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Peptidoglycan Tripeptide Content and Cross-Linking Are Altered in *Enterobacter cloacae* Induced To Produce AmpC β-Lactamase by Glycine and D-Amino Acids

ABRAMO C. OTTOLENGHI,[1]* MARTA CAPARRÓS,[2] AND MIGUEL A. DE PEDRO[2]

Department of Medical Microbiology and Immunology, The Ohio State University College of Medicine, Columbus, Ohio 43210,[1] and Instituto de Biologia Molecular, Consejo Superior de Investigaciones Cientificas-Universidad Autonoma de Madrid Canto Blanco, E-28049 Madrid, Spain[2]

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Induction of AmpC β-lactamase in *Enterobacter cloacae* ATCC 13047 by D-methionine, glycine, or D-tryptophan was accompanied by alterations in peptidoglycan composition and structure; in the case of D-methionine, it was also accompanied by morphologic changes. A decrease in peptidoglycan tripeptides was seen. With glycine, there was an increase in the proportion of diaminopimelic-diaminopimelic cross-links. The possible implications of these changes for β-lactamase induction are discussed.

Nonspecific inducers have been shown to induce class I β-lactamase (BLA) in gram-negative organisms. High levels of glycine (Gly), which are known to alter the structure of the peptidoglycan (9), induce BLA in *Enterobacter cloacae* (6, 7). Induction in liquid medium by Gly has been reported for *E. cloacae* (13) and for *Escherichia coli* transformed with pNU305 by Martin and Schmidt (12). D-Methionine (D-Met) and D-tryptophan (D-Trp) also induce BLA in *E. cloacae* (13). Other nonspecific additions to the medium have been shown to influence the induction of this enzyme (4, 5, 11).

In the present paper, we show that when *E. cloacae* is induced to produce AmpC BLA by Gly, D-Met, or D-Trp, there are alterations in the proportion and cross-linking of the tripeptide moieties of the peptidoglycan. When D-Met is used as the inducing agent, morphologic changes which eventually culminate in the production of large round bodies showing cytoplasmic but not cell division are seen.

E. cloacae ATCC 13047 grown overnight in modified Torriani medium (16) with 0.15 M NaH_2PO_4 rather than glycerol phosphate and 0.002 M KCl was diluted to an optical density at 550 nm of 0.04 to 0.06 in fresh prewarmed medium and was incubated with agitation at 37°C to an optical density at 550 nm of ca. 0.3. Baseline samples were taken. The culture was split, and D-Met (0.013 M), glycine (0.05 M), or D-Trp (0.02 M) was added. Aliquots were taken after 2 h, and the remaining cultures were diluted 1:2 in prewarmed medium containing the appropriate amino acid. Additional aliquots were taken after 1 h and again at 2 h. Experiments with D-Met were terminated at this point. Cultures with Gly or D-Trp were again diluted 1:2, and the incubation continued for 1 h more.

BLA was assayed in triplicate as previously described (14). Cephaloridine was used as the substrate for the assay of the enzyme induced by D-Met or glycine or under control conditions. Nitrocefin was used as the substrate for the experiments with D-Trp. Protein assay was by the method described by Bradford (2).

In early experiments, cultures maintained without dilution showed only a two- to threefold induction of BLA after 7 to 8 h (data not shown). In the cases of D-Met and D-Trp, exponential growth maintained by one twofold dilution of the culture increased the levels of BLA to final levels that were 10- to 20-fold higher than the base level. With Gly, significant increases were not seen until the 4th h of incubation (two dilutions), when the levels reached were similar to those attained with the D-amino acids (D-aas). In cultures with D-Trp, there was variability in what happened after the peak was reached. In two of five experiments, there was a decrease in specific activity to the initial level. In the other experiments, the higher level was maintained.

Peptidoglycan analysis was conducted by high-performance liquid chromatography (HPLC) according to the method described by Glauner and Höltje (8). The peaks were identified by comparison with chromatograms of *E. coli* peptidoglycan verified by mass spectroscopy. Calculations and groupings were according to the method described by Pisabarro et al. (15), which was modified to calculate the proportions of muropeptides with a tripeptide side chain and their cross-linking patterns as a separate group.

The HPLC chromatograms of the peptidoglycan from cells grown in D-Met or D-Trp (Fig. 1C and D) closely resembled those obtained for *E. coli* by Caparrós et al. (3), with the appearance within 2 h of new species containing the D-aa used. When Gly was used (Fig. 1B), the amounts of glycine containing monomers or dimers increased. An analysis of the proportions of the various components of the peptidoglycan revealed that in all three cases there was a decrease of approximately 50% in lipoprotein (GlucNAc–MurNAc–L-Ala–D-Glu–*m*–diaminopimelic acid [DAP]–Lys–Arg)-containing moieties, a decrease in cross-linking, and in the case of Gly and D-Trp, a decrease in the percentage of muropeptides with 1,6-anhydromuramic acid residues, giving rise to an increase in the average length of the glycan chains (Table 1).

With all three amino acids, changes in the proportions of tripeptides and the types of cross bridges involving tripeptides in the side chains were seen. Figure 2B through D illustrates the course of these changes. When Gly (Fig. 2B) was added to the medium, the proportion of tripeptide-containing muropeptides (*R*–L-Ala–D-Glu–*m*–DAP) decreased by ca. 25% and there was more than a twofold increase in the proportion of tripeptides which were cross-linked in a *meso*-diaminopimelyl-*meso*-DAP (DAP-DAP)

* Corresponding author.

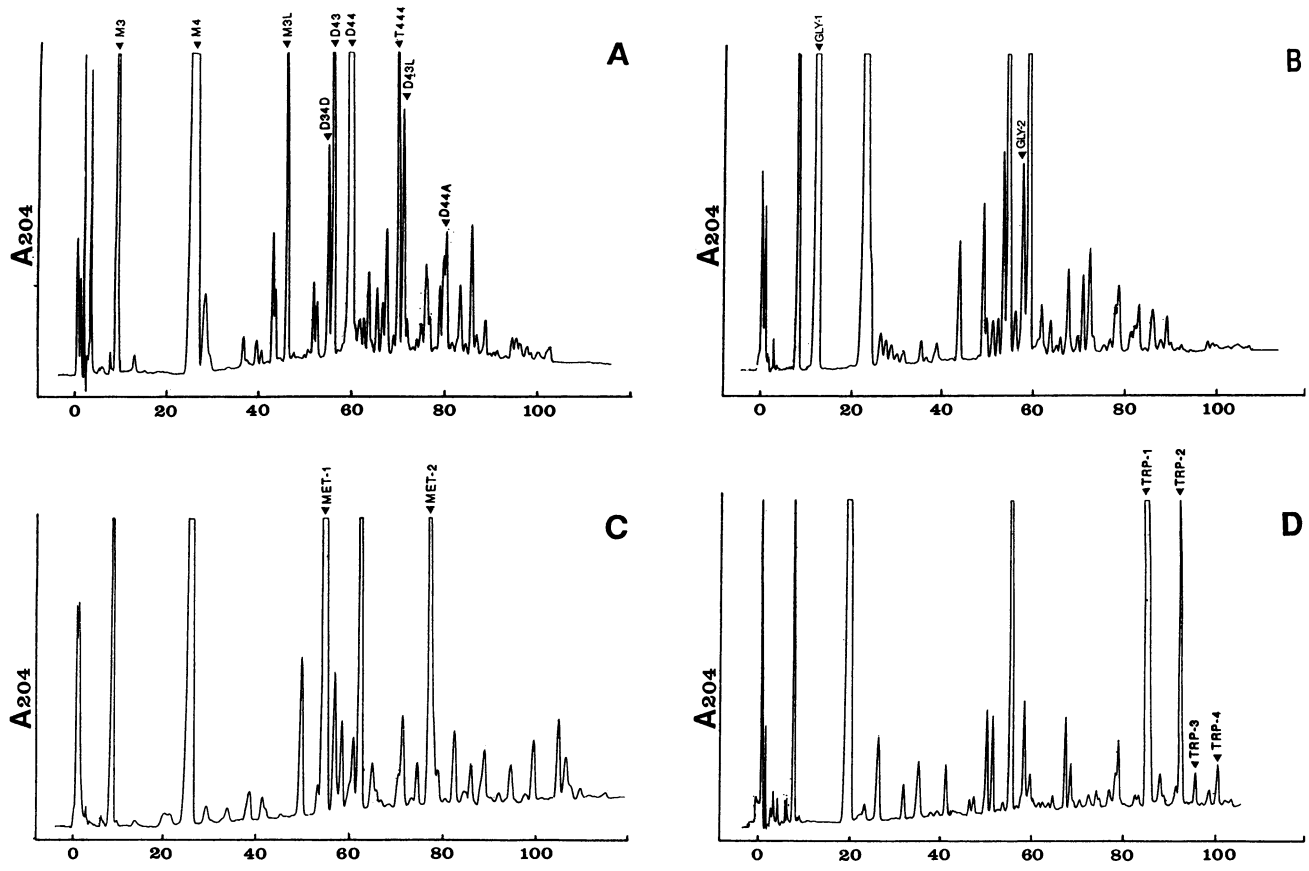

FIG. 1. HPLC analysis of peptidoglycan purified from cells grown in the presence of glycine or D-aas. Panels show UV adsorption profiles (A_{240}). (A) Untreated control. Arrows indicate the positions of the most-conspicuous muropeptides. M3, GlucNAc–MurNAc–L-Ala–D-Glu–*m*-DAP; M4, GlucNAc–MurNAc–L-Ala–D-Glu–*m*-DAP–D-Ala; M3L, GlucNAc–MurNAc–L-Ala–D-Glu–*m*-DAP–(ε)–L-Lys–L-Arg (lipoprotein-bound M3); D34D, LD DAP–DAP cross-linked dimer of M3 and M4; D43, cross-linked DD DAP–D-Ala dimer of M3 and M4; D44, cross-linked DD DAP–D-Ala dimer of M4; T444, trimer of M4; D34L, lipoprotein-bound D43; D44A, GlucNAc–MurNAc–L-Ala–D-Glu–*m*-DAP–D-Ala–GlucNAc–(1,6–anhydro)-MurNAc–L-Ala–D-Glu–*m*-DAP–D-Ala. (B) Cells grown in the presence of glycine. Arrows indicate the positions of enhanced glycine-containing peaks. GLY-1, GlucNAc–MurNAc–L-Ala–D-Glu–*m*–DAP–Gly; GLY-2, GlucNAc–MurNAc–L-Ala–D-Glu–*m*-DAP–D-Ala–GlucNAc–MurNAc–L-Ala–D-Glu–*m*-DAP–D-Ala–Gly (dimer of M4 and glycine-containing pentapeptide). (C and D) Cells grown in the presence of D-Met and D-Trp, respectively. Arrows indicate the positions of new peaks containing each amino acid used in the culture.

linkage of L-D configuration instead of in the more usual D-alanyl–*meso*–DAP (D-Ala–DAP) cross bridge of the D-D configuration. The total proportion of DAP-DAP bridges was consequently increased.

When D-Met was added (Fig. 2C), the changes observed were different in nature. There was a ca. 20% decrease in tripeptides and a 50% decrease in DAP-DAP cross-links. A still different picture was seen with D-Trp (Fig. 2D), which resulted in a decrease in tripeptides of more than 30%, with decreases of more than 50% being seen in the DAP-DAP cross-linked proportion. As a result, a concomitant proportional decrease in that type of cross-linkage in relation to the total bridges is also seen in the direction opposite to that seen with Gly.

Incorporation of the D-aas used in the culture into the peptidoglycan of *E. coli* has been reported by Tsuruoka et al. (17), who also showed that there was a decrease in the amount of lipoprotein attached to the peptidoglycan. Similar results have been reported by Caparrós et al. (3) and are seen with *E. cloacae* also (as shown in Fig. 1C and D). A similar pattern of increase in the amino acid incorporation and decrease in the lipoprotein content can be seen when excess glycine is added to the medium. This decrease in lipoprotein content is consistent with the increase in the amount of tetrapeptides and pentapeptides containing the D-aas or glycine, since the attachment of lipoprotein to murein requires the presence of a tetrapeptide as a donor. If, as indicated by Caparrós et al. (3), the enzyme implicated in this attachment does not recognize the tetrapeptides modified by the replacement of the terminal D-Ala by Gly or a D-aa, there would be be a decrease in the available donor substrate.

For phase-contrast and interference light microscopy, cells were fixed in 0.2% (vol/vol) formalin in 0.9% (wt/vol) NaCl, layered on top of glass slides covered with agarose, and photographed. For electron microscopy, cells were fixed

TABLE 1. Effect of the addition of glycine or D-aas on muropeptide composition

Addition	h	Relative abundance (mol%) of[a]:											
		Monomers	Dimers	Trimers	Gly MUR	Xaa MUR	DAP-DAP	LPP MUR	ANH MUR	Tripep	Tetrapep	Pentapep	% Cross-linked
None													
	0	64.7	30.0	5.3	3.9	NA[b]	4.3	8.3	5.9	19.5	71.6	2.7	40.5
	2	64.5	30.4	5.0	3.5	NA	4.9	9.2	5.5	20.2	71.6	2.9	40.6
	3	65.0	29.7	5.3	4.0	NA	4.0	9.8	6.4	21.7	67.8	3.8	40.3
	4	64.6	29.9	5.6	3.6	NA	4.6	11.8	6.3	23.8	66.0	3.1	41.0
	5	65.5	29.1	5.4	3.8	NA	3.6	9.8	6.3	22.8	67.7	3.5	39.8
Glycine													
	0	63.9	30.4	5.6	0.4	NA	4.4	7.6	3.8	17.2	79.5	1.7	41.6
	2	71.2	25.8	1.6	33.8	NA	4.1	3.4	2.7	12.0	79.0	1.9	29.3
	3	70.9	26.0	1.7	33.3	NA	4.6	3.8	1.5	11.8	80.3	2.3	28.8
	4	68.5	27.0	2.5	31.5	NA	6.0	4.5	2.9	15.0	75.9	2.2	32.7
	5	71.3	24.8	2.4	38.5	NA	5.2	4.3	1.5	15.1	74.7	2.5	30.1
D-Met													
	0	64.0	30.4	5.6	0.4	0.0	4.4	7.6	3.8	17.2	79.5	1.7	41.6
	2	70.9	26.8	2.3	0.5	36.6	1.4	3.9	2.6	12.3	87.4	0.8	31.4
	3	72.0	25.8	2.2	0.0	31.9	2.4	4.1	2.9	12.6	84.4	3.2	30.1
	4	76.0	21.2	2.7	1.3	29.5	2.9	4.5	3.4	15.0	74.6	0.9	26.7
D-Try													
	0	64.7	30.0	5.3	3.9	0.0	4.3	8.3	5.9	19.5	71.6	2.7	40.5
	2	77.8	20.9	1.3	1.4	25.9	2.0	4.5	2.8	12.4	77.3	1.8	23.4
	3	78.3	20.0	1.1	2.9	28.4	1.3	4.1	3.4	12.6	76.9	3.2	22.4
	4	76.6	21.4	1.2	1.9	32.1	1.3	5.0	3.3	11.9	77.9	2.2	24.1
	5	77.0	21.0	1.2	2.4	31.1	1.2	4.6	3.8	12.8	76.9	2.6	23.7

[a] Muropeptides were quantified and grouped in structurally related families as described in reference 15 (modified to also calculate proportions of muropeptides with tripeptide side chains and their cross-linking). For the estimation of cross-linkage, the D-aa-containing monomer was included with the disaccharide tetrapeptide monomers and the D-aa-containing dimer was included with the bis-disaccharide tetrapeptide. Both have therefore been included in the tetrapeptides group. Gly MUR, glycine-containing muropeptides; Xaa MUR, muropeptides incorporating the added D-aa; DAP-DAP, muropeptides cross-linked by DAP-DAP bridges; LPP MUR, lipoprotein-bound muropeptides; ANH MUR, muropeptides with a residue of the glycan-chain-terminating sugar (1,6-anhydro)-MurNAc; Tripep, tripeptides; Tetrapep, tetrapeptides; Pentapep, pentapeptides.

[b] NA, not applicable.

with glutaraldehyde, and the pellets were embedded in agar and sectioned after Epon embedding.

Very marked morphologic changes were evident in cells grown with D-Met (Fig. 3B to D). Following an initial blunting of the poles (starting at about 2 h [Fig. 3B]), forms in which septation may be seen to start but is not completed appear (ca. 3 h) (Fig. 3C to E). In some cases, it can be seen that the cytoplasm has divided without the occurrence of a completed division of the cell wall (Fig. 3C to D). In other cases (Fig. 3D), it appears that the cell wall has enlarged without division of the cytoplasm. By 4 h, ca. 85% of the cells showed altered morphology.

Lark and Lark (10) showed that D-aas induced the production in *Alcaligenes faecalis* of protoplast-like forms which they termed crescents. Both D-Met and D-Trp were effective in producing the morphologic alterations in that organism. Although Lark and Lark (10) did not present any photographs of their protoplast-like crescent cell, the morphologic changes that we have seen can be considered to be in general agreement with their description.

A. faecalis appeared to be more sensitive to the D-aas than *E. cloacae*, in that protoplast-like forms appeared earlier (by 20 min) and at lower D-Met concentrations (0.004 M). These differences may reflect the strains or the result of the growth conditions. Lark and Lark (10) showed that there were differences in the outcome, depending on the medium that they used. The absence of formation of aberrant forms when *E. cloacae* was induced to produce BLA in the presence of D-Trp may also be due to this apparently lower sensitivity. The concentration used in this study was close (0.02 versus 0.15 M) to the threshold level for crescent formation in *A. faecalis* (10).

Similar considerations may explain the absence of reports of aberrant forms in the work of researchers who have studied the incorporation of D-aas in the peptidoglycan of *E. coli* (3, 17). Lark and Lark (10) observed that the appearance of crescent forms in *E. coli* required a level of D-Met 15 times higher than that required for *A. faecalis*. This level is higher than that used by Caparrós et al. (3) and by Tsuruoka et al. (17).

The kinetics of BLA induction varies, depending on the inducing agent. When an antibiotic such as cefoxitin is used, induction is rapid (within 5 to 10 min [13a]), whereas with glycine or the D-aas it is much slower. The induction kinetics seen with each agent may simply reflect the mechanisms that an organism can call on in order to maintain homeostasis.

In the case of glycine, for instance, the delay in the appearance of BLA may be due to the presence of a system designed to maintain the proportions of glycine-containing peptides (which occur naturally) at a homeostatic level. Induction would occur only when the system available to maintain this homeostatic level is overwhelmed. With the D-aas used in this study, there may be no corrective system (3); BLA induction would occur earlier when the cumulative damage alters homeostasis beyond the tolerance point.

There is good evidence that tripeptides are the acceptors for the cross-links formed during septation, whereas pentapeptides are the acceptors for the cross-links formed

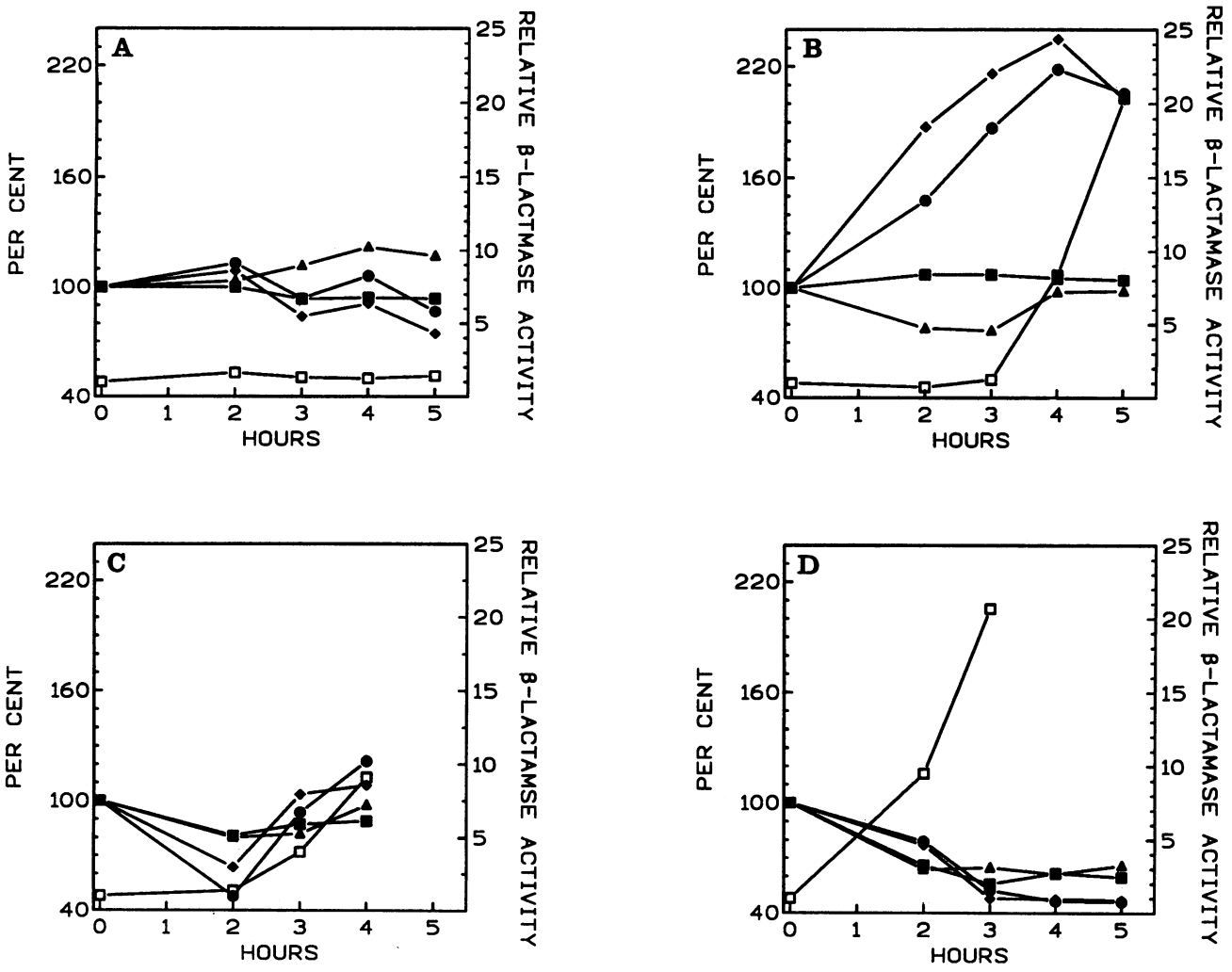

FIG. 2. Alterations in tripeptide content and cross-linking in the peptidoglycan and β-lactamase induction in *E. cloacae* ATCC 13047 grown in the presence of glycine and D-aas. For the tripeptides, 100% represents the values obtained from the analysis of peptidoglycan before the addition of the amino acids. (A) Untreated control; (B through D) changes in tripeptide composition of the peptidoglycan of cells grown in the presence of glycine, D-Met, and D-Trp, respectively. □, BLA (values relative to baseline before the addition of amino acids); ■, changes in the proportion of cross-linked tripeptides (all links); ◆, changes in the proportion of tripeptides cross-linked in a DD DAP-DAP cross bridge; ●, changes in DD DAP-DAP cross-links as a proportion of total cross-links; ▲, change in tripeptide content.

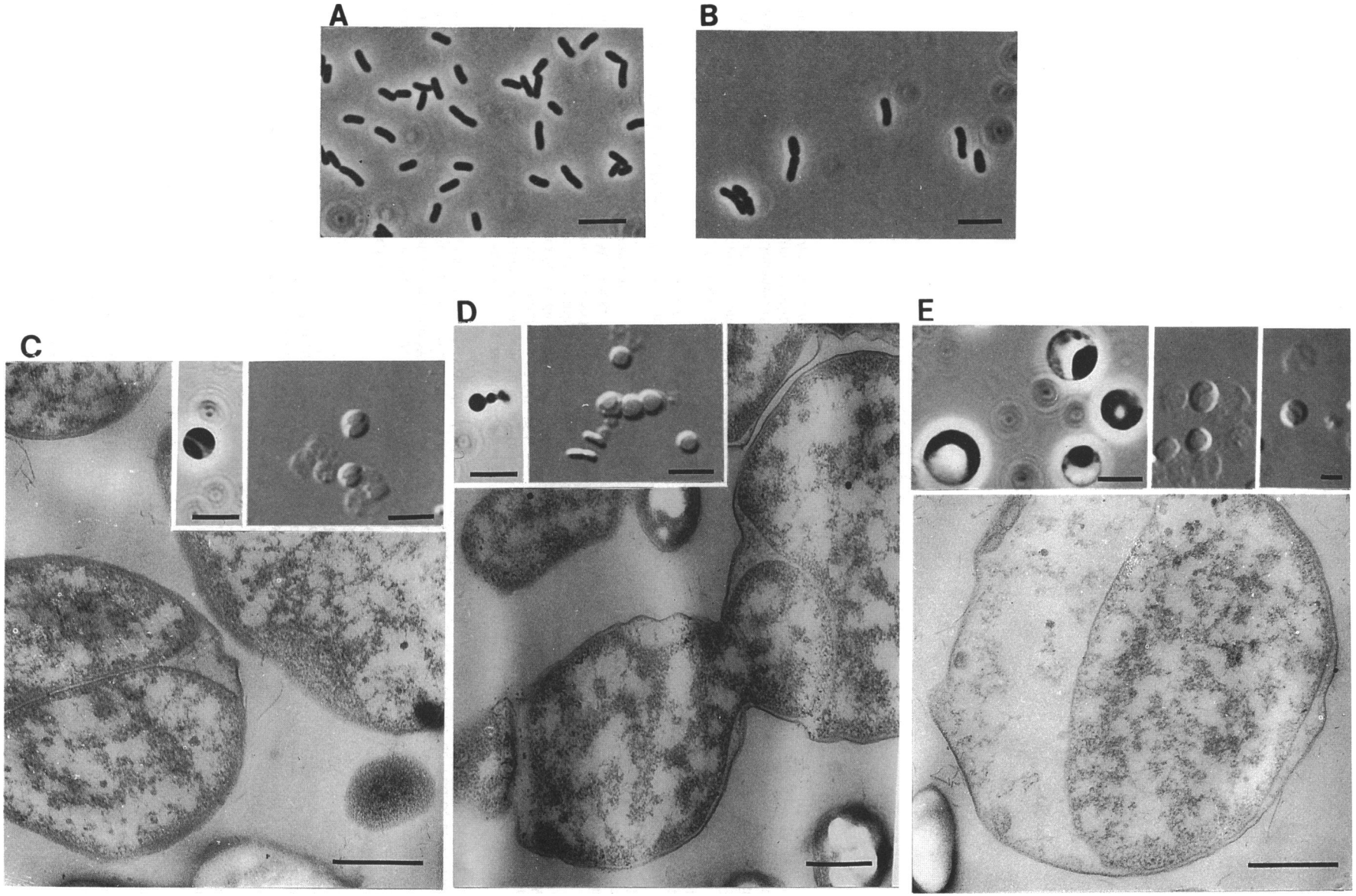

FIG. 3. Light and electron micrographs of *E. cloacae* ATCC 13047 cells cultured in the presence of D-Met. (A) Untreated control; (B) cells cultured for 2 h in the presence of the amino acid; (C through E) cells after 4 h of incubation. Inserts show both transmitted and interference phase micrographs of cells comparable to those seen in the electron micrographs. Bars represent 5 μm for the light micrographs and 0.5 μm for the electron micrographs.

during lateral peptidoglycan synthesis (1). In all of the cases that we have presented here, there is a decrease in tripeptides and an alteration in the nature and extent of crosslinking. These changes point to modifications in the peptidoglycan which might affect the efficiency of the septation-division mechanism. That septation is affected might also be deduced from the morphological observations.

We believe that these results make it possible to postulate that BLA induction is triggered by peptidoglycan alterations which are nonspecific in their nature but specific in their function because they alter the ability of the cell to septate and divide.

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