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Induction of Autolysis in Nongrowing *Escherichia coli*

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Unless relaxation of the stringent response is achieved, all nongrowing bacteria rapidly develop resistance to autolysis induced by a variety of agents, including all classes of cell wall synthesis inhibitors. We now describe inhibitors of cell wall synthesis which were unusual in that they could continue to effectively induce autolysis in *relA*$^+$ *Escherichia coli* even after prolonged amino acid starvation. The process of cell wall degradation seems to be catalyzed by similar hydrolytic enzymes in nongrowing and growing cells, yet the activity of these new agents capable of inducing autolysis in the nongrowing *relA*$^+$ cells did not involve relaxation of RNA or peptidoglycan synthesis. We propose that the suppression of autolysis characteristic of nongrowing cells can be bypassed by a novel mechanism of autolytic triggering which is independent of the *relA* locus.

Nongrowing bacteria, including bacteria starved of a required amino acid, rapidly become resistant to autolysis by cell wall synthesis inhibitors or chaotropic agents (4, 10). The only mechanism currently known by which this protection from autolysis may be temporarily overcome involves the *relA* gene; autolysis resistance develops only after a considerable delay following the onset of amino acid starvation in *relA* mutants (3) or in *relA*$^+$ cells treated with relaxing agents such as chloramphenicol (7). In studies aimed at a better understanding of this phenomenon, a number of cell wall synthesis inhibitors have been found with an unusual capacity to induce autolysis long after the onset of amino acid deprivation, even in *relA*$^+$ strains of *Escherichia coli*. In this report this process is compared to lysis in growing cells, and its independence from the relaxation phenomenon is determined.

E. coli W7 (*dap lys*) was grown in M9 simple salts medium supplemented with 10 μg of L-lysine per ml, 5 μg of DL-mesodiaminopimelic acid per ml, and 1 mM glycerol, as described before (3). Growth was halted by filtration and resuspension in lysine-free medium. We used these amino acid-starved (nongrowing) bacteria as a test system for evaluating the capacity of a large number of beta-lactam antibiotics (each at 10× their respective MIC equivalents) to induce autolysis and wall degradation in nongrowing *E. coli*. The compounds tested included such powerful inducers of autolysis of growing *E. coli* as benzylpenicillin, cephaloridine, cefsulodin, and inhibitors of penicillin-binding protein 2 or 3 (such as mecillinam or azthreonam and cephalexin), as well as over 70 structurally different cephalosporins, cephamycins, and penems. As expected, the vast majority of these agents could not induce more than marginal lysis (10% drop in optical density during 3 h of treatment) if the bacterial culture was transferred to a lysine-free medium 10 min before the addition of the antibiotics (Fig. 1, cephaloridine). It was surprising, however, to find several exceptional compounds that retained powerful lytic activity in the lysine-starved *E. coli* cells even after more prolonged periods of amino acid deprivation. Nocardicin A, MT 141, CGP 14233, and imipenem lysed *E. coli* cells that were starved for 20, 30, 30, and 30 min, respectively (Fig. 1). Bacteriolytic activity progressively diminished as the antibiotics were added to more extensively starved cells.

Several studies were performed to understand the mechanism of the unique autolysis-inducing capacity of these agents. The lytic activities of cephaloridine and nocardicin were compared in *relA*$^+$ and *relA*$^-$ strains. *E. coli* CP78 (*relA*$^+$) and CP79 (*relA*$^-$) were grown in supplemented M9 medium (3); growth was halted by addition of 500 μg of valine per ml, which resulted in isoleucine deprivation. As expected, cephaloridine (10× MIC) failed to kill nongrowing *relA*$^+$ cells (98% survival), while it could lyse nongrowing *relA*$^-$ cells if added during the initial 30 min of starvation (48% survival at 3 h). Unexpectedly, however, nocardicin lysed the nongrowing *relA*$^+$ and *relA*$^-$ strains up to but not beyond 30 min of starvation (58 and 45% survival, respectively, at 3 h). The fact that the period of susceptibility to nocardicin lysis for the *relA*$^-$ strain was not longer than that for the *relA*$^+$ strain was the first suggestion that the lytic activity of nocardicin against nongrowing cells involved a new mechanism not related to the relaxation phenomenon.

To establish whether the antibiotics capable of lysing nongrowing wild-type (*relA*$^+$) bacteria were relaxing agents, the rates of RNA, protein, and cell wall syntheses were monitored in nongrowing *E. coli* W7 treated with penicillin, nocardicin, or CGP 14233. The rates of macromolecular synthesis were determined by pulse-labeling according to established procedures (3, 9), using 5 μCi and 5 μg of [^{3}H]phenylalanine (15 Ci/mmol; New England Nuclear Corp., Boston, Mass.) to label protein, 10 μCi and 10 μg of [5-^{3}H]uridine (25.8 Ci/mmol; New England Nuclear) to label RNA, or 10 μCi and 10 μg of *N*-acetyl-D-[^{3}H]glucosamine (2.84 Ci/mmol; Amersham, Inc., Amersham, U.K.) to label the cell wall. The samples were frozen, and the rate of incorporation was determined by assessing the amount of radioactivity precipitable in cold 5% trichloroacetic acid (protein and RNA) or boiling 4% sodium dodecyl sulfate (SDS) (cell wall). For cumulative rates of synthesis, similar procedures were used, except that three parallel bacterial cultures were continuously labeled with one of each radiolabel during the sampling period.

Bacterial lysis was evident only after 30 min of antibiotic exposure in both growing and nongrowing cells. Thus, synthetic rates were compared in control and drug-treated cells over the first 30 min of starvation (Fig. 2). In control cultures, the rates of macromolecular syntheses decreased within 5 min of the onset of amino acid deprivation to approximately 25% for RNA, protein, and cell wall. Thereafter, this low level of synthesis was maintained indefinitely

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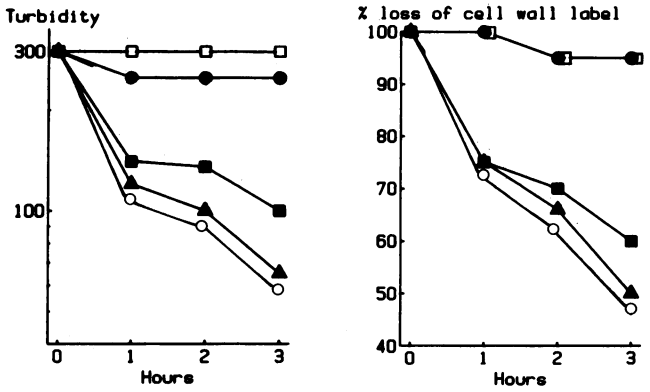

FIG. 1. Lysis and cell wall degradation in nongrowing *E. coli* W7 treated with beta-lactam antibiotics. *E. coli* W7 prelabeled in the cell wall with *N*-acetylglucosamine (3) was transferred at time zero to minimal medium lacking the essential amino acid lysine. Growth was halted within minutes in the control culture (□). The 10× MIC of cephaloridine (●), CGP 14233 (■), or imipenem (▲) was added at time 10 min, and cultures were monitored for lysis (Sequoia Turner nephelometer) (left panel) and autolytic activity (counts per minute retained in boiling SDS-precipitable material) (right panel). Nongrowing cells could not be induced to autolyze by cephaloridine, while growing cells resupplied at time zero with lysine lysed rapidly (○). By contrast, CGP 14233 and imipenem retained bacteriolytic activity against the nongrowing cells.

in the nongrowing state. Addition of 10× MIC equivalents of penicillin, CGP 14233, or nocardicin to these nongrowing cultures at time zero did not alter the decrease in synthetic rates observed in the drug-free controls. Relaxing agents would be expected to cause a significant increase in the rates of RNA and cell wall syntheses in amino acid-deprived cells. No such increase was observed with any of the agents tested. Similar results were obtained on comparison of the cumulative rates of RNA and cell wall syntheses in *relA*$^+$ *E. coli* CP78 starved of isoleucine upon treatment with cephaloridine, CGP 14233, or the relaxing agent chloramphenicol. While net RNA and wall synthesis decreased sharply in untreated isoleucine-starved control cells, RNA and wall syntheses resumed in starved cells treated with the relaxing agent chloramphenicol (data not shown). If the good lytic activity of CGP 14233 on nongrowing cells is related to relaxation of the stringent response, then addition of this drug to the isoleucine-starved cells should affect biosynthetic rates in a manner similar to that of chloramphenicol yet different from that of cephaloridine (which is not a good lytic agent for nongrowing cells). However, the rates of RNA and wall syntheses decreased markedly in the CGP 14233-treated cells, which is similar to what occurred in the cephaloridine-treated starved cells (data not shown). This decrease was similar to that observed for RNA synthesis in untreated starved cells but exceeded the decrease of control cultures for wall synthesis, a result expected for cell wall synthesis inhibitors. Thus, addition of CGP 14233 did not induce relaxation, as did chloramphenicol. In fact, when CGP 14233 or cephaloridine was added to chloramphenicol-treated starved cells, RNA synthesis persisted at the relaxed rate characteristic for chloramphenicol, and wall synthesis decreased to a rate characteristic of cells treated with wall synthesis inhibitors. The effects of chloramphenicol were, therefore, clearly distinct from those of CGP 14233 and cephaloridine.

The unusual capacity of a select group of agents to induce autolysis in amino acid-starved *E. coli* cells that were completely resistant to autolysis by most of the other cell wall synthesis inhibitors tested and that maintained an intact stringent response raises the possibility that the process of autolytic cell wall degradation in the nongrowing cells occurs by a mechanism different from that observable in growing bacteria. To compare autolysis in growing and lysine-starved cells, two parallel cultures of *E. coli* W7, one growing in full M9 medium and the other after 10 min of lysine starvation, were each treated with imipenem (10× MIC) for 2 h. During this time, both cultures underwent partial lysis, releasing

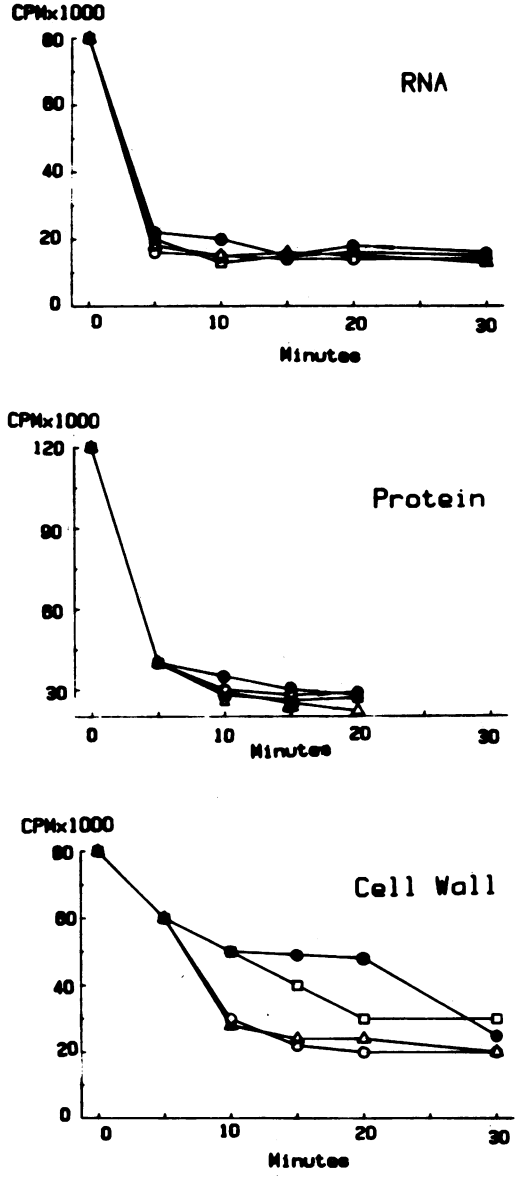

FIG. 2. Lack of relaxation of the stringent response of RNA, protein, and cell wall syntheses in lysine-starved *E. coli* cells treated with agents capable of inducing autolysis of the nongrowing cells. *E. coli* W7 cells were transferred to lysine-free minimal medium at time zero. Rates of synthesis of RNA, protein, and cell wall (determined by amount of incorporation of radiolabel during 5-min pulses of culture aliquots) decreased rapidly in control cultures (●). The 10× MIC of nocardicin (□), penicillin (△), or CGP 14233 (○) was added at time zero to separate culture aliquots. Rates of synthesis decreased in a manner parallel to controls despite drug addition.

TABLE 1. Comparison of composition of residual murein of *E. coli* W7 after partial autolysis by imipenem in growing and nongrowing cells

Autolysis	% Total muropeptides[a]						
	Monomer[b] family	Dimer family	Trimer family	Lipoprotein muropeptides	Anhydro-muropeptides	Monomer[c] tetrapeptide	Dimer[d] tetra/tetrapeptide
Control[e] (no drug)	51.4	43.8	4.8	3.2	3.2	48.7	37.6
Imipenim vs growing cells	42.9	40.0	17.1	9.4	12.1	35.2	30.5
Imipenem vs nongrowing cells[f]	54.4	40.1	5.5	4.2	5.6	36.6	29.4

[a] Calculated by grouping muropeptides as described in the text.
[b] (Area under curve of all muropeptides in the family/total area of monomers, dimers, and trimers) × 100.
[c] Major monomer component with peak retention time of 35 min in HPLC.
[d] Major dimer component with peak retention time of 73 min in HPLC.
[e] Growing cells and cells starved of lysine for 10 min have identical HPLC profiles.
[f] Imipenem added at 10 min of lysine deprivation.

about 25% in nongrowing and 40% in growing cells of cell wall murein label as fragments soluble in boiling 4% SDS. The cultures were centrifuged; residual (unhydrolyzed) cell wall murein was isolated, purified, and enzymatically digested with muramidase; and the enzymatic hydrolysates were then analyzed by high-performance liquid chromatography (HPLC) with a reverse-phase system (1, 2). The HPLC elution profiles of the digests of the residual walls left unautolyzed after imipenem treatment of growing and nongrowing cells were compared with the composition of control walls prepared from untreated normally growing cultures (peaks defined and analyzed as in references 2, 5, and 8) (Table 1). The major imipenem-induced loss of murein material occurred from the same muropeptides in growing and nongrowing cells: a decrease from 48 to 35% of monomer tetrapeptides and a decrease from 37 to 29% of dimer tetra/tetrapeptides. It is interesting, however, that the composition of nonhydrolyzed murein left intact in the sacculus was different in growing and nongrowing cells. The residual murein in growing cells after lysis was enriched for highly cross-linked species and lipoprotein-containing muropeptides, thus suggesting that autolysis induced by imipenem was selective, as suggested for penicillin (6). By contrast, however, the average composition of residual murein after lysis in starved cells did not change from that in the control cell wall. The increase in anhydromuropeptides in both imipenem-treated cultures is compatible with the appearance of new chain ends expected from transglycosylase activity (6). This was particularly evident in growing cells where more murein was solubilized.

The discovery of antibiotics which cause nongrowing bacterial strains to depart from their general property of surviving exposure to potentially lytic antibiotics has many important implications. Clearly, autolysis is not always strongly dependent on the *relA* gene product, since imipenem, nocardicin A, GCP 14233, and other compounds lyse nongrowing bacteria despite the generation of the stringent response. Thus, other mechanisms for overcoming the autolysis resistance of amino acid-starved bacteria, in addition to disruption of the stringent response, indeed exist. Chemical analysis of partially autolyzed walls of imipenem-treated *E. coli* cells indicates that, once induced, autolysis is remarkably similar in nongrowing and growing cells. The process of autolysis in amino acid-starved nongrowing cells as well as in actively growing cells involves the loss of similar murein components (and, therefore, presumably similar hydrolytic enzyme systems). However, when tested for efficiency of lysis induction, antibiotics which were outstanding lytic triggers in growing cells were, for the most part, not effective against nongrowing cells. While inhibitors of the penicillin-binding protein 1 complex have been associated with rapid lysis of growing *E. coli* (5), no particular penicillin-binding protein affinity pattern was noted for the good triggers of nongrowth lysis (Tuomanen, in press). At least some inhibitors of early events in the peptidoglycan synthetic pathway, such as the combination of cycloserine and β-chloro-D-alanine, also remained effective in both growth states.

The mechanism whereby this unusual group of cell wall synthesis inhibitors triggers lysis in nongrowing bacteria is not clear at this time. However, the fact that these agents appear to act independently of the constraints of the stringent response suggests a novel mechanism with important implications for putative antibacterial agents which may be able to overcome phenotypic tolerance (i.e., resistance to autolysis and to the bactericidal effect of antibiotics) of nongrowing cells in vitro and in vivo.

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