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NOTES

Autolysis-Resistant Peptidoglycan of Anomalous Composition in Amino-Acid-Starved *Escherichia coli*

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Nongrowing *Escherichia coli* deprived of an essential amino acid continued to produce peptidoglycan at a rate approximately 30% of that of growing cells. The composition of this peptidoglycan was very different from that of growing cells and resembled that of peptidoglycan left undegraded during partial autolysis of the bacteria. Synthesis of this peptidoglycan of anomalous composition began at once upon the removal of the amino acid from the medium. Fifteen minutes of amino acid deprivation was sufficient to virtually completely prevent penicillin-induced autolytic wall degradation in vivo. During this time, although the specific activities of soluble and membrane-bound hydrolytic transglycosylases and endopeptidases remained high, the peptidoglycan produced showed decreased sensitivity to degradation in vitro. After more extensive (2-h) starvation, triggering of autolysis by chaotropic agents was also blocked. Autolysis in growing cells may be selective for peptidoglycan representing the cylindrical portion of the sacculus. It is suggested that at least part of the mechanism of the well-known lysis resistance of nongrowing *E. coli* is related to the deposition of structurally anomalous and relatively autolysin-resistant peptidoglycan at some strategically located sites on the bacterial surface.

It is known that *Escherichia coli* deprived of an essential amino acid develops resistance to autolysis induced by cell wall synthesis inhibitors and other agents within minutes of a halt in growth (4, 13). This phenomenon, called phenotypic tolerance (13), is shared by all bacterial species under all conditions of growth limitation, which suggests that it involves a general protective mechanism. An earlier study established that the total autolysin content of nongrowing *E. coli* deprived of a required amino acid remains unchanged long past the appearance of a complete block in autolysis (3). On the other hand, susceptibility of peptidoglycan isolated from such cells to in vitro degradation by a crude autolytic extract decreased gradually with increasing duration of amino acid starvation. This suggested that a halt in growth rapidly causes some alteration in cell wall structure which may contribute to resistance to autolysis.

The purpose of the studies described here was to determine the composition of the peptidoglycan synthesized in nongrowing *E. coli* by means of a high-resolution analytical technique (2). The specific activities of four major autolysins and the autolysin susceptibility of the peptidoglycan produced during the amino acid starvation were also determined. The results suggest that one major mechanism of phenotypic tolerance involves a rapid modification of peptidoglycan structure that blocks the initiation of autolysis.

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Composition of peptidoglycan as determined by reverse-phase HPLC. *E. coli* W7 (*dap lys*) was grown in M9 minimal salts medium supplemented with 25 μg of L-lysine and 5 μg of diaminopimelic acid (DAP) per ml (3). To halt growth, bacteria were collected on a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.), washed, and suspended in medium without lysine. Peptidoglycan was prepared by the method of Glauner and Schwarz, which involves precipitation in boiling sodium dodecyl sulfate, digestion with α-amylase and pronase, reprecipitation in boiling sodium dodecyl sulfate, digestion with M1 muramidase, and reduction with borohydride (2). High-performance liquid chromatography (HPLC) was carried out under established conditions (6, 15). Peak identities were based on the pattern of *E. coli* standards (15).

We compared the compositions (HPLC profiles) of peptidoglycans prepared from batch cultures of the following three types of cells: fast-growing bacteria (doubling time, 45 min), cells extensively starved of lysine (24 h), and cells with the residual cell wall left undegraded (i.e., remaining sodium dodecyl sulfate precipitable) after fast-growing *E. coli* were exposed to penicillin at 10 times the MIC (50 μg/ml) until 55% of cell wall-associated radiolabel was released (3 h).

Peptidoglycan from extensively starved cells (Table 1, column NG) differed in composition from that of growing cells (Table 1, column G) as follows: (i) enrichment (from 16 to 31%) for tripeptide-containing species (especially peaks 1, 5, 6, and 7); (ii) large decreases in the major monomer, dimer, and lipoprotein-linked dimer fractions (peaks 2, 8, and 13); and (iii) enrichment from 3 to 13% in diaminopimelyl-DAP cross bridge (dap-dap)-containing species (especially peaks 5 and 6).

Most interestingly, after partial autolysis, residual peptidoglycan had a profoundly changed composition compared with that of the starting material and closely resembled that of nongrowing cells. Autolysis accounted for this dramatic

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TABLE 1. Peptidoglycan composition in growing and nongrowing cells before and after penicillin treatment

Peak no.	Muropeptide structure[a]	Amt of muropeptide (% total)[b] isolated		
		G	NG	G/Pen
1	Tri	3.0	10.3	10.4
2	Tetra	43.5	30.8	27.0
3	Di	1.5	0.7	1.0
4	Tri-LysArg	4.1	1.3	4.5
5	Tri-Tri(dap-dap)	0.4	3.0	3.7
6	Tetra-Tri(dap-dap)	1.4	10.2	10.0
7	Tri-Anh	2.6	5.5	3.2
8	Tetra-Tetra	33.3	29.0	22.1
9	Tetra-Penta	0.1	0.8	0.1
10	Tri-Tri(dap-dap)LysArg	1.3	0.1	3.0
11	Tetra-Anh	0.5	2.6	1.4
12	Tetra-Tetra-Tetra	3.5	2.6	1.8
13	Tetra-Tri-LysArg	1.4	0.1	2.7
14	Tetra-Tetra-Tetra-Tetra	0.3	0.3	1.0
15	Tetra-Tetra-Tri-LysArg	0.3	0.4	1.3
16	Tetra-Tetra-Anh	0.2	0.4	0.1
17	Tetra-Tetra-Tri-Anh	1.1	0.3	1.1
18	Tetra-Tetra-Tetra-Anh	1.3	0.8	1.7
19	Tetra-Tetra-Tetra-Tetra-Anh	0.1	0.7	0.7
20	Tetra-Tri-LysArg-Anh	0.1	0.1	3.2

[a] Peak identities were assigned as in reference 15. Abbreviations used for the muropeptides were based on their peptide chains; thus, Tri stands for *N*-acetylglucosaminyl-*N*-acetylmuramyl-tripeptide, and Tetra-Tri stands for the cross-linked dimer of *N*-acetylglucosaminyl-*N*-acetylmuramyl-tetrapeptide and *N*-acetylglucosaminyl-*N*-acetylmuramyl-tripeptide; the tripeptide consists of L-Ala-D-Glu-*m*-DAP, and the tetrapeptide consists of L-Ala-D-Glu-*m*-DAP-D-Ala. The peptide chains are cross-linked between *m*-DAP and D-Ala (in position 4) if not otherwise stated. Other abbreviations: Di, dimer; Anh, *N*-acetylglucosaminyl-1,6 anhydro-*N*-acetylmuramyl; Lys Arg, lysine and arginine residues of lipoprotein; dap-dap, an *m*-DAP-*m*-DAP cross bridge; Penta, pentapeptide.

[b] UV A_{205} corrected for molar extinction coefficient as described in reference 7. G, Growing cells; NG, nongrowing cells (24 h without lysine); G/Pen, undegraded (autolysis-resistant) portion of peptidoglycan from growing cells treated with penicillin (10 times the MIC, 3 h; 55% wall degradation).

shift, since the concentration of penicillin used (10 times the MIC) completely inhibited peptidoglycan incorporation into the sacculus (data not shown). The simplest interpretation of the striking compositional difference of the autolytic residue is that autolysis was selective and avoided parts of the wall rich in lipoprotein (peaks 4, 10, 13, 15, and 20) and tripeptides (peaks 1, 5, and 6), while the major loss of material occurred in tetrapeptide monomers (peak 2) and dimers (peak 8). These findings are consistent with earlier observations that lipoprotein-rich parts of the wall are relatively resistant to autolytic degradation (19), autolysis is catalyzed by the hydrolytic transglycosylase and an endopeptidase (5, 6), and material lost during autolysis has a composition most like that of a cylindrical cell wall (15).

Kinetics of the alteration of composition of peptidoglycan made in nongrowing *E. coli*. *E. coli* continues to make peptidoglycan in the amino-acid-starved state at a rate that is approximately 30% of the rate in growing cells (13, 18). Since phenotypic tolerance (resistance to autolysis triggered by wall inhibitors) arises within minutes of growth arrest, we examined the composition of newly incorporated peptidoglycan and the kinetics of alteration of the average peptidoglycan composition of the nongrowing cell as the new nongrowing-cell wall was incorporated. New cell wall structure was characterized by radiolabeling equal amounts of material in growing and nongrowing cells, i.e., incorporation during 5 min of normal growth versus incorporation during the first 15 min of lysine deprivation, respectively. In each case, fractions were collected from the HPLC column (1 ml every 2 min), mixed with Ultrafluor scintillation fluid (New England Nuclear Corp., Boston, Mass.), and counted on a scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

New cell wall made during early growth arrest was similar to the average extensively starved cell wall (Table 2): enriched in moieties containing dap-dap (peaks 5, 6, and 10) and tripeptides (peaks 1, 4–7, 10, 15, 17, and 20) and depleted in the major dimer (peak 8) and the lipoprotein-linked dimer (peak 13). The monomer (peak 2) remained stable. These experiments suggest that the anomalous nongrowing-cell wall began to be incorporated into the sacculus immediately upon growth arrest. Compositional values intermediate between those for growing cells and those for extensively starved cells were found for peptidoglycan from cells starved for only 1 h (HPLC UV analysis; data not shown), suggesting that in nongrowing cells, new cell wall of altered structure was being added to a large amount of normal (growing) cell wall, gradually shifting the average wall composition with time.

Murein hydrolase activity and in vitro hydrolyzability of peptidoglycan from nongrowing cells. Extracts from growing cells and cells starved of lysine for 15 min, 2 h, and 24 h were compared for murein hydrolase activity at equivalent concentrations of protein. Endopeptidase assays were carried out with [^{3}H]DAP-labeled bis-disaccharide peptide dimer (C_3; 2×10^3 cpm/pmol) as described by Markiewicz (8). Determinations of transglycosylase (murein:murein-6-muramyl transferase) activity with [^{3}H]DAP-labeled sacculi (4×10^4 cpm/μg) as a substrate were made by the method of Mett et al. (9). Over the first 15 min of growth arrest, three of the four enzyme activities remained stable, while soluble endopeptidase activity decreased to 70% of control levels (Table 3). Soluble and membrane-bound transglycosylase activity decreased slowly during prolonged starvation.

Susceptibility of peptidoglycan to autolytic wall degradation in vitro. To determine whether nongrowing murein was

TABLE 2. Relative amount of radiolabel in newly synthesized peptidoglycan

HPLC peak no.[a]	% of total cpm	
	Growing cell	Nongrowing cell
1	0.1	2.1[b]
2	49.8	49.4
3	2.2	1.5
4	1.0	3.1[b]
5	0.2	1.0[b]
6	0.2	2.2[b]
7	1.2	7.6[b]
8	34.2	11.2
9	1.6	0.5
10	1.0	2.8[b]
11	1.0	5.5[b]
12	0.9	1.2[b]
13	3.7	2.1
14/15	<0.01	1.5[b]
16	0.4	0.8[b]
17	0.4	0.8[b]
18	0.3	2.3[b]
19	1.3	2.7[b]
20	0.4	1.7[b]

[a] See Table 1 for structural assignments.

[b] Number in nongrowing cell was more than two times higher than in growing cell.

degraded at an altered rate in vitro, murein degradation by extracts of *E. coli* containing mainly membrane-bound and soluble transglycosylase activity (9) was determined by using peptidoglycan radiolabeled in growing cells (control) or during starvation ([^{3}H]DAP [1 μCi/ml of culture; specific activity, 7.8 mCi/mmol; New England Nuclear] for 15 min, 2 h, or 24 h of lysine deprivation) (3). When compared with control peptidoglycan (>90% release), peptidoglycan radiolabeled during 15 min, 2 h, or 24 h of starvation released only 18, 24, or 21% of radiolabel, respectively, indicating that even early in growth arrest, nongrowing-cell peptidoglycan was relatively resistant to hydrolysis in vitro.

Degradation of cell wall in growing and nongrowing cells in vivo. In contrast to labeling of cell wall synthesized in nongrowing cells described in the previous section, the effect of incorporation of nongrowing-cell wall on hydrolysis of preexisting normal cell wall in vivo was determined by radiolabeling growing *E. coli* cells with [^{3}H]DAP. Radiolabeled cells were then incubated in medium with or without lysine, and the release of radiolabel from prelabeled growing-cell wall (boiling sodium dodecyl sulfate-precipitable material) was determined in cells exposed to the following autolytic stimuli: 50 μg of benzylpenicillin per ml (10 times the MIC) for 10 min followed by suspension in buffer (5), three cycles of freeze-thaw (3), or 5% trichloroacetic acid (3).

While growing-cell wall underwent extensive autolytic degradation provided that the antibiotic or other agents were added to actively growing cells, radiolabel was not released from the same cells treated with penicillin after the cells were briefly (15 min) starved of lysine (Table 4). However, partial degradation of such growing-cell peptidoglycan could still be induced by trichloroacetic acid or by repeated freezing and thawing early in growth arrest. Resistance to autolysis by these harsher agents eventually appeared only after more extensive starvation (by 2 h). Thus, nongrowing cells appear to be a hybrid of old, growing-cell, hydrolyzable peptidoglycan and new, nongrowing-cell, hydrolysis-resistant peptidoglycan. Degradation of old cell wall could be triggered by chaotropic agents but not by penicillin.

Our observations together with those in the literature (3, 19) strongly suggest that an altered peptidoglycan composition may be a contributor to the mechanism of lysis resistance of nongrowing *E. coli*. Strong similarities exist among the composition of peptidoglycan resistant to hydrolysis in growing *E. coli*, the average composition of cell walls in amino-acid-starved (autolysis-resistant) *E. coli*, the composition of the autolysis-resistant slow-growing *E. coli* (cultivated in the chemostat with a generation time of 13.5 h) (1, 15, 16), and, in part, the composition of *E. coli* in the stationary phase of growth (10). In each of the three physiological conditions resulting in autolysis resistance (i.e., amino acid deprivation, slow growth rate, and stationary phase), unique but common chemical features include increases in dap-dap linkages and tripeptides and a decrease in the major tetrapeptide dimers. The fact that the composition of residual peptidoglycan left undegraded after partial autolysis of growing bacteria is also shifted in the same direction strongly suggests that peptidoglycan of this unique composition is relatively resistant to autolytic attack in vivo. This is supported by the in vitro degradation data. Our findings also suggest that this nongrowing-cell peptidoglycan is the product of the residual peptidoglycan synthesis that beings immediately and continues (at a reduced rate) in the amino acid-starved *E. coli* (13, 18) and that eventually makes up the majority of the cell wall of extensively starved cells. It seems that when greater than 90% of the sacculus is composed of nongrowing-cell wall (as in the cells deprived of lysine for >2 h), then autolytic wall degradation is not initiated by penicillin or even by the harsher membrane-disrupting treatments (i.e., trichloroacetic acid and freezing-thawing).

TABLE 3. Endopeptidase and transglycosylase activities in lysine-starved and growing *E. coli*[a]

Sample	Sp act of transglycosylase (U)[b]		Sp act of endopeptidase (U)[c]	
	Soluble	Membrane bound	Soluble	Membrane bound
Growing cells	1.0	1.0	1.0	1.0
Cells starved for:				
0.25 h	0.95	1.21	0.69	0.93
2.0 h	0.72	1.15	0.38	0.70
24.0 h	0.53	0.45	0.27	0.41

[a] Means of triplicate experiments.
[b] Specific activities of transglycosylase in control cells expressed in picomoles of [^{3}H]labeled murein solubilized by 1 μg of protein in 60 min at 37°C were 2.16 (soluble) and 17.71 (membrane bound).
[c] Specific activities of endopeptidase in control cells expressed in picomoles of C_3 split into C_6 by 1 mg of protein in 30 min at 37°C were 45 (soluble) and 69 (membrane bound).

TABLE 4. Percent release of cell wall radiolabeled with [^{3}H]DAP in growing *E. coli* upon subsequent treatment under growing or nongrowing incubation conditions

Growth condition during treatment	% Loss of cpm after treatment with[a]:			
	Nothing	Penicillin	Chaotropic agent	
			TCA[b]	Freeze-thaw
Growing cells (control)	1	85	63	82
15 min, no lysine	11	8	55	71
120 min, no lysine	2	7	35	1

[a] Percent loss of hot sodium dodecyl sulfate-insoluble counts per minute after 3 h; means of three experiments; maximum standard deviation ± 11%.
[b] TCA, Trichloroacetic acid.

Resistance to autolysis arose much more rapidly for penicillin than for chaotropic agents, i.e., within minutes of the onset of growth arrest. At this time, the majority of the cell wall was still hydrolyzable, since chaotropic agents readily initiated wall degradation. Autoradiographic (11) and biochemical (12, 14) evidence suggests that the incorporation of new wall material occurs at topographically limited areas of the *E. coli* cell wall. In amino-acid-starved *E. coli*, these areas presumably contain altered nonhydrolyzable wall material. If one assumes that penicillin-induced autolysis is initiated in these same areas, as suggested by recent observations (14), then the presence of only a small amount of strategically placed, new, nonhydrolyzable cell wall in the growth zone(s) may effectively limit penicillin-induced hydrolysis initiated from this site(s). In contrast, chaotropic agents are presumed to cause generalized membrane disruption and therefore would release autolysins all over the cell surface. A considerably longer period of incorporation of altered cell wall (estimated at >90% of the total cell wall in 2 h) was needed to deter autolysis when such nonlocalized membrane-disrupting agents were used.

It seems probable that the observed alteration of cell wall composition is, at least in part, responsible for the decreased susceptibility to autolytic degradation (phenotypic tolerance) in nongrowing *E. coli*. It has been suggested that PBP 7 may be particularly important in the generation of the altered structure of cell wall in nongrowing *E. coli*, since inhibition of PBP 7 promotes lysis of nongrowing cells (17).

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