)], and that it was extremely sensitive to 1% SDS even in the absence of mitomycin C treatment. Surprisingly, expression of $\Phi(\text{ceaB-lacZ})101$ was apparently normal in this strain, and derivatives carrying pAPIP421 (ColN) or pAPIP408 (ColE1) produced relatively large amounts of colicins N and E1 respectively, and released these colicins with concomitant lysis and activation of phospholipase (data not shown). Furthermore, *E. coli* O2(pAPIP219) produced normal amounts of colicin E2 when grown in M63 glycerol medium containing 0.5 $\mu\text{g ml}^{-1}$ mitomycin C, and $\Phi(\text{lacZp-celB})$ expression in derivatives carrying pCHAP23 provoked lysis and activation of phospholipase when the cultures were grown in minimal medium, but not in L broth (data not shown; see Methods). pAPIP219 was consistently present at approximately the same copy number as in *E. coli* K12 (data not shown).

E. coli O10(pAPIP219) produced normal amounts of colicin E2 when grown in L broth containing 0.5 $\mu\text{g ml}^{-1}$ mitomycin C, but the colicin which was released was apparently rapidly degraded. A similar phenomenon was reported previously in *E. coli* K12 derivatives carrying ColE4 or ColE7 plasmids (Pugsley, 1983a).

The two *Erwinia* strains (*Erwinia carotovora* 3937 and *Erwinia herbicola* H) were both incapable of producing large amounts of colicin when transformed with pAPIP219 and grown in the presence of 0.5 $\mu\text{g ml}^{-1}$ mitomycin C. However, the temperature at which these experiments were done was higher (37 °C) than the optimum growth temperature for these strains (30 °C), and they therefore grew very slowly (doubling times at 37 °C 90–100 min compared with 40–50 min for *E. coli* K12). Previous experiments with *E. coli* K12 strains have shown that reductions in growth rate severely reduce the amount of colicin produced. *E. coli* K12 mutants selected by their survival after mitomycin C induction of the wild-type colicin operon, or after induction of $\Phi(\text{lacZp-celB})$, usually grew at slower rates than the parent strains, and did not carry mutations directly related to colicin production and release (unpublished results, see also Pugsley & Schwartz, 1983b). Furthermore colicin production by *E. coli* K12 ColE2⁺ in cultures containing 0.5 $\mu\text{g ml}^{-1}$ mitomycin C was reduced to $<10\%$ by lowering the incubation temperature from 37 °C to 30 °C, and the colicin which was produced was not released.

DISCUSSION

This study was initiated in order to test the validity of *E. coli* K12 as the model strain for studying the mechanisms of colicin production and release. Our preliminary studies with the strains used here indicated that they represented a fairly homogeneous group with respect to envelope structure; only small variations in minor lipid content and outer membrane protein content were observed (data not shown). In contrast, similar analyses of *Aeromonas*, *Yersinia*, and to a lesser extent *Serratia* strains revealed more striking differences in lipid and protein composition. These strains could not be transformed with ColE2 plasmid derivatives.

Most of the strains we tested were able to produce and release large quantities of colicin E2 when induced with mitomycin C. This implies that they, like *E. coli* K12 (Pugsley, 1984a), have a LexA protein which represses colicin operon expression, and which is inactivated after induction of the SOS response. In addition, all of the elements necessary for efficient colicin release seem to be present in most of these strains. Variations in the extent of 'lysis' and PldA phospholipase activation may result in part from differences in the extent of ColE2 plasmid lysis gene expression. Measurements of colicin operon expression with operon fusions such as $\Phi(\text{ceaB-lacZ})101$ used here give only indirect indications of the expression of down-stream *celB* expression, but direct measurements of *celB* expression are complicated by the small size of its product, by the need for post-translational processing and by the fact that wild-type *celB* is essential for optimum expression of the entire operon (Oudega *et al.*, 1983; Cole *et al.*, 1985). Sequence analysis of the colicin E2 operon has revealed the existence of a possible transcription terminator (or attenuator) just up-stream from *celB* (Pugsley, 1984a; Cole *et al.*, 1985); variations in the efficiency of attenuation in the different strains could affect production of the lysis protein in the same way as the reduction in its copy number consequent upon introduction of the colicin E2 operon into the chromosome. Reduced lysis protein production would reduce phospholipase activation and hence colicin release (Pugsley & Schwartz, 1984).

On the basis of our results with *E. coli* K12, the appearance of lipid Y and lysoPE seems to result from the action of PldA phospholipase A on pre-existing phospholipid. ColE2⁺ *Salmonella typhimurium* produced only lipid Y, but this may reflect a lower level of phospholipase activity or increased lysoPE turnover. The identity of lipid Y and of lipid Z, which appeared in other strains when they were induced to produce colicin E2, remains unknown; they could correspond to lysoPG and lysoCL respectively, or to intermediates in phospholipid biosynthesis such as PS and lysoPS (Raetz, 1978). Further characterization of these lipids will require their purification and chemical analysis.

A striking difference between *E. coli* K12 and all other strains tested was that only *E. coli* K12 became lysozyme-sensitive after induction of colicin production (Tables 3 and 4). This may reflect the inability of this basic protein to penetrate the side chain LPS residues of 'smooth' strains of *Enterobacteriaceae*. In all other cases, the strains differed from *E. coli* K12 in specific details of their response to mitomycin C, some of which were not necessarily indicative of overall differences in mechanisms of colicin production and release. In general, we believe that the results are consistent with the model proposed to explain colicin release from *E. coli* K12 (Pugsley & Schwartz, 1984; Pugsley, 1984a), and justify the continued use of this strain in future studies.

The impermeability of the cytoplasmic membrane to proteins is apparently not substantially altered when cells release colicin from the cytoplasm. At least four proteins or protein complexes (RNA polymerase, β -galactosidase, colicin Ia and amyloamylase) remain in the cytoplasm when colicin and cytoplasmic EFTu protein are released (Pugsley & Rosenbusch, 1981; Pugsley, 1983b; M. A. Bloch & A. P. Pugsley, unpublished results). Colicin Ia and amyloamylase are approximately the same size as the colicin E2-immunity protein complex (mol. wt approximately 70000; M. A. Bloch, unpublished data), which makes it unlikely that the relatively high specificity of colicin release from the cytoplasm is due solely to its small size (Pugsley, 1984a; Pugsley & Schwartz, 1985). It seems likely that lysis protein plays a direct role in the transfer of colicin across the cytoplasmic membrane. In contrast, outer membrane permeability is substantially increased. The extremely high amounts of colicin which are released give the impression of high selectivity (Fig. 4), although in reality, most periplasmic

proteins are quantitatively released along with the colicin (Pugsley & Rosenbusch, 1981). It seems probable that the presence of increased amounts of lyso lipids is an important feature of this increase in outer membrane permeability (Pugsley & Schwartz, 1984), as it is in the case of membrane permeabilization by EDTA (Hardaway & Buller, 1979). It is not known whether other treatments which affect the permeability of the outer membrane to proteins do so by activating phospholipase (Hancock, 1984), but we have recently found that typical periplasmic leaky mutants of *E. coli* K12, which spontaneously release periplasmic proteins into the growth medium (Hancock, 1984), do not have elevated levels of lysoPE. These mutations (*rfa*, *tolA* and *tolB*) also have no effect on colicin E2 release in liquid culture (unpublished data). Thus, it would seem that activation of phospholipase is only one of the ways in which the permeability barrier of the Gram-negative outer membrane can be breached.

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