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# Colicin M Is an Inhibitor of Murein Biosynthesis

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Colicin M inhibited the incorporation of DL + *meso*-2,6-diamino[3,4,5-$^3$H]pimelic acid into the murein (peptidoglycan) of growing cells of *Escherichia coli* W7 *dap lys*. The inhibition of the UDP-*N*-acetylmuramyl pentapeptide-dependent incorporation of UDP-*N*-acetyl-D-[*U*-$^{14}$C]glucosamine into isolated cell envelopes indicated interference with a late step of murein biosynthesis. After the inhibition of murein biosynthesis, cells lysed, and they released lysis products of murein. In vitro, the murein biosynthesis of colicin M-tolerant mutants (*tolM*) was inhibited by colicin M. Therefore, tolerance is probably conferred by an impaired uptake or an altered fixation close to the target site and not by a mutation of the target itself. Preliminary studies with beta-lactam antibiotics and with mutants in penicillin-binding proteins did not reveal a specific enzymatic step inhibited by colicin M. The unique action among the colicins renders colicin M a potentially useful tool for studying murein biosynthesis.

The definition of colicin M was based on the cross-resistance between colicin M and phage T5- and T1-resistant strains of *Escherichia coli* (7). It was later shown that all three agents adsorb to the isolated receptor protein encoded by the *tonA* (*fhuA*) gene (1, 3, 4, 13). Among the colicins (16), colicin M is unique in that it induces the lysis of cells (2). Under osmotic protection, colicin M leads to the formation of spheroplasts (2). This penicillin-like action suggested murein (peptidoglycan) as the target. Lysis could be due to the degradation of existing murein or to the inhibition of murein biosynthesis. Previous attempts to determine the mode of action of colicin M were hampered by a contaminating murein endopeptidase and a protease activity (22) in the colicin preparation. After we had managed to isolate pure colicin M (22), its mode of action could be studied. We report here that colicin M inhibits murein biosynthesis both in vivo and in vitro.

## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* W7 *dap lys* is a double mutant unable to synthesize diaminopimelic acid and to decarboxylate it to lysine (9). *E. coli* AB2847 *aroB thi tsx malT* was the parent of the spontaneous *tolM* derivative K450 and of the spontaneous *tonA* mutant P8. *E. coli* 3-19 HfrC *ponB dacB tsx* Δ*lac proA tonA mal tsx gal thy his metB rpsL mtl* had no active penicillin-binding proteins 1b and 4. *E. coli* JE5637 HfrC *ponB* 1085 *metB proA malA* Δ*lac tonA* was lacking penicillin-binding protein 1b. *E. coli* m3 *ponA dacA mal rpsL thy his tonA* had no active penicillin-binding proteins 1a, 4, and 5. In vitro, murein synthesis was measured with envelopes prepared from *E. coli* PA3092 F$^-$ *tonA thr leu his arg trp thi thy lacY xyl malA mtl rpsL supE*$^+$. The latter four strains were kindly given by Y. Hirota. These strains were converted into *tonA*$^+$ derivatives by P1 transduction of the *tonA*$^+$ gene of strain H1020 *lac trp his metB tsx zad*::Tn*10*, together with the Tn*10* transposon (K. Hantke, personal communication).

**Media and growth conditions.** Cells were grown at 37°C in a gyratory water bath shaker in LB medium (19) or in Penassay broth (antibiotic medium 3; Difco Laboratories, Detroit, Mich.).

**Incorporation of [$^3$H]diaminopimelic acid into murein.** For the study of cell lysis induced by colicin M, cells of *E. coli* W7 were grown overnight in M9 minimal medium (19) supplemented with 0.4% glucose, L-lysine (20 μg/ml), *meso*-DL-diaminopimelic acid (3 μg/ml), and [$^3$H]diaminopimelic acid (0.5 mCi/100 ml of cell culture). Cells were washed and then grown in the same medium without diaminopimelic acid until cell mass had doubled. Then 200 μg of diaminopimelic acid per ml was added. The cells were cultured for two generations, harvested, and used immediately for the experiment. This procedure depleted the internal pool of free [$^3$H]diaminopimelic acid and of labeled precursors.

The pulse-labeling of murein (6) was performed with a logarithmically growing culture of *E. coli* W7 in Penassay broth to which 0.1% Triton X-100 and 0.1 mM $CaCl_2$ were added. One culture contained colicin M. Two samples of 1 ml from each culture were taken and incubated at 37°C for 3 min with 5 μl of DL + *meso*-2,6-diamino[3,4,5-$^3$H]pimelic acid (40 Ci/mmol,

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2 mCi/ml; Commissariat à l'Energie Atomique, Gif-sur-Yvette, France). The incorporation was terminated by adding 1 ml of boiling 8% sodium dodecyl sulfate, and the samples were further boiled for 10 min. The SDS-insoluble material was separated on membrane filters (BA 90/1; pore size, 0.6 μm; Schleicher & Schüll, Dassel, West Germany) and washed three times with 10 ml of water. The filters were dried, and the radioactivity on the filters was determined with 5 ml of toluene-based scintillation fluid in a Packard Tri-Carb scintillation counter.

**Preparation of cell envelopes.** *E. coli* K-12 PA3092 was grown in Penassay broth, supplemented with *meso*-diaminopimelic acid (20 μg/ml), L-lysine (20 μg/ml), and thymine (40 μg/ml) at 37°C with aeration in a water bath shaker. Cells from the logarithmic growth phase ($6 \times 10^8$/ml) were harvested and washed once with and suspended in 0.01 M Tris-maleate buffer–0.01 M $MgCl_2$ (pH 7.8; 0.4 g [wet weight] of cells per ml). Cell envelopes were prepared by mechanical disruption of the bacteria in a cell mill. The bacteria were shaken with glass beads (0.17 μm in diameter) in the presence of 5 μg of DNase per ml for 15 min in the cold. After the separation of whole cells by centrifugation at 5,000 × *g* for 15 min, the cell envelopes were centrifuged for 40 min at 100,000 × *g*, washed once in 0.05 M Tris-hydrochloride buffer–0.1 mM $MgCl_2$–1 mM β-mercaptoethanol (pH 7.4), and suspended in this buffer at a concentration of 20 mg of protein per ml.

**In vitro murein synthesis.** The UDP-*N*-acetylmuramyl pentapeptide-dependent incorporation of [$^{14}$C]UDP-*N*-acetylglucosamine into high-molecular-weight material was determined by the method of Izaki et al. (11) with the following modifications. Cell envelopes (10 μl), prepared as described above, were incubated in the presence of 80 nmol of UDP-*N*-acetylmuramyl pentapeptide and 0.723 nmol of UDP-*N*-acetyl-D-[*U*-$^{14}$C]glucosamine (346 mCi/nmol) in 0.05 Tris-hydrochloride buffer–0.01 M $MgCl_2$–0.01 M β-mercaptoethanol (pH 8.0), made up to a total volume of 40 μl. The samples were incubated at 37°C for 30 min. After inactivation by boiling for 3 min, the synthesis products were separated by paper chromatography. The incubation mixture was chromatographed on Whatman no. 3 chromatography paper for 20 h in isobutyric acid–1 M ammonium hydroxide (5:3) (11). The radioactivity which had remained at the origin was determined. The cut-out paper strip was placed into a toluene-based scintillation fluid and measured.

**Preparation of colicin M.** Pure colicin M was isolated as previously described (22). Its purity was further tested by determining the sequence of the amino acids at the amino-terminal end with an automatic sequencer (Beckman Instruments, Inc., Palo Alto, Calif.). The sequence of the first 30 amino acids was determined without interference by contaminating amino acids (V. Braun and B. Wittmann-Liebold, unpublished data).

## RESULTS

**Inhibition of murein biosynthesis in vivo.** The effect of colicin M on the rate of incorporation of diaminopimelic acid into SDS-insoluble murein was investigated by employing short pulses (3 min) of tritium-labeled diaminopimelic acid with a very high specific radioactivity. A decrease in the amount of diaminopimelic acid incorporated into SDS-insoluble material was apparent as early as 4 min after treatment with colicin M (Fig. 1). A comparison of the inhibition of murein synthesis with the decrease in the absorbance of the cell culture shows that murein synthesis was inhibited before cells started to lyse (Fig. 1).

**Inhibition of murein biosynthesis in vitro.** The effect of colicin M on the synthesis of murein was studied in an in vitro system consisting of a crude cell envelope fraction and the precursors UDP-*N*-acetylmuramyl pentapeptide and UDP-*N*-acetylglucosamine. Under these conditions, cross-linked high-molecular-weight murein is synthesized (11). Colicin M inhibited the formation of murein in this system (Fig. 2). Fifty percent inhibition of murein synthesis was achieved by 4 μg of colicin M. To compare the inhibition of murein synthesis in vitro with the lysis of growing cells by colicin M, we related the amount of colicin needed to the number of cells used for the assays. The membranes em-

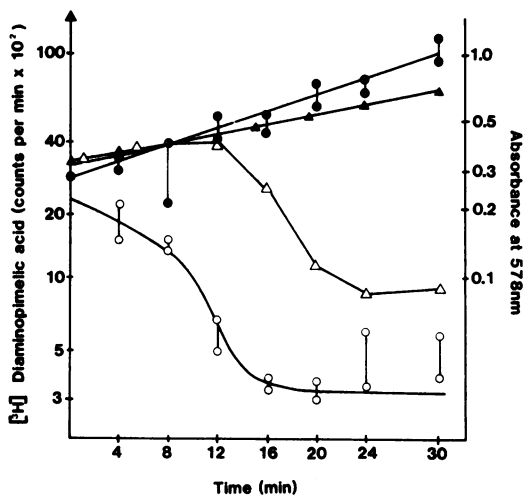


FIG. 1. Inhibition of the incorporation of diaminopimelic acid into high-molecular-weight murein by colicin M. Colicin M (1.2 μg/ml) was added at zero time to a culture of logarithmically growing cells of *E. coli* W7 in Penassay broth. A second culture served as a control. Both cultures contained 0.1% Triton X-100 to stabilize colicin M. $CaCl_2$ (0.1 mM) was added to enhance the action of colicin M. At the indicated time intervals, two samples of 1 ml were withdrawn and incubated at 37°C in the presence of 10 μCi of [$^3$H]diaminopimelic acid (40 Ci/nmol) for 3 min. The amount of label incorporated into 4% SDS-insoluble material was determined. Diaminopimelic acid incorporation in the presence (○) and in the absence (●) of colicin M and absorbance of the culture in the presence (△) and in the absence (▲) of colicin M are shown.

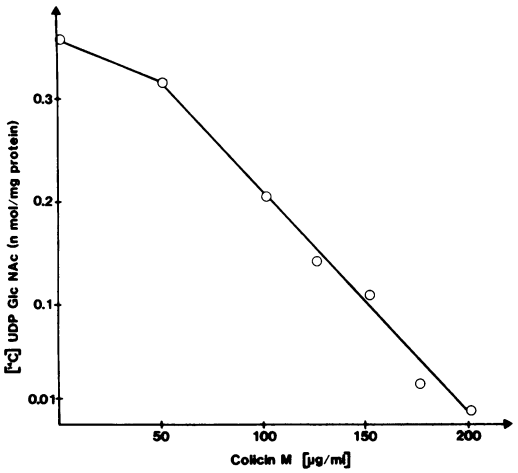


FIG. 2. Inhibition of the in vitro synthesis of murein by colicin M. In vitro murein synthesis was performed in the presence of the indicated concentrations of colicin M. The radioactivity which remained at the origin of the paper chromatogram was taken as a measure of murein synthesis. GlcNAc, *N*-Acetylglucosamine.

ployed in the study of murein synthesis were isolated from the cells of a 20-ml culture ($7 \times 10^8$ cells per ml). To achieve a 50% decrease in the number of colonies without the stabilization of colicin M with Triton X-100, 0.02 µg of colicin M had to be added to a 20-ml culture of logarithmically growing cells of *E. coli* W7. Thus, the killing effect of colicin M in vivo and the inhibition of murein synthesis in vitro were achieved with amounts that differed by a factor of 200.

Colicin M turned out to be a rather peculiar protein. Boiling for 5 min completely destroyed its in vivo activity, but decreased the in vitro activity only by 20 to 50%. This behavior may indicate that colicin M consists of two domains, one of which is necessary for the specific interaction with the TonA receptor protein in the outer membrane, whereas the other domain takes part in the inhibition of the murein synthesis. A difference in the heat sensitivity of these two domains would then explain the different biological activities of heat-denatured colicin M in vivo and in vitro. In fact, colicin M could be cleaved with proteases into fragments of which one only inhibited the binding of colicin M to cells, whereas another fragment still induced the lysis of cells (R. Dreher and V. Braun, unpublished data).

**Degradation of murein.** It was of interest whether colicin M not only inhibited murein synthesis but also caused murein hydrolysis. The murein of *E. coli* W7 was prelabeled with radioactive diaminopimelic acid. At 10 to 15 min after colicin M had been added, radioactive label was found in the supernatant of the cells (Fig. 3). This was about the time cells started to lyse (Fig. 1). About 10% of the total label was solubilized after 40 min. With ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA), which inhibits the $Ca^{2+}$-dependent action of colicin M up to the onset of cell lysis (22, 23), the release of [$^3$H]diaminopimelic acid-labeled material could still be partially prevented when it was added 16 min after the administration of colicin M to the growing cell culture.

Previously, we have shown (22, 23) that SDS prevents cell lysis induced by colicin M as long as colicin M is still accessible at the cell surface (bound to the receptor protein). At a later stage, after about 5 to 8 min, SDS, as well as trypsin- or colicin M-specific antibodies, could no longer prevent cell lysis. The action of SDS upon the colicin M-induced release of [$^3$H]diaminopimelic

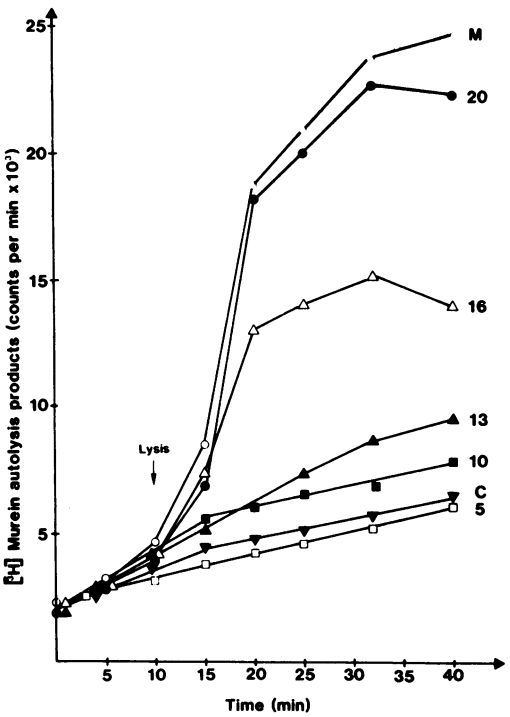


FIG. 3. Release of murein autolysis products by cells of *E. coli* W7 treated with colicin M and inhibition by EGTA. Cells labeled with [$^3$H]diaminopimelic acid were grown in LB medium at 37°C. Colicin M with bovine serum albumin (final concentration, 4 mg/ml) was added at zero time. Released radioactivity was measured in the supernatant fraction after the centrifugation of 1-ml samples. EGTA (0.2 mM, final concentration) was added at 5, 10, 13, 16, and 20 min after the addition of colicin M. Curve M indicates autolysis induced by colicin M without the addition of EGTA, and in the assay of curve C, no colicin and no EGTA have been added. The colicin concentration was 0.04 µg/ml.

acid-labeled material (Fig. 4) followed the time course of its action upon cell lysis.

**Joint action of colicin M and beta-lactam antibiotics.** Since both colicin M and beta-lactam antibiotics inhibit murein synthesis, we tested whether there was an additive or synergistic action between these inhibitors. Ampicillin and colicin M, both administered at concentrations at which one inhibitor did not induce cell lysis, caused rapid lysis when supplied together (Fig. 5). The effect of both inhibitors was not additive since double the concentration of ampicillin (6 μg/ml) did not induce cell lysis.

Mecillinam shows a unique biological activity among penicillins. *E. coli* cells are converted to large ovoids without the inhibition of the cross-linking reaction (12, 17, 18, 21). Overall murein synthesis is inhibited only by 50%. Mecillinam specifically interacts with penicillin-binding protein 2, which has been calculated to be present in about 20 molecules per cell (26). A similarly low concentration of colicin M (on the order of 10 molecules) has been estimated to kill one cell (22). When cells were treated with mecillinam and colicin M, an antagonistic effect was observed (Fig. 6). Growing cells were incubated for 52 min with 0.1, 0.2, 0.3, and 0.4 μg of mecillinam per ml before colicin M was added. Lysis induced by colicin M was largely prevented by mecillinam. However, no interference of mecillinam with the action of colicin M could be demonstrated in the in vitro assay (Table 1). Mecillinam administered together with colicin M did not prevent the inhibition of murein synthesis by colicin M in vitro. Furthermore, murein synthesis in membranes from cells which had been converted to ovoids by pretreatment with mecillinam was as sensitive to colicin M as in membranes derived from untreated control cells. Thus, under the conditions used for the determination of murein biosynthesis in vitro,

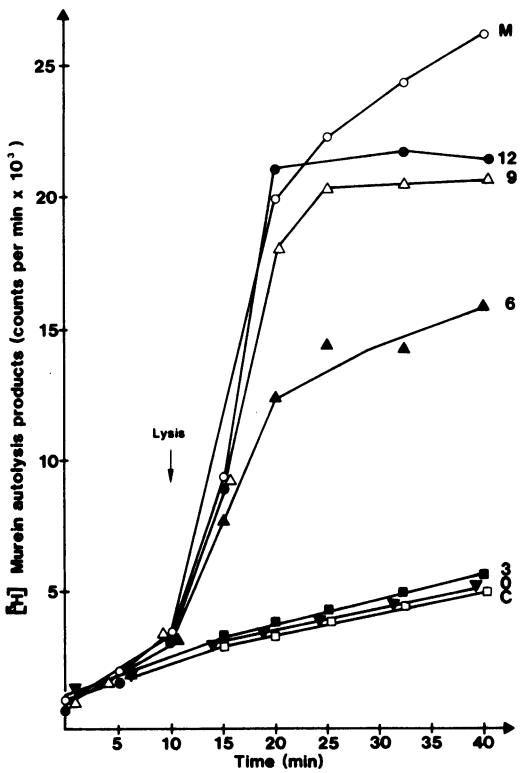


FIG. 4. Release of murein autolysis products by *E. coli* W7 treated with colicin M and inhibition by SDS. The experiment was performed as described in the legend to Fig. 3. Instead of EGTA, SDS (final concentration, 0.1%) was added together with colicin M at zero time and at 3, 6, 9, or 12 min after the addition of colicin M. Curve M represents treatment with colicin M without SDS. Curve C represents a control culture without any addition.

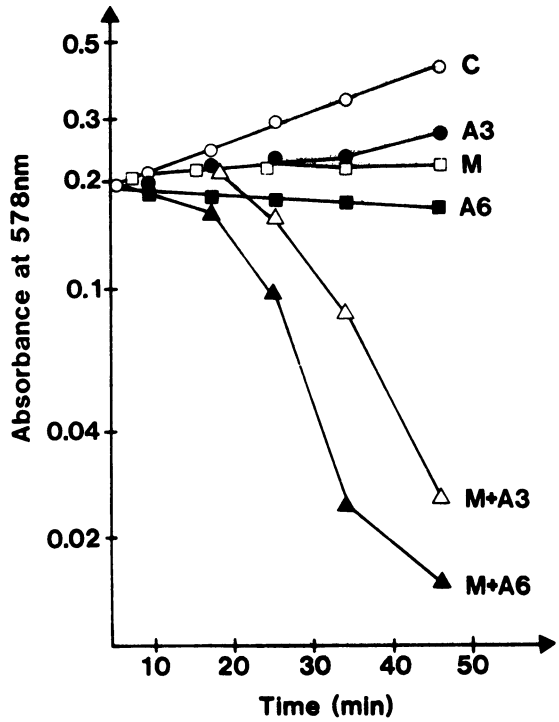


FIG. 5. Synergistic effect of colicin M and ampicillin upon cells of *E. coli* W7. Ampicillin was added to logarithmically growing cells in LB medium at a final concentration of 3 μg/ml (curve A3) and 6 μg/ml (curve A6). Colicin M was added to a third culture (curve M), and two additional cultures received colicin M plus 3 μg (curve M + A3) or 6 μg (curve M + A6) of ampicillin. Nothing was added to the control culture (curve C). The colicin concentration was 0.01 μg/ml. The colicin was stabilized by 0.1% Triton X-100 present in all cultures. Ampicillin was added 5 min before colicin M (zero time on the abscissa).

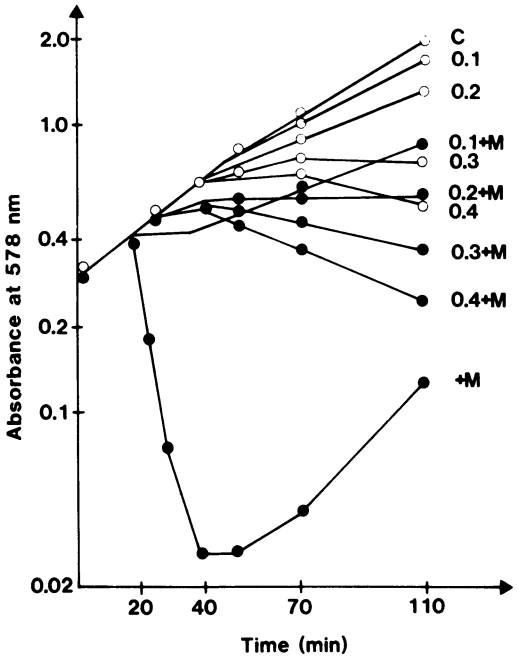


FIG. 6. Antagonistic effect of colicin M and mecillinam upon cells of *E. coli* W7. The experimental conditions were as described in the legend to Fig. 5. Mecillinam was added to final concentrations of 0.1, 0.2, 0.3, and 0.4 μg/ml without colicin M (curves marked 0.1, 0.2, 0.3, 0.4) and with colicin M (curves marked 0.1 + M, 0.2 + M, 0.3 + M, 0.4 + M). The same concentration of colicin M (0.03 μg/ml) yielded the curve marked +M. The control culture (C) contained no colicin and no mecillinam. Mecillinam was added 52 min before colicin M (zero time on the abscissa).

no antagonistic effect of mecillinam and colicin M could be observed. In this context, it is of interest that mecillinam-pretreated cells transported iron(III) as ferrichrome complex with the same rate as untreated cells (data not shown). Since this transport depends on specific translocation steps across the outer and the cytoplasmic membrane (13, 30), this observation indicates that both membranes of the ovoid cells were still active.

**Colicin M sensitivity of penicillin-binding protein 1a- versus 1b-catalyzed murein synthesis.** It has been shown that penicillin-binding protein 1a, as well as 1b, catalyzes the synthesis of cross-linked high-molecular-weight murein although with different efficiency (10, 20, 27). Both reaction pathways are inhibited by penicillins. We determined, therefore, in vitro whether colicin M could distinguish between murein synthesis catalyzed by penicillin-binding proteins 1a and 1b. This, however, seemed not to be the case (Table 2). A similar result was obtained when the effect of colicin M on the absorbance of growing cultures of *tonA*$^+$ derivatives of strains PA3092, 3-19, JE5637, and m3 was measured (data not shown). The cells lysed, showing that they were colicin M sensitive. However, a 10-times-higher concentration of colicin M was required to obtain the same rate of absorbance decrease as with control strain W7. The slower lysis was due to the slower growth rate of these strains compared with that of W7 since it was also observed with PA3092, which contains a complete set of penicillin-binding proteins.

**Effect of mutations in the *tonA* and *tolM* genes on the inhibition of murein synthesis by colicin M.** The *tonA* gene product functions as a receptor in the outer membrane for colicin M (1, 3, 6). It was, therefore, quite unlikely that a defect in this protein would affect the action of colicin M in the in vitro murein synthesis system where permeation through the outer membrane does not have to occur. In fact, there was no difference when the activity of membranes of the *tonA* mutant P8 was compared with that of *tonA*$^+$ cells (Table 2).

Different classes of colicin M-insensitive mutants of *E. coli* have been described (1, 23). The mutants can either be inactive of colicin uptake, a prerequisite for the in vivo interaction with the target, or have a defective target site. Regarding the *tolM* mutation, an alteration at the target site was conceivable. The experimental results presented here demonstrate that colicin M inhibited the in vitro synthesis of murein in membranes isolated from *tolM* tolerant cells with the same efficiency as in membranes derived from wild-type cells. Therefore, a mutation of the target site for colicin M can be excluded for the *tolM* mutation.

## DISCUSSION

The lysis of growing cells of *E. coli* and the formation of spheroplasts under osmotic protection by colicin M suggested a beta-lactam-like mode of action (2). Indeed, the pulse-labeling of murein in growing cells and the incorporation of murein precursors into isolated cell envelopes revealed the bactericidal mode of action of colicin M. The inhibition of the murein synthesis of cells occurred almost instantly and was followed 10 to 15 min later by the release of autolysis products of murein into the medium. We found no evidence that colicin M itself is a murein hydrolase (22). Like many beta-lactam antibiotics, colicin M probably triggers autolysis upon the inhibition of murein biosynthesis (14, 15, 29) or shifts the balance between the activity of synthetic and lytic enzymes in favor of the latter (24, 28). The observation that mecillinam antagonized cell lysis induced by colicin M but did not

TABLE 1. Interaction of mecillinam and colicin M in murein biosynthesis in vitro

| Expt | Mecillinam (μg/ml) | Colicin M (μg/ml) | [$^{14}$C]UDP-*N*-acetylglucosamine incorporated (nmol/mg) | Inhibition (%) |
|---|---|---|---|---|
| I[a] | 0 | 0 | 0.35 | 0 |
| | 0 | 180 | 0.02 | 94 |
| | 1 | 0 | 0.26 | 26 |
| | 1 | 180 | 0.02 | 94 |
| II[b] | 0 | 0 | 0.48 | 0 |
| | 0 | 180 | 0.08 | 83 |
| | 1 | 0 | 0.42 | 13 |
| | 1 | 180 | 0.12 | 75 |
| | 5 | 0 | 0.47 | 2 |
| | 5 | 180 | 0.11 | 77 |

[a] Cells were grown in the presence of 0.25 μg of mecillinam per ml until 80% of the cells had become ovoid (after about 100 min of growth in Penassay broth) before colicin M was added.

[b] Mecillinam was added at the same time as colicin M.

prevent the colicin M-mediated inhibition of murein synthesis fits into the rather complicated picture that emerged from the extensive study of the mode of action of beta-lactams (28, 29). Inhibition of growth, loss of viability, and cell lysis can be separate events, depending on the beta-lactam used and the experimental conditions employed (8, 14, 28, 29). The triggering of the autolysis induced by the colicin M-mediated inhibition of murein biosynthesis may not take place in the ovoid cells formed by pretreatment with mecillinam. Mecillinam belongs to the beta-lactams that do not induce cell lysis (5, 12, 17, 18, 21). It inhibits murein synthesis over a large concentration range (1 to 1,000 μg/ml) only by 50% (5, 21).

For the inhibition of murein biosynthesis in vitro, a 200-times-higher concentration of colicin M (related to an equivalent cell mass) was required than for the induction of cell lysis. The difference may be due to the lower efficiency of the in vitro assay compared with the synthesis in growing cells. In vivo, colicin M is actively taken up into whole cells and reaches the target site in the proper orientation, whereas in isolated cell envelopes, it probably reaches the target by a random movement and adsorbs to all kinds of hydrophobic components in the membranes (2, 22). It is, therefore, not unexpected that the inhibitory concentration in the cell-free system is higher than is required in living cells.

The molecular mechanism of the action of colicin M cannot be deduced from the present state of knowledge. It could act as the inhibitor of an enzymatic reaction of murein biosynthesis. In our studies with sensitive cells, we usually employed colicin concentrations between 2,000 and 6,000 molecules per cell. However, a single lethal unit deduced from single-hit kinetics was estimated to be 10 molecules per cell (22). These numbers are comparable with the amounts of the known biosynthetic penicillin-binding proteins 1a, 1b, 2, and 3, which have been estimated to be in the range of 20 to 200 molecules per cell (26). The low number of colicin molecules required to kill cells would be sufficient to inhibit stoichiometrically the biosynthetic enzymes to an extent that murein hydrolysis prevails and cells lyse.

It remains to be established whether colicin M acts as an inhibitor of a single enzymatic step in murein biosynthesis. Immersed into certain sites of the cytoplasmic membrane, it could perturb murein biosynthesis without direct interaction with an enzyme of murein biosynthesis. The observed enhancement of the action of the antibiotics erythromycin, actinomycin, and kirromycin (data not shown) points to an increase in the permeability of the outer membrane and perhaps also of the cytoplasmic membrane by colicin M. If this holds true, colicin M would be related to the membrane-active colicins like A, B, E1, and K (16), but its mode of action differs from that of these colicins.

An important finding was that colicin M inhibited murein synthesis in isolated cell envelopes of *tonA* and *tolM* mutants as efficiently as in *tonA*$^+$ and *tolM*$^+$ strains. Apparently, colicin M does not have to be fixed to the TonA receptor protein of the outer membrane to be active. Colicin M could be shocked into *tonA* and *tonB* mutants by a rapid change in the osmolarity of the buffer in which the cells had been suspended (1). However, *tolM* mutants remained insensitive when treated by this procedure (1). A temperature-dependent *tolM* mutant was completely insensitive at 42°C and sensitive at 30°C. When the temperature was raised from 30 to 42°C before cells started to lyse, all cells survived when they were maintained at 42°C. Colicin M stayed bound to the cells since, upon lowering the temperature from 42 to 30°C, cells lysed. At 42°C colicin M could be degraded by trypsin and was inactivated by SDS or by anti-

TABLE 2. In vitro inhibition of murein synthesis by colicin M[a] in various *E. coli* strains

| Strain | Relevant phenotype | Inhibition (%) |
|---|---|---|
| AB2847 | Colicin M sensitive | 89 |
| K450 | Colicin M tolerant | 79 |
| P8 | T5, T1, colicin M resistant | 83 |
| 3-19 | Deficient in proteins 1b, 4 | 97 |
| JE5637 | Deficient in protein 1b | 62 |
| m3 | Deficient in proteins 1a, 4, 5 | 99 |

[a] The colicin M concentration used was 170 μg/ml.

bodies specific for colicin M (23). These agents also inactivated colicin M when the temperature was shifted from 30 to 42°C. From these data we concluded that colicin M could be translocated reversibly across the outer membrane and that a binding site close to the target is temperature sensitive (23). The results presented in this paper are consistent with this hypothesis if one assumes that colicin M has to be fixed to the TolM function only in whole cells.

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