JOURNAL OF BACTERIOLOGY, Nov. 1987, p. 5308–5310
0021-9193/87/115308-04$02.00/0
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Vol. 169, No. 11

Changes in Peptidoglycan Composition and Penicillin-Binding Proteins in Slowly Growing *Escherichia coli*

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Received 4 May 1987/Accepted 15 August 1987

The composition of peptidoglycan of chemostat-grown cultures of *Escherichia coli* was investigated as a function of growth rate. As the generation time was lengthened from 0.8 to 13.8 h, there was a decrease in the major monomer (disaccharide tetrapeptide) and dimer (bis-disaccharide tetrapeptide), while disaccharide tripeptide moieties increased to >50% of the total wall. The average chain length became much shorter; lipoprotein density tripled, and the number of unusual diaminopimelyl-diaminopimelic acid crossbridges increased fivefold. As cells grew more slowly, amounts of penicillin-binding proteins (PBPs) 1a-1b complex and 4 decreased, while amounts of PBPs 3 and the 5-6 complex increased. We propose that the chemical composition of *E. coli* cell walls changes with growth rate in a manner consistent with alterations in the activities of PBPs and cell shape.

The chemical composition of bacterial cell walls is modulated as a function of the composition of the growth medium and the phase of growth of the organism (10, 12). It has been suggested that peptidoglycan composition may also vary directly as a function of growth rate (4). Our studies were prompted by our recent observation that chemostat-grown *Escherichia coli* cells undergo a striking gradual decrease in sensitivity to the bactericidal and bacteriolytic effects of beta-lactam antibiotics in parallel with decreasing growth rates (16). In an effort to explore possible mechanisms of this phenomenon, we measured the chemical composition of *E. coli* peptidoglycan and the profile of penicillin-binding proteins (PBPs) in chemostat-grown cultures with a range of generation times from 0.8 to 13.8 h.

(This work was presented in part at the Annual Meeting of the American Society for Microbiology, Washington, D.C., 23–28 March 1986 [E. Tuomanen, R. Cozens, K. Gilbert, O. Zak, and A. Tomasz, Abstract Annu. Meet. Am. Soc. Microbiol. 1986, A-109, p. 19].)

Preparation of peptidoglycan for reverse-phase HPLC. *E. coli* W7 (*dap lys*) was grown in M9 minimal salts medium supplemented with 25 μg of L-lysine per ml, 5 μg of diaminopimelic acid (DAP) per ml, and 1 mM glycerol (6). Batch cultures (generation time, 0.8 h) were maintained at 37°C in a shaking water bath. Reductions in growth rate to 1.5, 3, 6, and 13.8 h were achieved by growing the bacteria in 50-ml, all-glass, aerated chemostats at 37°C, with glycerol as the limiting nutrient (3, 16). The cultures were stabilized at 1×10^8 to 5×10^8 CFU/ml for 10 generations.

Peptidoglycan was prepared by the method of Glauner and Schwarz (5). A portion of each sample was reserved for amino acid analysis with a Durram D-500 amino acid analyzer after hydrolysis with 6 N HCl at 100°C for 16 h. Peptidoglycan was digested with *Streptomyces globisporus* muramidase, reduced with borohydride (5), and subjected to high-performance liquid chromatography (HPLC) at room temperature under established conditions (7). Chromatograms were compared by converting the peak area (UV A_{205}) to the molar amount of muropeptide, expressed as a percentage of the total amount for all peaks. An empirically derived correction factor, K, was used to determine the molar extinction coefficient for each muropeptide as follows: K = disaccharide/ (0.6 disaccharide + 0.1 amide bonds − 0.1 anhydro ends + 0.2 lipoprotein) (8). Structural assignments of peaks were made on the basis of comparison with standards supplied by B. Glauner, Max Planck Institute, Tubingen, Federal Republic of Germany (4, 5, 12). The relative amount of each family of muropeptides, the percentage of cross-linkage, and the average chain length in disaccharide units were calculated as described elsewhere (5, 12).

Alterations in peptidoglycan composition in slowly growing cells. Three separate samples from each generation time were prepared, and each sample was analyzed by HPLC at least twice. Data shown are from representative chromatograms; the amounts of muropeptides varied by <10% of a given value from run to run. Amino acid analysis confirmed that the average composition of stem peptides did not change as the generation time was lengthened (2 mol of alanine, 1 mol of glutamic acid, and 1 mol of DAP).

HPLC profiles of cell walls of rapidly growing and slowly growing cultures were compared (Fig. 1). Retention times of all peaks remained unchanged as growth rate was reduced; no new peaks were found. Major changes did occur, however, in the relative sizes of various peaks. Traditional datum analysis with muropeptide families was misleading, since individual members of a family often changed in opposite directions as a function of growth rate (e.g., monomer peak 1 versus peak 2, Table 1). Thus, all analyses were performed by comparing individual peaks.

The major shifts in cell wall composition when generation time increased from 0.8 to 13.8 h involved (i) a decrease in the major monomer (Fig. 1, peak 2, down 27%) and dimer (peak 9, down 9%); (ii) a gradual increase in tripeptide-containing muropeptides from 16 to 52%; (iii) a threefold increase in lipoprotein-containing species, especially dimers, to 20%; (iv) a fivefold increase in DAP-DAP-containing moieties to 15%; (v) a large decrease in average chain length from 17 to 5 disaccharide residues; and (vi) a small increase in cross-linkage from 23 to 27%. Increases in lipoprotein and DAP-DAP-containing species confirm previous findings (4). In logarithmically growing cells, 1 lipoprotein residue was attached per 13.8 disaccharide residues, a ratio intermediate between published ratios of 16 (4) and 10 (1), respectively.

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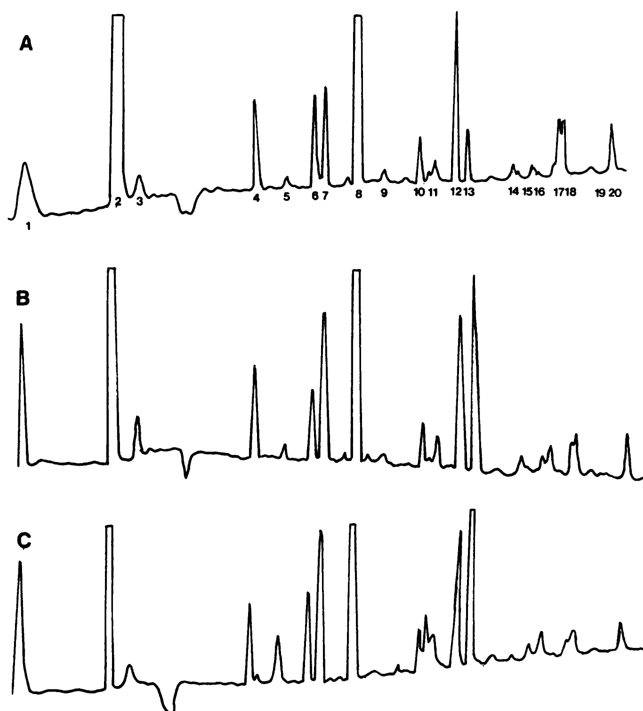

FIG. 1. HPLC profiles of rapidly and slowly growing *E. coli* W7. Analyses of 20-μg samples of *E. coli* peptidoglycan at generation times of 0.8 h (A), 3 h (B), and 13.8 h (C). Muropeptide peak numbers are identified in Table 1.

Slowly growing chemostat-grown cultures demonstrate a gradual decrease in susceptibility to the bacteriolytic and bactericidal actions of a group of cephalosporins as generation time increases (16). Alterations in cell wall composition as described here may modify substrate susceptibility to endogenous murein hydrolases leading to phenotypic tolerance in slowly growing and nongrowing cells (E. Tuomanen et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1986). The peptidoglycan composition of slowly growing cells resembles that of stationary-phase cells (12) in that both types are more highly cross-linked, have a shorter average glycan chain length, and are more enriched with lipoprotein than is the peptidoglycan of exponentially growing cells. Thus, our analysis of 13.8-h-generation-time cells suggests that a relationship may exist between cell wall composition of slowly growing cells and of cells entering stationary phase.

Profile of PBPs as a function of growth rate. It is reasonable to suggest that changes in cell wall composition with growth rate could involve alteration of the balance between synthetases and hydrolases. The PBP profile of intact cells from batch-grown and chemostat-grown cultures was determined in whole cells (2) normalized for protein concentration with 1.2 μg (70 μCi) and 0.12 μg (7 μCi) of [^{3}H]benzylpenicillin (courtesy of E. Stapley, Merck Sharp & Dohme, Rahway, N.J.). To quantitatively compare PBP band densities, gels were scanned with a scanning densitometer (Helena Instruments, Beaumont, Tex.). While the numbers and molecular sizes of PBPs did not change with growth rate, the amounts of individual PBPs changed markedly (batch-grown cultures resembled 0.8-h chemostat-grown cultures; Table 2). Band densities for the PBP 1 complex and PBP 4 decreased with longer generation time, while densities for PBPs 3 and the 5-6 complex increased. PBP 2 was not well resolved in the whole-cell assay. Since increasing the amount of [^{3}H]penicillin from 10 to 100 times the MIC did not change the maximal band densities (data not shown), the altered band densities of PBPs most likely reflected an alteration in copy numbers of PBPs, rather than in affinities for the drug. The near-disappearance of the PBP 1 complex should not be interpreted as an absence of these PBPs, since the sensitivity of detection of PBPs with the fluorographic assay is limited (15).

The interpretation of coincident changes in murein composition and the relative amounts of PBPs and hydrolases will require additional experimentation. However, an increase in tripeptide-containing muropeptides is consistent with both an increase in carboxypeptidase activity (PBP 5-6) and an increase in PBP 3, which is said to incorporate such units into the sacculus (13). A decrease in PBP 1a-1b is consistent with the decrease in the dimer tetra tetrapeptide fraction. The decrease in PBP 4 (endopeptidase) in slowly growing cells is consistent with the increase in cross-linkage. Since cell wall incorporation in *E. coli* may be catalyzed by

TABLE 1. Peptidoglycan composition at four generation times

Peak no.[a]	Muropeptide[b]	% Total peptidoglycan (A_{205}) at generation time of: 0.8 h	3 h	6 h	13.8 h
1[c]	Tri	3.0	6.6	9.5	13.0
2[c]	Tetra	43.5	31.0	27.5	16.4
3	Di	1.5	1.9	1.1	1.7
4	Tri-LysArg	4.1	3.0	2.5	3.1
5[c]	Tri-Tri (DAP-DAP)	0.4	0.6	2.3	3.2
6[c]	Tetra-Tri (DAP-DAP)	1.4	2.1	2.9	4.0
7[c]	Tri-Anh	2.6	6.2	7.7	10.2
8[c]	Tetra-Tetra	33.3	27.9	25.0	20.8
9	Tetra-Penta	0.1	0.1	0.1	0.1
10[c]	Tri-Tri (DAP-DAP) LysArg	1.3	1.8	3.2	8.0
11	Tetra-Anh	0.5	1.5	1.1	3.1
12	Tetra-Tetra-Tetra	3.5	4.8	4.8	3.5
13	Tetra-Tri-LysArg	1.4	6.8[d]	7.0	7.5
14	Tetra-Tetra-Tetra-Tetra	0.3	0.3	0.1	0.1
15	Tetra-Tetra-Tri-LysArg	0.3	0.3	0.1	0.1
16	Tetra-Tetra-Anh	0.2	0.7[d]	1.1	0.7
17	Tetra-Tetra-Tri-Anh	1.1	2.1[d]	2.0	2.0
18	Tetra-Tetra-Tetra-Anh	1.3	0.1[d]	0.1	0.3
19	Tetra-Tetra-Tetra-Tetra-Anh	0.1	0.6[d]	0.8	1.0
20	Tetra-Tetra-Tri-LysArg	0.1	1.6[d]	1.1	1.3

[a] Peak numbers correspond to those given in Fig. 1.
[b] Abbreviations used for muropeptides were based on their peptide chains; e.g., Tri, *N*-acetylglucosaminyl (NAcGlc)-*N*-acetyl-muramyl (NAcMur) tripeptide; Tetra-Tri, cross-linked dimer of NAcGlc-NAcMur tetrapeptide and NAcGlc-NAcMur tripeptide (the tripeptide consists of L-Ala-D-Glu-m-DAP, the tetrapeptide 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: Anh, NAcGlc-1.6 anhydroNAcMur; LysArg, lysine and arginine residues of lipoprotein; DAP-DAP, an *m*-DAP-*m*-DAP crossbridge.
[c] Changes gradually as generation time increases from 0.8 to 13.6 h.
[d] Abrupt change in comparison to next-fastest generation time.

TABLE 2. Alterations of relative densities of PBPs as a function of growth rate[a]

Generation time (h)	% of total [^{3}H]penicillin bound by: PBP 1a-b	PBP 2	PBP 3	PBP 4	PBP 5-6
0.8	8.4	0.4	5.6	25.7	59.9
3	2.4	0.1	6.4	21.1	69.9
6	<0.1	<0.1	9.2	9.3	81.5

[a] From densitometer tracings of autoradiographs of [^{3}H]benzylpenicillin bound to PBPs.

two enzyme systems (9, 14), changes in peptidoglycan composition may reflect a shift in relative activity from one to the other as bacterial growth rate slows down. *E. coli* cells are known to become progressively smaller, less elongated, and enriched with polar material as generation time lengthens (4). These same shape changes (data not shown), coupled to the substantial change in the ratio of carboxypeptidase (PBP 5-6) to transpeptidase (PBP 1a-b) activity which was observed in the slowly growing cells, is consistent with the hypothesis of Mirelman et al. (11). On the basis of the individual peak analysis data shown in Table 1, the cylindrical wall might be expected to contain mainly monomer tetrapeptide and dimer tetra tetrapeptide, while the septal wall might be enriched for species with tripeptides and DAP-DAP cross-links.

We thank A. Tomasz for continued discussion and for critical review of the manuscript, J. Schwartz and K. Gilbert for technical assistance, and M. Geller for preparation of the manuscript. We are grateful to B. Glauner and J.-V. Holtje for providing standards to calibrate the HPLC system.

This research was supported by a Career Investigator Award to E.T. from the Irma T. Hirschl Trust and by Public Health Service grants AI16794, AI23459, and RR07065 from the National Institutes of Health.

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