COLICIN M EXERTS ITS BACTERIOLYTIC EFFECT VIA ENZYMATIC DEGRADATION OF UNDECAPRENYL PHOSPHATE-LINKED PEPTIDOGLYCAN PRECURSORS*

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Running Title: Colicin M bacteriolytic action mechanism

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Colicin M was earlier demonstrated to provoke Escherichia coli cell lysis via inhibition of cell-wall peptidoglycan (murein) biosynthesis. As the formation of the O-antigen moiety of lipopolysaccharides was concomitantly blocked, it was hypothesized that the metabolism of undecaprenyl phosphate, an essential carrier lipid shared by these two pathways, should be the target of this colicin. However, the exact target and mechanism of action of colicin M remained unknown. Colicin M was now purified to near homogeneity and its effects on cell-wall peptidoglycan metabolism reinvestigated. It is demonstrated that colicin M exhibits both in vitro and in vivo enzymatic properties of degradation of lipid I and lipid II peptidoglycan intermediates. Free undecaprenol and either 1-pyrophospho-MurNAc-pentapeptide or 1-pyrophospho-MurNAc-(pentapeptide)-GlcNAc were identified as the lipid I and lipid II degradation products, respectively, showing that the cleavage occurred between the lipid moiety and the pyrophosphoryl group. This is the first time such an activity is described. Neither undecaprenyl pyrophosphate nor the peptidoglycan nucleotide precursors were substrates of colicin M, indicating that both undecaprenyl and sugar moieties were essential for activity. The bacteriolytic effect of colicin M therefore appears to be the consequence of an arrest of peptidoglycan polymerization steps provoked by enzymatic degradation of the undecaprenyl phosphate-linked peptidoglycan precursors.

The lethal action of colicins can be divided into three steps: binding to a specific outer membrane receptor protein, translocation through the cell envelope, and finally interaction with the target and killing effect. To each of these steps corresponds a specific protein domain, the different colicins showing a similar three-domain structural organization. Depending on the import pathway they parasitize to enter the cells, the Tol system or the TonB system, colicins are classified into two groups A and B, respectively (3). The mode of action of colicins is variable: formation of voltage-gated pores in the cytoplasmic membrane (e.g., colicins A, B, E1, Ia, N), inhibition of protein synthesis (e.g., colicins D and E3), enzymatic degradation of cellular DNA or 16S rRNA (e.g., colicin E2) and inhibition of cell-envelope biosynthesis (colicin M, see below).

Various bacterial cell-envelope polysaccharides (peptidoglycan, O-antigen, teichoic acid, capsular polysaccharide…) in both Gram-negative and Gram-positive bacteria have a lipid-linked intermediary stage in their biosynthesis that is dependent on the essential carrier lipid undecaprenyl phosphate (C55-P)1 (4-15) (scheme 1). In the peptidoglycan pathway, this lipid is needed for the synthesis and transport of hydrophilic GlcNAc-MurNAc-peptide monomeric motifs across the cytoplasmic membrane to the external sites of polymerization. In other pathways, it can serve as an acceptor for oligosaccharide repeating units or, in some cases, possibly for completed polymers. C55-P is generated from the dephosphorylation of undecaprenyl pyrophosphate (C55-PP) which itself is the product of eight sequential condensations of isopentenyl pyrophosphate with farnesyl pyrophosphate, a reaction catalyzed by the C55-PP synthase UppS (16,17). Several integral membrane proteins exhibiting a C55-PP phosphatase (UppP) activity were recently identified in E. coli (18,19): the BacA protein and three members from the PAP2 super-family of phosphatases, YbjG, YeiU and PgpB. Only the inactivation of these multiple genes was lethal.
and therefore required to completely deplete cells of the latter essential activity (19). C55-P phosphatase and undecaprenyl kinase activities that catalyzed the interconversion of C55-P into undecaprenol (C55-OH) were also earlier detected and purified from extracts of some Gram-positive bacteria but their genes have not been identified to date (20-23).

In the metabolism of C55-P, the dephosphorylation of C55-PP is the target of bacitracin, a cyclic polypeptide antibiotic which acts by sequestration of the C55-PP substrate (24-26). It was earlier hypothesized that the same step could also be the target of colicin M (27), a class B colicin requiring the outer membrane protein FhuA as a receptor (28-30). Colicin M exhibits a unique mode of action as it is the only colicin known to inhibit peptidoglycan synthesis and cause cell lysis (31). Interestingly, it was shown to also interfere with lipopolysaccharide O-antigen synthesis (32), suggesting an inhibition of a common step shared by the two pathways. As the C55-P carrier lipid is required in both processes, Harkness and Braun earlier hypothesized that the synthesis or recycling of this compound could be the target of colicin M (32). In order to identify the step of peptidoglycan synthesis blocked by colicin M, these authors analyzed the distribution of radiolabeled diaminopimelic acid (A2pm) in pulse-labeled colicin-treated cultures of E. coli and they observed an accumulation of the soluble peptidoglycan precursors (mainly UDP-MurNAc-pentapeptide) and the depletion of the pools of the two lipid intermediates I and II. It was thus concluded that colicin M acted by preventing the regeneration of the lipid carrier but the exact mechanism of action remained unknown and whether this inhibition was direct (e.g. inhibition of C55-PP phosphatase) or indirect was not determined (27).

In the present paper, the effects of colicin M on peptidoglycan metabolism were reinvestigated. Colicin M was shown to exhibit both in vitro and in vivo enzymatic properties of degradation of the lipid I and lipid II peptidoglycan intermediates. The depletion of the pools of these precursors results in an inhibition of peptidoglycan polymerization steps and cell lysis. To the best of our knowledge, this is the first time such an activity is described to date.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids and Growth Conditions** - The E. coli strains DH5α (supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 Φ80 lacZ ΔM15) (Bethesda Research Laboratories) was used as the host for plasmids. The FB8r lysA::KmR strain (33) was used to study the incorporation of radiolabeled meso-A2pm into peptidoglycan. The plasmid vector pTrc99A was from Pharmacia and the construction of pTrcHis30 and pTrcHis60 plasmids has been previously described (34). The pTO4 plasmid carrying the cma gene encoding colicin M and the cmi immunity gene has been previously described (35). The BW25113 strain and the pKD3 and pKD46 plasmids used for gene disruption experiments (36) were kindly provided by B. Wanner via the E. coli Genetic Stock Center (Yale University, New Haven). 2YT medium or minimal medium M63 (37) was used for growing cells. Ampicillin, kanamycin and chloramphenicol were used at 100, 35, and 25 µg.ml⁻¹, respectively.

**General DNA Techniques and E. coli Cell Transformation** - Polymerase chain reaction (PCR) amplification of genes from the E. coli chromosome were performed in a Thermocycler 60 apparatus (Bio-med) using the Expand-Fidelity polymerase from Roche. The DNA fragments were purified using the Wizard PCR Preps DNA purification kit (Promega). Standard procedures for endonuclease digestions, ligation and agarose electrophoresis were used (38). Small- and large-scale plasmid isolations were carried out by the alkaline lysis method (38). E. coli cells were made competent for transformation with plasmid DNA by the method of Daget and Ehrlich (39) or by electroporation.

**Construction of Expression Plasmids** - A plasmid allowing overexpression of the colicin M cma gene was constructed as follows: PCR primers Cma1 and Cma2 (see Table 1) were designed to incorporate an NcoI site 5’ to the initiation codon of the gene and a PstI site 3’ to the gene after the stop codon, respectively. The pTO4 plasmid (35) was used as the source of cma gene for the amplification and the resulting DNA fragment was treated with NcoI and PstI and ligated between the same sites of vector pTrc99A. The resulting plasmid, pMLD188, allowed expression of the cma gene under the control of the strong isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible trc promoter.

For expression of N-terminal His₆-tagged colicin M, the gene was amplified with primers Cma2 and Cma3 and the resulting fragment was cut with BamHI and PstI and inserted into pTrcHis30, generating plasmid pMLD189. For expression of C-terminal His₆-tagged colicin M, the gene was amplified with primers Cma1 and Cma4 and the resulting fragment was cut with NcoI and BglII and inserted into pTrcHis60, generating plasmid pMLD190.

A plasmid carrying both the cma gene and the cmi immunity gene was also constructed: in that case oligonucleotides Cma1 and Cmi were used for PCR amplification from the pTO4 plasmid and the 1.3-Kb
resulting fragment was cut by NcoI and PstI and inserted between the same sites of the vector pTrc99A, generating pMLD170.

In all cases DNA sequencing was performed to control that the sequence of the cloned fragments was correct.

Construction of a Null fhuA Mutant - The E. coli mutant strain BW25113 ΔfhuA::Cm\textsuperscript{R}, carrying a deletion of the chromosomal fhuA gene (an internal 1,819-bp fragment of the 2,244-bp gene was deleted and replaced by the chloramphenicol resistance gene), was created by following the method of Datsenko and Wanner (36). The FhuA-Inact1 and FhuA-Inact2 oligonucleotides (Table 1) were used for PCR amplification of the antibiotic resistance gene from pKD3 flanked by sequences designed for specific disruption of the fhuA gene. The 1.1-Kb PCR product was transformed by electroporation into BW25113(pKD46) cells that express phage lambda Red recombinase (36). Chloramphenicol-resistant clones were isolated and the inactivation of the fhuA gene was verified by PCR using the FhuA1 and FhuA2 primers.

Preparation of Crude Extracts and Purification of Colicin M - BW25113 ΔfhuA::Cm\textsuperscript{R} cells carrying plasmids pMLD189 or pMLD190 were grown at 37°C in 2YT-ampicillin medium (2-liter cultures). When the optical density of the culture reached 1, IPTG was added at a final concentration of 1 mM and growth was continued for 3 h. Cells were harvested and washed with 40 ml of cold 20 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM MgCl\textsubscript{2}, 2 mM β-mercaptoethanol and 150 mM NaCl (buffer A). The cell pellet (~8 g wet wt) was suspended in 25 ml of the same buffer and cells were disrupted by sonication in the cold (Bioblock). The resulting disrupted by sonication in the cold (Bioblock) suspension was centrifuged at 4°C for 30 min at 20,000 × g in a TL100 Beckman centrifuge. The supernatant (~400 mg of proteins) was stored at -20°C.

The His\textsubscript{6}-tagged colicin M was purified basically following the manufacturer’s recommendations (Qiagen). Soluble fractions described above were incubated for 1 hour at 4°C with nickel-nitritotriacetate (Ni\textsuperscript{2+}-NTA) agarose pre-equilibrated in buffer A. The polymer was then washed extensively with buffer A containing 20 mM imidazole and elution of the protein was obtained by increasing step by step the concentration of imidazole, from 40 to 300 mM, in buffer A. Pure protein-containing fractions (100-200 mM imidazole) were concentrated to 8 ml on a Millipore PM10 membrane and then dialyzed overnight against 100 volumes of buffer A. The final preparations were stored at -20°C after addition of 15% glycerol.

Determination of Colicin M Activity - The bacterioclytic activity of the crude soluble extracts prepared from the colicin M-expressing BW25113 fhuA strain and of the purified His\textsubscript{6}-tagged colicin M was tested both on plates and in liquid medium. 2YT-agar plates were overlaid with 3 ml of soft nutrient agar containing approximately 10\textsuperscript{8} indicator bacteria (BW25113 or DH5α). Serial dilutions (×3) of colicin M-containing solutions were made in buffer A containing 150 mM NaCl and 2-µl samples were spotted on the overlay. Plates were incubated overnight at 37°C and the titer of the colicin was expressed as the reciprocal of the final dilution still giving a clear spot. When assayed in liquid medium, dilutions of colicin M were added to exponentially growing cells when the optical density at 600 nm reached the value of 0.6.

Preparation of UDP-MurNAc-pentapeptide and Derivatives – Unlabeled and radiolabeled forms of UDP-MurNAc-L-[\textsuperscript{14}C]Ala-γ-D-Glu-meso-A2pm-D-Ala-D-Ala were prepared as previously described (40). 1-Pyrophospho-MurNAc-pentapeptide and 1-phospho-MurNAc-pentapeptide were obtained by treatment of UDP-MurNAc-pentapeptide with periodate (41,42) and nucleotide pyrophosphatase (43), respectively. MurNAc-pentapeptidase was obtained by mild-acid hydrolysis of UDP-MurNAc-pentapeptide (44). These compounds were purified by reverse-phase high-performance liquid chromatography (HPLC) and analyzed by mass spectrometry. 1-Pyrophospho-MurNAc-tetrapeptide and 1-pyrophospho-MurNAc-tripeptide were generated by treatments of 1-pyrophospho-MurNAc-pentapeptide with purified penicillin-binding protein PBP5 (45) and L,D-carboxypeptidase LdeA (46), respectively.

Synthesis of Radiolabeled C\textsubscript{55}-PP, Lipid I and Lipid II - Radiolabeled C\textsubscript{55}-PP was produced enzymatically by incubating farnesyl pyrophosphate and [\textsuperscript{14}C]isopentenyl pyrophosphate in the presence of pure UppS synthase, as previously described (18). [\textsuperscript{14}C]C\textsubscript{55}-P was obtained by treatment of [\textsuperscript{14}C]C\textsubscript{55}-PP with purified Baca phosphatase (18). The reaction mixture (200 µl) containing 100 mM Tris-HCl buffer, pH 7.5, 10 mM MgCl\textsubscript{2}, 3.9 mM n-dodecyl-β-D-maltoside (DDM), 5 µM [\textsuperscript{14}C]C\textsubscript{55}-PP (20 kBq) and Baca enzyme (5 µg). The radiolabeled C\textsubscript{55}-P was recovered by extraction with 1-butanol and dried under vacuum. The synthesis of lipid I was performed in a reaction mixture (400 µl) consisting in 100 mM Tris-HCl, pH 7.5, 40 mM MgCl\textsubscript{2}, 1.25 mM C\textsubscript{55}-P, 150 mM NaCl, 2 mM UDP-MurNAc-pentapeptide, and 8.4 mM N-lauroyl
sarcosine. The reaction was initiated by the addition of pure MraY enzyme (40) (10 µg), and the mixture was incubated for 2 h at 37 °C under shaking with a thermomixer (Eppendorf). [¹⁴C]lipid I labeled either in the pentapeptide moiety or the C₅₅-P moiety was synthesized as described above for the unlabeled lipid I except that the reaction volume was 20 µl and the concentrations of radiolabeled [¹⁴C]C₅₅-P and UDP-MurNAc-[¹⁴C]pentapeptide were 0.3 mM and 0.6 mM, respectively. The synthesis of lipid II was performed in a reaction mixture (200 µl) containing 200 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 1 mM lipid I, 2 mM UDP-GlcNAc, and 35% (v/v) Me₂SO (47). The reaction was initiated by the addition of pure UppS enzyme (1 µg) and when required, colicin M (2.7 µg), and the mixture was incubated for 3 h at 25 °C.

**Assay:**
The reaction was initiated by the addition of MurG enzyme (48)(10 µg), and the mixture was incubated for 2 h at 37 °C under shaking with a thermomixer (Eppendorf). [¹⁴C]lipid II labeled in the GlcNAc moiety was synthesized as described above for the unlabeled lipid II except that the reaction volume was 20 µl and the concentrations of lipid I and radiolabeled UDP-[¹⁴C]GlcNAc were 0.3 mM and 0.6 mM, respectively. In all cases, the extraction, analysis and quantification of lipid I and lipid II were performed as described previously (40).

**Enzymatic Assays - (i) C₅₅-PP Synthase Assay:** the assay was performed in a reaction mixture (20 µl) containing 100 mM HEPES buffer, pH 7.5, 50 mM KCl, 0.5 mM MgCl₂, 0.1% Triton X-100, 5 µM farnesyl pyrophosphate, and 50 µM [¹⁴C]isopentenyl pyrophosphate (2 kBq). The reaction was initiated by the addition of pure UppS enzyme (1 µg) and when required, colicin M (2.7 µg), and the mixture was incubated for 3 h at 25 °C. (ii) C₅₅-PP Phosphatase Assay: the assay was performed in a reaction mixture (20 µl) containing 100 mM Tris-Cl buffer, pH 7.5, 10 mM MgCl₂, 3.9 mM DDM, 5 µM [¹⁴C]C₅₅-PP (2,035 Bq), and enzyme: 5 µl of an appropriate dilution (in buffer A supplemented with 3.9 mM DDM) of membranes or pure BacA protein (0.1 µg). When required, colicin M (2.7 µg) was added and the mixture was incubated for 1 h at 37 °C. (iii) MraY Translocase Assay: the assay was performed in a reaction mixture (20 µl) containing 100 mM Tris-Cl buffer, pH 7.5, 40 mM MgCl₂, 1.1 mM C₅₅-P, 150 mM NaCl, 12.5 µM UDP-MurNAc-[¹⁴C]pentapeptide (800 Bq), and 0.2% DDM. The reaction was initiated by the addition of pure MraY enzyme (0.01 µg). When required, 2.7 µg of colicin M was added and the mixture was incubated for 30 min at 37°C under shaking with a thermomixer (Eppendorf). When E. coli membranes instead of pure MraY enzyme were used, C₅₅-P was omitted. (iv) Standard Assay for Colicin M Activity: unless otherwise noted, the activity of colicin M was tested in a reaction mixture (20 µl) containing 100 mM Tris-Cl, pH 7.5, 20 mM MgCl₂, 150 mM NaCl, 40 µM of [¹⁴C]radiolabeled substrate (400 Bq) and 0.2% DDM. The reaction was initiated by the addition of pure colicin M (2.7 µg), and the mixture was incubated for 30 min at 37 °C under shaking with a thermomixer (Eppendorf).

In all cases, the reaction was stopped by heating at 100 °C for 1 min, and the mixture was analyzed by thin-layer chromatography (TLC) on silica gel plates LK6D (Whatman) using either 1-propanol/ammonium hydroxide/water (6:3:1; v/v/v) (solvent system I) or diisobutyl ketone/acetic acid/water (8:5:1; v/v/v) (solvent system II) as a mobile phase. The radioactive spots were located and quantified with a radioactivity scanner (model Multi-Tracemaster LB285; Berthold France).

**Purification of Lipid I and Lipid II Degradation Products by HPLC -** Standard colicin M assays were performed as described above except that lipid I and lipid II (1 nmol) were used in unlabeled form. The reaction mixtures were diluted into 1 ml of 50 mM sodium phosphate buffer, pH 4.5, and applied to a Nucleosil 100C18 5 µm (4.6 × 250 mm, Alltech) column. Elution was performed with the phosphate buffer for 30 min, followed by a linear gradient of methanol from 0 to 20% during the next 30 min, at a flow rate of 0.6 ml min⁻¹. Peaks were detected at 215 nm. In these conditions the 1-pyrophospho-MurNAc-pentapeptide and 1-pyrophospho-MurNAc-(pentapeptide)-GlcNAc degradation products were eluted at 18 and 40 min, respectively. In the same conditions, pure 1-phospho-MurNAc-pentapeptide and the two α and β anomers of MurNAc-pentapeptide were eluted at 28, 34 and 50 min, respectively. The two degradation products were collected, lyophilized, and desalted by gel-filtration on a column of Sephadex G-25 fine. After lyophilization, these compounds were taken up in 10 µl of water and analyzed by mass spectrometry as described below.

**Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry -** MALDI-TOF mass spectra were recorded in the linear mode with delayed extraction on a PerSeptive Voyager-DE STR instrument (Applied Biosystems) equipped with a 337-nm laser. (i) 1-Pyrophospho derivatives: 1 µl of matrix solution (10 mg.ml⁻¹ 2,5-dihydroxybenzoic acid in 0.1 M citric acid) was deposited on the plate followed with 2 µl of sample solution. After evaporation of water, spectra were recorded in the negative mode at an acceleration voltage of -20 kV and an extraction delay time of 100 ns. A mixture of UDP-MurNAc, UDP-MurNAc-dipeptide, and UDP-MurNAc-pentapeptide was used as an external calibrant. (ii) C₅₅-OH: the sample solution was mixed with an equal volume of 0.5 M 2,5-dihydroxybenzoic acid in methanol containing
and radioactivity was counted in a liquid scintillation of Unisafe 1 mixture (Zinsser Analytic) were added dried, and immersed in 2 ml of 0.1 M NaOH. 13 ml Whatman). The filters were washed with 5.5% TCA, acid (TCA). Suspensions were kept at 0°C for 60 min and labeled peptidoglycan (TCA insoluble were added to 9.5 ml of ice-cold 5.5% trichloroacetic medium (50 ml cultures) supplemented with 0.4% glucose, and 100 µg.ml\(^{-1}\) each of lysine, threonine and methionine (33). At an optical density of 0.2, cultures were either treated or not with colicin M at 50 ng.ml\(^{-1}\), and \(^{3}H\)A\(_{2}pm\) was added ten minutes later (2.5 kBq.ml\(^{-1}\)). After designated time intervals, 500-µl samples were removed and centrifuged for 2 min with an Eppendorf centrifuge. The supernatants were analyzed for radioactivity in order to estimate the A\(_{2}pm\) uptake. In parallel, other 500-µl samples were added to 9.5 ml of ice-cold 5.5% trichloroacetic acid (TCA). Suspensions were kept at 0°C for 60 min and labeled peptidoglycan (TCA insoluble material) was filtered over glass fiber filters (GF/C, Whatman). The filters were washed with 5.5% TCA, dried, and immersed in 2 ml of 0.1 M NaOH. 13 ml of Unisafe 1 mixture (Zinsser Analytic) were added and radioactivity was counted in a liquid scintillation spectrophotometer.

**Uptake of Radiolabeled meso-Diaminopimelic Acid (A\(_{2}pm\)) and its Incorporation into Peptidoglycan** - The incorporation of meso-[\(^{3}H\)]A\(_{2}pm\) into the peptidoglycan of strain FB8r lysA was followed essentially as described earlier (33). The strain was grown exponentially in minimal M63 medium (50 ml cultures) supplemented with 0.4% glucose, and 100 µg.ml\(^{-1}\) each of lysine, threonine and methionine (33). At an optical density of 0.2, cultures were either treated or not with colicin M at 50 ng.ml\(^{-1}\), and \(^{3}H\)A\(_{2}pm\) was added ten minutes later (2.5 kBq.ml\(^{-1}\)). After designated time intervals, 500-µl samples were removed and centrifuged for 2 min with an Eppendorf centrifuge. The supernatants were analyzed for radioactivity in order to estimate the A\(_{2}pm\) uptake. In parallel, other 500-µl samples were added to 9.5 ml of ice-cold 5.5% trichloroacetic acid (TCA). Suspensions were kept at 0°C for 60 min and labeled peptidoglycan (TCA insoluble material) was filtered over glass fiber filters (GF/C, Whatman). The filters were washed with 5.5% TCA, dried, and immersed in 2 ml of 0.1 M NaOH. 13 ml of Unisafe 1 mixture (Zinsser Analytic) were added and radioactivity was counted in a liquid scintillation spectrophotometer.

**Cellular Distribution of \(^{3}H\)A\(_{2}pm\)** - In order to identify the step in peptidoglycan metabolism that was blocked by colicin M, the cellular distribution of \(^{3}H\)A\(_{2}pm\) incorporated in strain FB8r lysA was determined. The strain was grown, treated or not with colicin M (at t = 0), and labeled with \(^{3}H\)A\(_{2}pm\) (at t = 10 min) as described above, except that more radioactivity was used (50 kBq.ml\(^{-1}\)). Cells were harvested 15 min later, i.e. 25 min after the addition of colicin M and just before the onset of cell lysis. The cell pellets were resuspended in 0.1 M Tris-HCl, pH 7.4, and disrupted by sonication in the cold. The resulting suspensions were centrifuged at 4°C for 30 min at 200,000 × g. Membrane pellets were resuspended in phosphate-buffer saline, pH 7.4, and the lipids were extracted by the chloroform-methanol method (52). The chloroform extracts containing C\(_{55}\)-OH and its phosphorylated derivatives were dried, dissolved in the HPLC eluent and analyzed on the Nucleosil column. Elution was performed with methanol-2-propanol (4:1, v/v) containing 10 mM phosphoric acid (17), at a flow rate of 0.6 ml.min\(^{-1}\). Peaks were detected at 210 nm. Quantitation of C\(_{55}\)-OH in E. coli Cell Membranes - Cultures (300 ml) of BW25113 were treated or not with colicin M (at O.D. = 0.8) as described above and cells were harvested 40 min thereafter, just before the onset of cell lysis. The cell pellets were resuspended in 0.1 M Tris-HCl, pH 7.4, and disrupted by sonication in the cold. The resulting suspensions were centrifuged at 4°C for 30 min at 200,000 × g. Membrane pellets were resuspended in phosphate-buffer saline, pH 7.4, and the lipids were extracted by the chloroform-methanol method (52). The chloroform extracts containing C\(_{55}\)-OH and its phosphorylated derivatives were dried, dissolved in the HPLC eluent and analyzed on the Nucleosil column. Elution was performed with methanol-2-propanol (4:1, v/v) containing 10 mM phosphoric acid (17), at a flow rate of 0.6 ml.min\(^{-1}\). Peaks were detected at 210 nm. Quantitation of C\(_{55}\)-OH and its phosphorylated derivatives was performed with respect to commercial standards injected in the same conditions. The C\(_{55}\)-OH peak was collected and the solvents were evaporated. The residue was taken up in ether and the organic solution was washed with water to remove phosphoric acid. After evaporation of ether and lyophilization to remove residual water, the material was dissolved in 20 µl of methanol-2-propanol (1:1, system I). Under these conditions, peptidoglycan remains at the origin and the mixture of lipids I and II migrates with an R\(_{f}\) value of 0.6. The analysis of the soluble fractions by TLC allowed the separation of A\(_{2}pm\) from the pool of UDP-MurNAc-peptides and from the mixture of lipid I and II degradation products (see Results). An additional step of HPLC was then required to determine the relative amounts of the different UDP-MurNAc-peptides as well as the ratio of lipid I and II degradation products. This was achieved by using the Nucleosil column and isocratic elution with a mixture of 50 mM triethylammonium formate, pH 4.6, and methanol (94:6; v/v), at a flow rate of 0.6 ml.min\(^{-1}\). Under these conditions, A\(_{2}pm\), UDP-MurNAc-tripeptide, UDP-MurNAc-tetrapeptide and UDP-MurNAc-pentapeptide were eluted at 5, 35, 47 and 62 min, respectively. 1-pyrophospho-MurNAc-tripeptide, 1-pyrophospho-MurNAc-pentapeptide and 1-pyrophospho-MurNAc-(pentapeptide)-GlcNAc, which were detected in extracts from colicin M-treated cells, eluted at 22, 38 and 60 min, respectively. Detection of radioactivity in HPLC efﬂuents was performed with a radioactive ﬂow detector (model LB-506-C1, Berthold France) using the Quicksafe Flow 2 scintillator (Zinsser Analytic) at 0.6 ml.min\(^{-1}\). Quantitation was carried out with the Winflow software (Berthold France).
**Protein Monitoring - SDS-PAGE analysis of proteins** was performed as described by Laemmli and Favre (53). Protein concentrations were determined by using the Bradford procedure (54) with bovine serum albumin as the standard, and/or by quantitative amino acid analysis with a Hitachi model L8800 analyzer (ScienceTec) after hydrolysis of samples in 6 M HCl for 24 h at 105 °C.

**Chemicals - DNA restriction enzymes** were obtained from New England Biolabs. Oligonucleotides and DNA sequencing were done by MWG-Biotech and DNA purification kits were provided by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences. UDP-[U-14C]GlcNAc (9.85-11.1 GBq.mmol⁻¹) was purchased from Amersham Pharmacia Biotech, [4-³H]A2pm (1.11-2.22 TBq.mmol⁻¹) from ARC. [¹⁴C]isopentenyl-pyrophosphate (1.5 - 2.2 GBq.mmol⁻¹) from NEN Life Science Products, and [⁴⁰K]tritiated muramylpentapeptide was purchased from Amersham Pharmacia Biotech. D-camphorsultam (1.1-2.2 GBq.mmol⁻¹) from DuPont NEN. L-[¹⁴C]alanine (9.85-11.1 GBq.mmol⁻¹) was purchased from NEN Life Science Products, and 

**RESULTS**

*Expression and Purification of Colicin M - The cma gene* encoding colicin M was cloned into plasmid pTrc99A and derivative vectors allowing its expression under the control of the strong IPTG-inducible trc promoter in either wild-type (pMLD188), N-terminal- (pMLD189) or C-terminal (pMLD190) His6-tagged form. These plasmids appeared to be toxic in a classical *E. coli* host strain (DH5α) as only transformants carrying the empty vector were isolated on 2YT-ampicillin plates and complete lysis of the cell population occurred when transformation mixtures were grown in liquid medium. This toxic effect reflected the sensitivity of host cells to the colicin M they release in the growth medium. It indicated that a significant expression of the *cma* gene already occurred in the absence of inducer (IPTG) and further suggested that all three forms of colicin M were active and that the presence of a His-tag at the N- or C-terminal extremity did not abolish its biological activity. The problem of toxicity was overcome either by co-expressing the *cmi* immunity gene on the same plasmid (pMLD170), or by using BW25113 Δ*huA::Cm* that does not express the colicin M-outer membrane receptor as the host strain. Plasmids pMLD188, pMLD189 and pMLD190 were effectively successfully isolated in the latter genetic background, in total agreement with previous data from Braun's laboratory which showed that colicin M should enter cells from the outside to exert its lethal effect (56).

BW25113 Δ*huA::Cm* cells carrying the different expression plasmids were grown in 2YT-ampicillin medium and induced with IPTG for 3 h. Induced cells exhibited some morphological differences when observed by phase-contrast microscopy: they appeared as greatly enlarged rods and cell lysis frequently occurred after prolonged incubation. Accumulation in the cell content of a protein species of about 30 kDa was detected (Fig. 1), a value in agreement with that (29,453) calculated from the DNA sequence of *cm* (29). A typical fractionation procedure of cell extracts showed that colicin M was found mainly in the soluble fraction, but significant amounts (10 to 20%) were also detected in the particulate fraction. The N-terminal- and C-terminal His6-tagged versions of colicin M were apparently overproduced to similar levels (Fig. 1) and their migrations on SDS-PAGE gels were consistent with their slightly higher molecular mass, 30,580 (Met-His6-Gly-Ser-extension) and 30,548 (~Arg-Ser-His6 extension), respectively.

In order to roughly estimate and compare the activity of the three forms of colicin M present in these soluble fractions, BW25113 indicator cells were plated onto 2YT-agar plates and 2 µl of serial dilutions of the crude extracts were spotted on the lawn of bacteria. After overnight incubation at 37°C, final dilutions giving a clear spot were in all cases equivalent (~8 ng of proteins of crude extracts), confirming that the presence of a His-tag on the protein had no effect on its activity.

A large scale preparation of the two His6-tagged versions of colicin M was made from 2-liter cultures of BW25113 Δ*huA::Cm* cells carrying either the pMLD189 or pMLD190 plasmid. Crude soluble extracts were purified on Ni²⁺-NTA agarose and pure fractions, as judged by SDS-PAGE (Fig. 1), were dialyzed, concentrated to 1 mg.ml⁻¹ of protein, and then conserved at -20°C in the presence of 15% glycerol. This procedure yielded about 10 mg of either form of pure His6-tagged colicin M.

Pure colicin M preparations were assayed for bactericolytic activity on *E. coli* growing cells. Both His6-tagged forms behaved similarly and induced rapid lysis of BW25113 cells at a concentration of about 10 ng.ml⁻¹ (Fig. 2). It confirmed that the
presence of the His-tag at either the N-terminal or C-terminal end of the protein had no significant effect on the activity of the colicin.

**Enzymatic Properties of Colicin M** - We first tested, *in vitro*, the inhibitory effect of colicin M on the step of dephosphorylation of C55-PP, taking advantage of the recent identification of the first gene (named *bacA*) encoding such a C55-PP phosphatase activity in *E. coli* (18). Using purified BacA protein, no inhibition was observed in the presence of up to 300 µg.ml⁻¹ of colicin M in typical assay conditions. As multiple genes encoding C55-PP phosphatase activities were recently identified in *E. coli* (19), namely *ybjG*, *pgpB* and *yetU*, it was possible that a phosphatase other than BacA was the specific target of colicin M. The finding that colicin M did not inhibit the overall C55-PP phosphatase activity detected in crude cell membrane extracts prepared either from wild-type cells or from cells overexpressing these individual genes made this hypothesis unlikely (data not shown). These results suggested that colicin M might interfere with another step of the metabolism of C55-P. Assays using pure *E. coli* C55-PP synthase UppS showed that the preceding step of formation of C55-PP was not inhibited by colicin M either (data not shown).

We thus envisaged that the downstream steps leading to the formation of peptidoglycan lipid intermediates I and II could be targeted by the colicin. Interestingly, when a MraY translocase assay that consists in following the formation of radiolabeled lipid I from C55-P and UDP-MurNAc-[¹⁴C]pentapeptide was performed, an additional radioactive spot of unidentified product was observed when colicin M was present (Fig. 3B). Its absence when either the enzyme or the C55-P substrate was omitted from the reaction mixture, strongly suggested that it was a degradation product of lipid I. Incubation of purified [¹⁴C]lipid I (radiolabeled in the peptide moiety) with colicin M confirmed this assumption as a complete conversion of lipid I into the latter product was observed (Fig. 3C). The low migration of this product in the TLC conditions used suggested the loss of the C55 lipid moiety. To localize the site of cleavage, MurNAc-pentapeptide and its 1-phospho and 1-pyrophospho derivatives were generated by mild-acid hydrolysis or treatments with pyrophosphatase or periodate of UDP-MurNAc-[¹⁴C]pentapeptide, respectively. From the behavior of these different compounds on HPLC (details in Experimental Procedures), it was concluded that the degradation product was 1-pyrophospho-MurNAc-pentapeptide. The other product resulting from the degradation of lipid I by colicin M was therefore C55-OH but this product was not labeled and consequently could not be detected in these experiments. To confirm the data, [¹⁴C]lipid I labeled this time in the lipid moiety was synthesized. Incubation of this substrate, [¹⁴C]C55-PP-MurNAc-pentapeptide, with colicin M effectively resulted in its quantitative conversion into [¹⁴C]C55-OH, as unambiguously confirmed by TLC analysis (in solvent system II, the *Rₚ* values of lipid I and its product were 0.20 and 0.98, and those of authentic standards of C55-PP, C55-P, and C55-OH were 0.36, 0.50, and 0.98, respectively).

Colicin M thus appeared to be an enzyme hydrolyzing the peptidoglycan lipid intermediate I into C55-OH and 1-pyrophospho-MurNAc-pentapeptide. We observed that C55-PP was not a substrate of colicin M, suggesting that the presence of a bound MurNAc residue was critical for activity or at least that the beta phosphate group should not be free. The fact that UDP-MurNAc-pentapeptide was not a substrate either clearly showed that colicin M was specific of C55-linked peptidoglycan precursors. We also analyzed the effects of colicin M on [¹⁴C]lipid II synthesized from lipid I and UDP-[¹⁴C]GlcNAc by purified MurG transferase. The same phenomena were observed, namely the cleavage of this lipid into two products, which were identified in that case as C55-OH and 1-pyrophospho-MurNAc(pentapeptide)-GlcNAc, and the absence of hydrolysis of the nucleotide precursor UDP-GlcNAc (data not shown). In the *in vitro* assay conditions used, the specific activities of colicin M for lipid I and lipid II substrates were estimated at about 0.2 and 0.4 nmol.min⁻¹.mg⁻¹ of protein, respectively.

To further confirm the nature of the degradation products, pure unlabeled lipids I and II were treated by colicin M and the degradation products were purified by HPLC (Experimental Procedures) and analyzed by MALDI-TOF mass spectrometry. One main peak was observed in each case (Fig. 4), with *m/z* ratios of 967.2 and 1170.3, that perfectly matched the expected values for the [M-H]⁻ ions of 1-pyrophospho-MurNAc-pentapeptide (C₁₂H₁₅N₄O₃₃P₂, average molecular mass of 967.8 g.mol⁻¹) and 1-pyrophospho-MurNAc(pentapeptide)-GlcNAc (C₄₀H₆₄N₄O₃₃P₂, average molecular mass of 1171.0 g.mol⁻¹), respectively. In both spectra, smaller peaks corresponding to cation adducts were present.

**In Vivo Activity of Colicin M** – Our data suggested that the effects of colicin M on *E. coli* cells, *i.e.*, the inhibition of peptidoglycan synthesis followed by cell lysis, might result from the specific degradation of lipid intermediates I and II. To control the physiological significance of the phenomena observed *in vitro*, cell labeling experiments were performed that used radiolabeled
A₂pm as a specific marker of peptidoglycan metabolism. As previously reported, to ensure a specific and efficient labeling, a hysA mutant strain and particular growth conditions in minimal medium were used to reduce the internal pool of A₂pm and block its conversion into lysine (33,57). A rapid uptake of [³H]A₂pm was observed after its addition to cultures of FB8r hysA cells. This was followed by a rapid incorporation of radioactivity into TCA-precipitable material (peptidoglycan), which was completed in about 30 minutes (Fig. 5). When colicin M at a final concentration of 50 ng.ml⁻¹ was added to the culture 10 min before the addition of [³H]A₂pm, the incorporation of radioactivity into peptidoglycan was first similar to that detected in untreated cells during the first 5 min of labeling but it rapidly stopped thereafter. The arrest of peptidoglycan synthesis was followed by the onset of cell lysis about 10 min later (Fig. 5). When precipitable material (peptidoglycan), which was a rapid incorporation of radioactivity into TCA-precipitable material: although the molecular ion peak (calculated m/z ratio 768) was not visible, a peak of m/z ratio of 750.9, corresponding to the molecular ion after loss of water, was present (Fig. 4C). Such an elimination reaction in mass spectrometry conditions has already been observed for C55-OH (20,60). This peak was absent in the spectrum of the similar fraction collected from the control membranes (data not shown). All these data clearly validated the physiological significance of the in vitro established enzymatic properties of colicin M and definitely demonstrated that this antibiotic protein acts by destroying lipid intermediates involved in peptidoglycan biosynthesis.

**DISCUSSION**

Colicin M was earlier shown to induce *E. coli* cell lysis by interfering with cell-wall peptidoglycan biosynthesis. It was shown not to have a peptidoglycan-degrading activity (61) and to act synergistically with β-lactam antibiotics that inhibit polymerization steps catalyzed by penicillin-binding proteins (31). As lipopolysaccharide O-antigen synthesis was concomitantly blocked, the metabolism of the carrier lipid C₅₅-P that is shared by the two pathways clearly appeared as the target for colicin M (27,32). However, the inhibited metabolic step was not precisely identified and the mechanism of action of colicin M remained unknown. We now revisited this question by purifying this protein to near homogeneity and analyzing in details its effects on cell wall peptidoglycan metabolism, using both in vitro and in vivo experiments. Our data show that purified colicin M exhibits enzymatic properties and catalyzes in vitro the hydrolysis of peptidoglycan lipid intermediates I and II. This is clearly the mode of action of colicin M as the degradation of the latter lipid intermediates was confirmed in *E. coli* growing cells by appropriate radiolabeling experiments. The enzymatic cleavage of the peptidoglycan precursors occurs between the undecaprenyl and 1-pyrophospho-MurNAc moieties. This is to our knowledge the first time such an activity is reported.
The degradation products, namely C55-OH and either 1-pyrophospho-MurNac-pentapeptide or 1-pyrophospho-MurNac-(pentapeptide)-GlcNAc, are expected to be end products as they a priori could not be directly reused for de novo peptidoglycan synthesis. The fact that no C55-OH kinase activity has been identified to date in E. coli suggests that C55-OH either does not normally exist or could not be recycled into C55-P in this bacterial species. This could explain why C55-OH readily accumulates in colicin M-treated cells. However, a further degradation of the pyrophosphorylated degradation products into free MurNac and peptides by specific glucosaminidase (NagZ), amidase (AmpD) and carboxypeptidase (LdcA) activities involved in the peptidoglycan recycling process (see (62) for references) was expected to occur. The present identification of 1-pyrophospho-MurNac-tripeptide as a secondary degradation product supports this assumption.

Colicin M was shown in vitro to cleave lipid I and lipid II with quite similar efficiencies. The pool levels of these two peptidoglycan lipid intermediates were previously estimated at about 700 and 2000 molecules per cell, respectively (51,58,59). The present radiolabeling experiments showed, however, that the main degradation product isolated from colicin M-treated cells was 1-pyrophospho-MurNac-pentapeptide, i.e. the lipid I degradation product. 1-Pyrophospho-MurNac-(pentapeptide)-GlcNAc, the lipid II degradation product, was also detected, although at a much lower level (<2%). The apparent prevalence of the lipid I degradation product could have several origins: (i) lipid I is the first lipid intermediate in the pathway and consequently the first target made accessible to colicin M; (ii) as already mentioned, peptidoglycan recycling enzymes are present in the cytoplasm, in particular the glucosaminidase NagZ that cleaves between MurNac and GlcNAc residues; therefore, conversion by this enzyme of the lipid II degradation product into what could be interpreted as a lipid I degradation product is expected to occur; (iii) whether colicin M exerts its deleterious activity on the inner or outer side of the membrane is at present unknown but this localization could privilege in some way the access of colicin M to the lipid intermediate I.

Only a few reports on the effects of colicin M on peptidoglycan metabolism had been previously published (27,31). Using similar cell radiolabeling experiments, these authors also observed an accumulation of UDP-MurNac-pentapeptide and the depletion of both lipid intermediates I and II in treated cells, and naturally came to the conclusion that colicin M inhibited the recycling of the carrier lipid C55-P. However they could not discriminate at that time between different hypotheses they envisaged (27): an inhibition of the C55-PP phosphatase, a colicin M-catalyzed conversion of C55-P into C55-OH, or a sequestration of C55-P by colicin M. Why they failed to detect the here observed additional spots of lipids I and II degradation products is unclear. Most likely the paper chromatography technique they used at that time to resolve the mixture of labeled products did not allow the separation of these minor compounds from the UDP-MurNac-peptides. These authors also tried to estimate the pools of C55-PP and C55-P in membranes of control and colicin M-treated cells. The analytical procedure they used (separation by paper chromatography followed by quantitation based on the determination of phosphate) was not very conclusive but they found that a treatment with colicin M resulted in an increase of the C55-PP/C55-P ratio, from 0.6 to 2.3, consistent with an arrest of C55-P recycling (32). However, their procedure based on phosphate determination did not allow detection of C55-OH, the other main degradation product resulting from colicin M action identified in the present report.

The pool level of C55-P is generally assumed to be low (17,51,63) although it is required for the synthesis of multiple cell envelope components, and although huge amounts of precursors (several millions molecules per generation time) are translocated via this lipid across the cytoplasmic membrane. It was earlier considered as a potential regulatory factor controlling the flow of metabolites going through these different pathways (64,65). The pool levels of the peptidoglycan lipid intermediates I and II are also quite low (51,58,59). Both the low-size pool of the C55-P carrier lipid and the enzymatic mode of action of colicin M that results in the interruption of its recycling likely explain the powerful effect of colicin M. We effectively observed that pure colicin M provoked lysis of sensitive E. coli cells at extremely low concentrations, in the ng ml⁻¹ range (~50 to 200 molecules per cell). It should be noted that Braun and coworkers previously showed that colicin M rapidly lost activity (within 20 min) after its dilution in cell growth medium, due to a denaturation that could be partly overcome by addition of bovine serum albumin or nonionic detergents such as Triton X-100 (61). As we did not check the stability of colicin M in our growth conditions and performed all our experiments in the absence of any additives, the minimal number of molecules needed to kill a single cell could probably still be lower. This is perfectly consistent with the earlier estimate that a single lethal unit, deduced from single-hit kinetics, corresponded to 10 molecules of added colicin M (61). Amounts of colicin M that are accumulated in
the cell content of the *fhuA* mutant following expression from the pMLD188 to pMLD190 plasmids are enormous as compared to those needed to kill cells when externally supplied in the growth medium. It confirms the previous observation that colicin M exhibits its bactericidal properties only when entering the cell from outside (56). The lipid I and lipid II intermediates, the colicin M targets, are synthesized by the MraY and MurG enzymes on the inner side of the cytoplasmic membrane and then translocated to the outer side of the membrane where the polymerization steps occur (10). The absence of deleterious effects observed for colicin M molecules produced inside cells is therefore surprising. This could be interpreted as a non accessibility of this enzyme to its targets from the cytoplasm side, suggesting that the latter could be either protected within MraY and MurG enzymes active sites or rapidly transferred to the outer leaflet of the membrane once synthesized.

Neither the nucleotide precursors UDP-MurNAc-pentapeptide and UDP-GlcNAc nor C55-PP are cleaved by colicin M, indicating a requirement for both the lipid and sugar moieties present in the structure of lipids I and II. Interestingly, colicin M was earlier shown to interfere with the biosynthesis of the O-antigen moiety of lipopolysaccharides (32). This could simply be an indirect effect of the depletion of the C55-P pool that itself results from the degradation of the peptidoglycan lipid intermediates by colicin M. Alternatively, it is tempting to speculate that the arrest of O-antigen synthesis also results from the degradation by colicin M of its respective lipid-linked precursor, C55-PP-GlcNAc, the *wecA* gene product (66). Experiments previously reported by Harkness and Braun did not allow to discriminate between the two hypotheses (32). Further experiments with the purified protein and different synthetic C55-PP-sugar derivatives are underway to analyze in more details the kinetic properties and substrate specificity of colicin M.

As is the case for all colicins, colicin M is organized in three distinct domains, the C-terminal one being responsible of its lethal activity (28). Interestingly, an alignment of amino acid sequences had revealed some homology between the C-terminal domain of colicin M (215-249 region) and the region around the active-site serine residue in the penicillin-binding proteins (29). Whether this discrete homology could be related to the presently identified activity of colicin M remains to be demonstrated. It is noteworthy that these two classes of proteins bind to (and compete for) the same substrate, lipid II, but however they catalyze quite different reactions of degradation and polymerization, respectively. The local sequence homologies could then reflect some evolutionary conserved binding domain for this complex substrate. Interestingly in this respect, multiple hydroxylamine-generated inactive forms of colicin M were previously described that carried mutations on residues 193-197 and 223-252, suggesting that residues important for substrate binding or catalysis might effectively reside in the latter regions. The deletion of the two terminal amino acid residues (Lys-Arg) at the C-terminal extremity of the protein was shown to abolish its activity (67), confirming the importance of the integrity of the C-terminal domain. Addition of a His-tag at this extremity had however no apparent effect on its activity, as shown in the present report. All these available informations will be very useful in future work aiming at characterizing this protein in more details, both biochemically and structurally, in the light of its newly identified activity.
REFERENCES


**FOOTNOTES**

* We thank Muriel Crouvoisier for providing pure MurG enzyme, Mireille Hervé for pure PBP5, and Gregory Moeck for the pTO4 plasmid. This work was supported by grants from the European Community (FP6, COBRA project, LSHM-CT-2003-503-335) and from the Centre National de la Recherche Scientifique (UMR 8619). M.E.G. was recipient of a scholarship from the Ministère de l’Education Nationale, de la Recherche et de la Technologie (Ecole Doctorale Innovation Thérapeutique, du Fondamental à l’Appliqué) and H.B. was supported by the European Community.

1 The abbreviations used are: GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; C55-P, undecaprenyl phosphate; C55-PP, undecaprenyl pyrophosphate; C55-OH, undecaprenol; PP, pyrophosphoryl; A2pm, diaminopimelic acid; lipid I, C55-PP-MurNAc-pentapeptide; lipid II, C55-PP-MurNAc(pentapeptide)-GlcNAc; Ni2+-NTA agarose, nickel-nitriolotriacetate agarose; DDM, n-dodecyl-β-D-maltoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; O.D., optical density; IPTG, isopropyl-β-D-thiogalactopyranoside; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.
SCHEME AND FIGURE LEGENDS

Scheme 1. Biosynthesis of undecaprenyl phosphate and its use for peptidoglycan synthesis. The targets of bacitracin and colicin M are indicated.

Fig. 1. Overproduction and purification of colicin M. DH5α cells carrying plasmids pMLD188, pMLD189 and pMLD190 for expression of the cma gene in wild-type, N-terminal- or C-terminal His6-tagged form, respectively, were grown in 2YT-ampicillin medium (50-ml cultures) and induced for 3 hours with 1 mM IPTG. Crude extracts were prepared and both soluble and membrane fractions obtained after high-speed centrifugation were analyzed by SDS-PAGE. The two His6-tagged versions of colicin M were overproduced from 2-liter cultures and purified by using Ni2+-NTA agarose as described in the text. Lanes A to D: soluble extracts from control DH5α and IPTG-induced DH5α(pMLD188), DH5α(pMLD189) and DH5α(pMLD190) cells, respectively. Lanes E and F: pure colicin M prepared with a N-terminal- and a C-terminal His6-tag, respectively. The faint band of protein observed about 68 kDa in the latter lanes does not correspond to a dimer of colicin. It is a minor native protein from E. coli that is commonly co-purified with histidine-tagged proteins due to its affinity for Ni2+-NTA (68). Molecular mass standards (M) indicated on the left (kilodaltons) are bovine serum albumin, 67; ovalbumin, 43; carbonic anhydrase, 30; and soybean trypsin inhibitor, 20. Staining was performed with Coomassie brilliant blue R250 (Merek).

Fig. 2. Bacteriolytic effect of colicin M. DH5α cells were grown at 37°C in 2YT medium. At the time indicated by the arrow (zero time; O.D. = 0.7), pure colicin M (ColM), in either N-terminal (closed symbols) or C-terminal (open symbols) His6-tagged form was added at different concentrations (triangles, diamonds and squares: 7.9, 10 and 13.6 ng.ml−1, respectively; circles, no addition) and growth was followed at 600 nm.

Fig. 3. In vitro degradation of peptidoglycan lipid intermediate I by colicin M. (A-B) An MraY assay that consists in following the formation of C55-PP-MurNAc-[14C]pentapeptide (lipid I) from UDP-MurNAc-[14C]pentapeptide (800 Bq) and C55-P was performed in the absence (A) or presence (B) of colicin M. Conditions that result in ~50% conversion of the radiolabeled substrate into product were used. In C, purified [14C]lipid I labeled in the peptide moiety (400 Bq) was incubated with or without colicin M (dashed and solid lines, respectively). Substrate and reaction products were separated by TLC (solvent system I) and the corresponding spots were detected with a radioactivity scanner, as detailed in the text (Rf values for lipid I and UDP-MurNAc-pentapeptide were 0.6 and 0.26, respectively). In both cases, an additional product with an Rf value of 0.09 was observed when colicin M was present.

Fig. 4. MALDI-TOF mass spectrometry analysis of the purified degradation products generated by colicin M. In the case of the 1-pyrophospho-MurNAc-pentapeptide product resulting from treatment of lipid I by colicin M (A), peaks of m/z 967.2, 989.2 and 1005.0 were observed, that were assigned to be the [M-H]−, [M-2H+Na]− and [M-2H+K]− ions, respectively. In the case of 1-pyrophospho-MurNAc-(pentapeptide)-GlcNAc (B), the lipid II-degradation product, peaks of m/z 1170.3, 1192.3 and 1214.3 were observed, that were assigned to be the [M-H]−, [M-2H+Na]− and [M-3H+2Na]− ions, respectively. In the case of C55-OH purified by HPLC from membranes of colicin M-treated E. coli cells (C), a peak of m/z 750.9 was observed, that was assigned to be the [MH-H2O]+ ion.

Fig. 5. Effect of colicin M on peptidoglycan synthesis. FB8r lysA cells (50 ml cultures) were grown in M63-glucose minimal medium supplemented with lysine, threonine and methionine. At an O.D. of 0.2, colicin M was added at a final concentration of 50 ng.ml−1. [3H]A2pm (2.5 kBq.ml−1) was added 10 min later (zero time) and its incorporation into peptidoglycan was followed as described in the text. Square and circle symbols correspond to control and colicin-treated cells, respectively. Open symbols: O.D. at 600 nm; closed symbols: radiolabel (cpm) incorporated into peptidoglycan.

Fig. 6. Effect of colicin M on the cellular distribution of [3H]A2pm incorporated in E. coli growing cells. The FB8r lysA strain (50 ml cultures) was grown in minimal medium, treated or not with colicin M, and 10 min later labeled for 15 min with [3H]A2pm (50 kBq.ml−1). Cells were harvested and treated with boiling water (see Experimental Procedures) and aliquots (1/5) of soluble fractions were analyzed by TLC as described in
the legend of Fig. 3. (A) soluble fraction from untreated cells; (B) soluble fraction from colicin M-treated cells. Compounds present in the different peaks were identified as described in the text.

**Fig. 7. Identification of degradation products formed in colicin M-treated cells.** (A) The mixture of degradation products formed in colicin M-treated cells was eluted from the silica gel after TLC analysis (Fig. 6B) and analyzed by HPLC on a Nucleosil 100C18 5µm (4.6 × 250 mm) column, using a mixture of 50 mM triethylammonium formate, pH 4.6, and methanol (94:6, v/v) as a mobile phase, at a flow rate of 0.6 ml.min⁻¹. (B) Identification of compound in peak X as 1-pyrophospho-MurNAc-tripeptide: purified 1-pyrophospho-MurNAc-pentapeptide (500 Bq) was treated (dashed line) or not (solid line) with the PBP5 and LdcA enzymes that catalyze its conversion into 1-pyrophospho-MurNAc-tripeptide, and the reaction mixtures were analyzed by HPLC as described in (A).
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<td>GTTACCGCTGCACCTGCACGCAAGAAAGCGCATGGGGCCTGCTGCAACGTGTagGGCTGAGCTGCTTCC</td>
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a Restriction sites (in bold) introduced in oligonucleotides are indicated in parentheses and the initiation codon of 

*cm* gene is underlined.
TABLE 2

Effects of colicin M on the cellular distribution of [3H]A2pm incorporated into E. coli cells

FB8r lysA cells (50 ml cultures) were grown in minimal medium supplemented with 0.2% glucose and 100 µg.ml⁻¹ each of lysine, threonine and methionine. At an optical density of 0.2, cultures were treated or not with colicin M at 50 ng.ml⁻¹ (at t = 0) and labeled with [3H]A2pm (50 kBq.ml⁻¹) at t = 10 min. Cells were harvested 15 min later, i.e. 25 min after the addition of colicin M and just before the onset of cell lysis. They were treated with boiling water and the distribution of the radioactivity incorporated into peptidoglycan and its precursors was determined using the different analytical procedures described in the text.

<table>
<thead>
<tr>
<th>Distribution of incorporated [3H]A2pm as measured by radioactivity (cpm)⁸</th>
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<tr>
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<tr>
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</tr>
<tr>
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<tr>
<td>1-PP-MurNAc-tripeptide ³</td>
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</tbody>
</table>

⁸ Amounts of radioactivity incorporated in the different compounds are those found in the total 50-ml culture. Variations observed in colicin M-treated cells, as compared to control cells, are indicated in parentheses (in percentages).

⁵ Not detected.

³ Peak X in Fig. 7A.
Scheme 1

farnesyl pyrophosphate + 8 isopentenyl pyrophosphate

\[ \xrightarrow{\text{UppS}} \]

undecaprenyl pyrophosphate

\[ \xrightarrow{\text{UppP (BacA)}} \]

undecaprenyl phosphate

\[ \xrightarrow{\text{MraY}} \]

undecaprenyl-PP-MurNAc-pentapeptide (lipid I)

\[ \xrightarrow{\text{Colicin M}} \]

undecaprenyl-PP-MurNAc-(pentapeptide)-GlcNAc (lipid II)

peptidoglycan

bacitracin

Other cell wall components

Scheme 1
Figure 2

O.D. (600 nm)

Time (min)

ColM
Figure 3

Radioactivity counts

Distance from the origin (cm)

A

UDP-MurNAc-pentapeptide
Lipid I

B

UDP-MurNAc-pentapeptide

C

1-pyrophospho-MurNAc-pentapeptide
Lipid I

Figure 3
Figure 4
Figure 5

- [3H]A2pm incorporated into peptidoglycan (cpm x 10^5)

- O.D. (600 nm)

- Time (min)

- [3H]A2pm

- ColM

- Peptidoglycan (cpm x 10^-5) incorporated into [3H]A2pm
Figure 6
Figure 7
Colicin M exerts its bacteriolytic effect via enzymatic degradation of undecaprenyl phosphate-linked peptidoglycan precursors
Meriem El Ghachi, Ahmed Bouhss, Hélène Barreteau, Thierry Touzé, Geneviève Auger, Didier Blanot and Dominique Mengin-Lecreulx

J. Biol. Chem. published online June 15, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M602834200

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