In Vitro Peptidoglycan Synthesis by Envelopes from *Escherichia coli* tolM Mutants Is Inhibited by Colicin M

ROBIN E. HARKNESS* AND VOLKMAR BRAUN

*Corresponding author.

Mikrobiologie II, Universität Tübingen, D-7400 Tübingen, Federal Republic of Germany

Received 12 July 1989/Accepted 9 October 1989

An in vitro peptidoglycan synthesis reaction was employed to further characterize the role of the tolM product in colicin M-induced inhibition of peptidoglycan synthesis. It was found that the tolM product is not the colicin M target and that this gene product does not play a role in the interaction of the colicin with its target. Colicin M remained associated with envelopes prepared from colicin-treated tolM mutants. These findings suggested that the tolM product most likely is involved with the internalization of colicin M.

The lethal action of colicins can be divided into three stages. Stage 1 requires binding of the colicin to a specific cell surface receptor (outer membrane protein), stage 2 involves translocation through the cell envelope, and stage 3 involves the lethal interaction of the colicin with its target. In the case of colicin M, stage 1 requires the FhuA protein (4), stage 2 requires the TonB and ExbB proteins (3, 6), and stage 3 requires an unidentified target involved in the regeneration (dephosphorylation) of the bactoprenyl carrier lipid (9, 9a). For the action of some colicins, a group of gene products encoded by the tol genes is also required (11). Colicin M requires the tolM product. Mutants in this gene are specifically tolerant to killing by colicin M (2, 15). This specificity differentiates tolM from the other tol mutants which are insensitive to more than one colicin (1, 10, 16). Both the function and identity of the tolM product are unknown.

The molecular events occurring during colicin uptake are ill defined. Colicin M in solution is resistant to digestion by trypsin. However, when bound to the cell surface, it becomes sensitive to trypsin, indicating that the colicin has assumed a new conformation susceptible to trypsin cleavage (13). Both free and cell-bound colicin are inactivated by sodium dodecyl sulfate (13). After translocation into the cell, the toxin becomes inaccessible to both trypsin and sodium dodecyl sulfate. At this stage, colicin-induced lysis can still be prevented by the addition of ethylene-bis(β-aminomethyl ether)-N,N,N′,N′-tetraacetate (EGTA; 13). This chelating agent removes the Ca²⁺ required for colicin M activity. Following treatment with EGTA, internalized colicin is displaced back to the cell surface, where it again becomes sensitive to trypsin. The addition of excess Ca²⁺ reverses the protection from lysis by EGTA. An effect similar to that observed with EGTA is found with temperature-sensitive tolM mutants (15). These mutants are sensitive to colicin M at 30°C but insensitive at 42°C. Mutants treated with colicin M at 30°C and then shifted to 42°C are protected from colicin-caused lysis. The colicin is trypsin sensitive following the temperature shift, indicating that it has been displaced to the cell surface. In mutant cells treated with colicin M at 42°C, the colicin remains bound to the cell surface, where it can be inactivated by both trypsin and antiserum against colicin M. In these temperature-sensitive mutants, the lethal process induced by colicin M has been initiated at 42°C but is stopped before completion. This is seen in mutant cultures treated with colicin at 42°C and then shifted to 30°C. Such downshifted cultures lyse earlier than cultures treated at 30°C. These findings suggest that the tolM product is required for the translocation of colicin M to its target or for a target-colicin interaction (15).

We used an in vitro peptidoglycan synthesis system to further examine the role of the tolM product. The results of this study are presented here. The assay has been described in detail elsewhere (14). Crude cell envelopes used for the assays were prepared from *Escherichia coli* K-12 strains AB2847 (aroB thi tss malT [8]) K458 (a temperature-sensitive tolM derivative of AB2847 [15]), and Mo3 (a tolM derivative of AB2847 [2]). UDP-N-acetylmuramyl-penta-peptide was provided by U. Kohlrausch (Max-Planck-Institut für Entwicklungbiologie, Tübingen, Federal Republic of Germany), and UDP-N-acetyl-[U-14C]glucosamine (11.2 GBq/mmol) was from Amersham-Buchler (Braunschweig, Federal Republic of Germany). Reactions were incubated at 28, 37, or 42°C as indicated in Table 1. Pure colicin M was isolated as described previously (13). The colicin concentrations we used inhibited peptidoglycan synthesis by 30 to 50% (final concentration in assay, 158 µg/ml). In the presence of this amount of colicin, minimal amounts of radioactivity were associated with the lipid intermediate fraction. As shown elsewhere (9), in vitro peptidoglycan synthesis takes place (at reduced levels) even when minimal amounts of lipid intermediate are present (lipid must be turned over very rapidly). Only data on peptidoglycan synthesis are presented here (see below), since inhibition of peptidoglycan and lipid intermediate synthesis followed the same trend in the parent and tolM extracts. Protein was determined by the method of Sanderman and Strominger (12).

In agreement with published findings (9, 9a, 14), the results presented in Table 1 show that colicin M inhibited in vitro peptidoglycan synthesis catalyzed by cell envelopes from the tolM" strain (strain AB2847). In vitro peptidoglycan synthesis by envelopes from a tolM mutant, strain Mo3, was also inhibited. Synthesis by envelopes from the parent and the mutant was inhibited 40 and 30%, respectively, in the presence of colicin M. Since in vitro peptidoglycan synthesis was inhibited when colicin M was added to envelopes from the tolM mutant, the tolM product must not be the target of colicin. The results further suggest that the tolM product does not play a role in a colicin-target interaction. Colicin M added to in vitro reactions containing envelopes isolated from a temperature-sensitive tolM mutant (strain K458) grown at either the permissive or nonpermissive
TABLE 1. Effect of colicin M on in vitro peptidoglycan synthesis catalyzed by cell envelopes prepared from tolM mutants and their parent strain

| Source of envelopes (strain) | Temp (°C) | Growth | Assay | Colicin* | Peptidoglycan synthesis* |
|-----------------------------|-----------|--------|-------|----------|--------------------------|
| AB2847                      | 37        | 37     |       | -        | 52.0 (1.0)               |
| AB2847                      | 37        | 37     | +     | 32.2 (0.6) |                          |
| Mo3                        | 37        | 37     | -     | 53.9 (1.0) |                          |
| Mo3 (col–)                  | 37        | 37     | +     | 38.3 (0.7) |                          |
| Mo3 (col+)                  | 37        | 37     | -     | 25.8 (1.0) |                          |
| K458                      | 28        | 28     |       | -        | 46.1 (1.0)               |
| K458                      | 28        | 28     | +     | 22.9 (0.5) |                          |
| K458                      | 28        | 42     |       | -        | 52.0 (1.0)               |
| K458                      | 28        | 42     | +     | 16.1 (0.3) |                          |
| K458                      | 42        | 28     |       | -        | 44.1 (1.0)               |
| K458                      | 42        | 42     | +     | 33.0 (0.7) |                          |
| K458                      | 42        | 42     | +     | 50.6 (1.0) |                          |
| K458                      | 42        | 42     | +     | 23.5 (0.5) |                          |

* +, Colicin added; -, colicin not added.
* In picomoles of N-acetyl-[1-14C]glucosamine incorporated per milligram of protein. Relative values in parentheses.
* Culture treated with colicin M (0.1 µg/ml) for 10 min prior to harvest.

temperature (28 and 42°C, respectively) inhibited peptidoglycan synthesis when reactions were incubated at 28 and 42°C (Table 1). These results further support the conclusion that tolM does not code for the colicin M target site. These data suggest that tolM codes for a product involved in a step between receptor binding and target interaction of the colicin.

Strain Mo3 shows no sensitivity to colicin M in vivo. However, cell envelopes prepared from cultures treated with colicin M synthesized about 50% less peptidoglycan than envelopes from untreated cells (Table 1). This suggests that the colicin M of pretreated tolM mutants was bound to the cell envelope and that during envelope preparation and the subsequent in vitro assay, this prebound colicin interacted with its target and inhibited the in vitro reaction. The addition of colicin to the in vitro reaction derived from treated cells resulted in only 10% additional inhibition. These findings support previous observations (15) that colicin M bound to the cell surface of tolM mutants. During either preparation of cell envelopes or subsequent in vitro incubation, the prebound colicin could interact with its target to inhibit peptidoglycan synthesis. This situation parallels that of the temperature-sensitive tolM mutants treated with colicin at the nonpermissive temperature (15). Shifting these treated mutants to the permissive temperature resulted in lysis. Clearly, in both of these situations colicin M was associated with the cell envelope but was unable to interact with its target until either envelopes were prepared (this study) or the cells were shifted to the permissive temperature (15). In comparison, when cell envelopes from strain AB2847 (tolM+) were used, cells were pretreated with colicin M, and envelopes were isolated under the same conditions, only 0.7 pmol of N-acetyl-[1-14C]glucosamine was incorporated per mg of protein (that is, 98.8% less than that incorporated by untreated envelopes). Clearly, in these cells, although cell lysis had not occurred prior to envelope preparation, colicin M reacted and lethally interacted with its target. These results are in agreement with in vivo studies (9) which show that although cell lysis occurs approximately 20 to 30 min after the addition of colicin M, the inhibition of peptidoglycan biosynthesis occurs within the first 5 min of treatment with colicin. The in vitro findings further support the proposal that the tolM product is involved with colicin uptake.

Previous work (13) found that the CaCl2 enhanced the in vivo activity of colicin M. The addition of CaCl2 (0.1 to 0.4 mM) to the in vitro peptidoglycan synthesis system had neither an enhancing nor an inhibitory effect on colicin M action. This was true for envelopes obtained from both a tolM mutant and the parent strain (results not shown). This indicated that the observed Ca2+ enhancement of in vivo activity was not due to an interaction between the colicin and its target. If this were the case, greater inhibition of in vitro peptidoglycan synthesis would have been expected in reactions in which both colicin M and CaCl2 were included. These data suggested that Ca2+ played a role in the interaction of colicin M with its cell surface receptor or in the internalization of the colicin. In whole cells, the removal of Ca2+ by treatment with EGTA displaced the internalized colicin to the cell surface, making it susceptible to inactivation by trypsin or colicin M antiserum (15). This in vivo observation and the in vitro results presented here indicate that Ca2+ is involved in translation of the colicin. Since the presence of Ca2+ has no effect on the stability of purified colicin preparations (unpublished observations), a stabilizing role by this cation can be excluded.

Previous results (2, 15) suggested that the tolM product participates in the uptake of colicin M or in the interaction of the colicin with its target or that it was itself the target site of the colicin. The in vitro results presented here show that the tolM product is not the colicin M target but rather somehow involved in the internalization of this colicin. Observations that tolM mutants are insensitive to colicin M following osmotic shock, whereas flaA and tonB mutants are made sensitive by such treatment, suggested that the tolM product might be the colicin M target (2). The in vitro results presented here rule out this possibility. Colicin M introduced into the cell by osmotic shock still requires the tolM product to lethally interact with its target, a function which can be bypassed in vitro. The in vitro data also showed that colicin M bound and remained bound to tolM mutants during envelope preparation. Possibly the tolM product plays a role similar to that of the Cet protein. This inner membrane protein is involved in the uptake of colicin E2, and cet mutants are tolerant to colicin E2 (5, 7). Although tolM has been positioned on the E. coli linkage map (72 min [2, 15]), attempts to clone the gene have been unsuccessful (M. Brombach, unpublished data). Further studies to identify the role of this gene product in colicin M lethality are required.

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