Alterations of murein structure and of penicillin-binding proteins in minicells from *Escherichia coli*

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Minicells, as compared with a whole cell preparation of a *minA/B* mutant of *Escherichia coli*, showed a number of changes in the structure of the murein sacculus. Minicell murein was enriched in LD-A₂pm-A₂pm crossbridges by about 66% and reduced in the amount of L-Ala-D-Glu dipeptide moieties by about 55%. In addition, the length distribution of the glycan strands in the murein was shifted to shorter lengths. In particular, the relative amount of the shortest possible strand, the size of a disaccharide, was more than doubled. Minicells were also found to have an altered penicillin-binding protein (PBP) pattern. Whereas PBP4 and PBP6 were greatly diminished, PBP8 was significantly increased. We consider it unlikely that the sort of changes observed in murein structure reflect the fact that minicells are composed of two hemispherical polar caps.

Keywords: *Escherichia coli*, murein, penicillin-binding proteins, minicells, cell division

INTRODUCTION

During the cell cycle of bacteria, morphological changes occur that are due to corresponding alterations in the shape of the bacterial exoskeleton, the murein sacculus (Weidel & Pelzer, 1964; Schwarz *et al.*, 1969; Nanninga, 1988). In the case of rod-shaped bacteria such as *Escherichia coli*, cell elongation alternates with septum formation which is followed by splitting of the septum to allow separation of the daughter cells. These processes are accompanied by alterations in the metabolism of murein. During cell elongation cylindrical murein is synthesized, whereas for cell division to take place septal murein is formed which – after cleavage – gives rise to two new hemispherical polar caps. Specific enzyme systems are

assumed to be involved in these processes (Schwarz *et al.*, 1969; Park & Burman, 1973; Spratt, 1975; Botta & Park, 1981; Kraus & Höltje, 1987; Lleo *et al.*, 1990; Nanninga *et al.*, 1990; Nanninga, 1991). The question that then arises is whether the different shapes of polar caps and the cylindrical part of the sacculus are paralleled by changes in the chemistry of the murein. Up to now, such a correlation has not been proven experimentally; rather, it has recently been suggested that it does not exist (Driehuis & Wouters, 1987; de Jonge *et al.*, 1989).

Therefore, we addressed the problem with a novel analytical method that allowed us to determine the length distribution of the glycan strands from which the sacculus is made (Harz *et al.*, 1990). As a source for polar cap material we decided to isolate minicells, which are formed in *minB* mutants of *E. coli* as a result of a failure in placing the site of division at the equator of the rod-shaped bacterium (de Boer *et al.*, 1989). Instead, a potential division site at one of the poles is chosen erroneously by the cell. PBP3, which is the septum synthesizing enzyme (Botta & Park, 1981), together with FtsZ (de Boer *et al.*, 1992; Raychaudhuri & Park, 1992; Mukherjee *et al.*, 1993) are required for both normal cell division at the midpoint and minicell formation at the poles of the cell (Ward & Lutkenhaus, 1985). Thus, it seems likely that the septum that splits off a minicell is made of normal septal murein. Minicells, which lack cylindrical murein, may therefore indeed consist of regular polar cap murein only.

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Abbreviations: A₂pm, diaminopimelic acid; PBP, penicillin-binding protein; Abbreviations for mucopeptides (Glauner *et al.*, 1988) are as follows: Di, GlcNAc(β1-4)MurNAC-L-Ala-D-Glu; Tri, GlcNAc(β1-4)MurNAC-L-Ala-D-Glu-A₂pm; Tetra, GlcNAc(β1-4)MurNAC-L-Ala-D-Glu-A₂pm-D-Ala; Penta, GlcNAc(β1-4)MurNAC-L-Ala-D-Glu-A₂pm-D-Ala-D-Ala; Anh, 1,6-anhydromuramic acid present; Gly4/Gly5, D-Ala in position 4 or 5 of the peptidyl residue is replaced by Gly; Tetra-Tri, Tetra-Tetra, Tetra-Penta, dimers of the indicated substituents formed by a D-Ala-m-A₂pm interpeptide bond; Tetra-Tetra-Tri, Tetra-Tetra-Tetra, trimers consisting of the indicated substituents formed by two D-Ala-m-A₂pm interpeptide bonds; A₂pm, an m-A₂pm-m-A₂pm interpeptide bond is present instead of the D-Ala-m-A₂pm crossbridge.

Using the minicell producing strain *E. coli* P678-54, both the murein of the rod-shaped whole cells and the murein of the spherical minicells can be compared directly with each other.

Minicells cannot easily be obtained in sufficiently high quantities because yield from the minicell producing strain *E. coli* P678-54 is less than 1%. Therefore, we established a procedure that allows the introduction of a label with a high specific radioactivity to murein after its preparation. This can be done by specific galactosylation of the terminal glucosamine residues of the polysaccharides using galactosyltransferase and labelled UDP-galactose (Schanbacher & Ebner, 1970; Schindler *et al.*, 1976). Not only the composition of the murein but also the pattern of PBPs was found to differ between minicells and whole cell preparations.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* MC 4100 and the minicell producing strain P678-54 (*minA minB*) (Adler *et al.*, 1967) were grown at 37 °C with vigorous shaking in LB-medium containing (l⁻¹) 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl. Growth of the cultures was followed by measuring the optical density at 578 nm (OD₅₇₈) in an Eppendorf photometer. Exponentially growing cells at an OD₅₇₈ of about 0.7 were harvested by centrifugation in the cold. Radiolabelling of murein was done by growth of the cells in the presence of 0.74 kBq D-[1-³H]glucosamine hydrochloride ml⁻¹ (185 GBq mmol⁻¹; Amersham).

Isolation of minicells. Minicells of *E. coli* P678-54 were isolated by isopycnic Percoll gradient sedimentation according to the method of Bellemare *et al.* (1986) (see also Markiewicz & Höltje, 1992).

PBP assay. The profile of PBPs in the membranes of whole cells and minicells of *E. coli* P678-54 was basically determined according to the procedure described by Spratt (1975). However, a ¹²⁵I-labelled derivative of ampicillin, prepared following the method of Schwarz *et al.* (1981), was used. Membranes (10 µg protein) suspended in 20 µl 0.05 M potassium phosphate buffer containing 0.01 M MgCl₂, pH 7.0 were incubated in the presence of ¹²⁵I-labelled ampicillin (5 µl of freshly prepared material; about 37 kBq) as described by Schwarz *et al.* (1981). After solubilization of the membrane proteins according to Spratt & Pardee (1975), they were separated by SDS-10% (w/v) polyacrylamide gel electrophoresis (Laemmli, 1970) and the labelled penicillin-enzyme complexes were made visible by autoradiography.

Preparation of murein sacculi. Murein sacculi were prepared as described by Harz *et al.* (1990).

Analysis of the muropeptide composition. Murein from minicells and whole cells of *E. coli* P678-54 was degraded with Cellosyl, a muramidase from *Streptomyces coelicolor*, as described by Glauner (1988). The muropeptides were reduced with NaBH₄ and separated at 55 °C at a flow rate of 0.5 ml min⁻¹ on a Hypersil ODS C₁₈ column using a linear gradient from 50 mM sodium phosphate, pH 4.31, to 75 mM sodium phosphate,

pH 4.95 containing 15% methanol, following the procedure of Glauner *et al.* (1988).

Analysis of the length distribution of the glycan strands.

Glycan strands were released from murein sacculi by digestion with human serum amidase as described by Harz *et al.* (1990). To introduce a radioactive label, the *N*-acetylglucosamine ends of the sugar chains were substituted with [³H]- or [¹⁴C]galactose by the galactosyltransferase reaction according to the method of Schindler *et al.* (1976). The reaction mixture consisted of the glycan strands (0.05 mM) released from isolated murein sacculi by amidase digestion, uridine diphospho-D-[6-³H]- or -[U-¹⁴C]galactose (Amersham; 74 kBq, adjusted with unlabelled UDP-Gal to a final concentration of 0.5 mM), 0.1 mM MnCl₂, 50 mM MOPS, pH 7.4, and galactosyltransferase (1.8 × 10⁻³ U ml⁻¹; Sigma). The mixture was incubated at 37 °C for 5 h, adjusted with H₃PO₄ to pH 4.5 and boiled for 8 min. Denatured protein was removed by centrifugation for 10 min in an Eppendorf centrifuge. The supernatant was concentrated and stored frozen at -20 °C. Prior to HPLC fractionation of the glycan strands, the peptide moieties had to be removed by chromatography on a Mono S HR 5/5 column (Pharmacia) as described by Harz *et al.* (1990). Fractionation of the glycan strands according to their chain length was performed by HPLC on Nucleosil 300 C₁₈. Elution was done at a flow rate of 0.5 ml min⁻¹ at 50 °C using a convex gradient from 5% to 11% (w/v) acetonitrile in 100 mM sodium phosphate, pH 2.00 (Harz *et al.*, 1990).

RESULTS

Specific labelling of isolated glycan strands

Galactosylation of the *N*-acetylglucosamine residues was used as a means to label isolated murein specifically. This

Fig. 1. Co-chromatography of [³H]GlcNAc- and [¹⁴C]Gal-[³H]GlcNAc-double-labelled glycan strands. *E. coli* MC4100 was grown in LB medium in the presence of 7.4 kBq [³H]GlcNAc ml⁻¹ (185 GBq mmol⁻¹) and harvested during exponential growth. Murein was isolated, the glycan strands prepared and one portion of the glycans was galactosylated with 74 kBq UDP-[¹⁴C]Gal ml⁻¹ (1.2 Bq mmol⁻¹) as described in Methods to yield double-labelled glycan strands. The glycans were separated by reversed-phase HPLC on an ODS Nucleosil (5 µm) column using a convex acetonitrile gradient in phosphate buffer, also as described in Methods., ³H label; —, ¹⁴C label.

Fig. 2. Comparison of the length distribution of glycan strands from [³H]GlcNAc-labelled murein before and after galactosylation with [¹⁴C]Gal. The double-labelled glycans were quantified on the basis of their ¹⁴C label only. The preparation procedures and the conditions for reversed-phase HPLC of the glycans on ODS Nucleosil are given in Methods. ■, [³H]GlcNAc-labelled glycans; □, [¹⁴C]Gal label of double-labelled glycans.

method adds one galactose molecule to the nonreducing end of the glycan strands without otherwise affecting the material (Schindler *et al.*, 1976). It became apparent that glycan strands released from murein by amidase treatment are a better substrate for the galactosyltransferase than intact murein sacculi. Accordingly, prior to galactosylation the murein was digested with human serum amidase following the established procedure (Harz *et al.*, 1990). Another problem was caused by the relatively high Mn²⁺ concentration which, although optimal for the enzyme (Schindler *et al.*, 1976) results in precipitation of the glycan strands. Analysis of the dependency of the reaction on Mn²⁺ revealed that a decrease to 0.1 mM did not affect the galactosyltransferase activity but avoided precipitation of glycan strands.

The reliability of the galactosylation procedure for labelling isolated glycan strands was investigated by comparing this method with *in vivo* labelling of murein by growth in the presence of a specific label. Murein was prepared from cells grown in the presence of [³H]GlcNAc, and the glycan strands were isolated. One aliquot was galactosylated with UDP-[¹⁴C]Gal as described in Methods, yielding double-labelled glycans. No separation into ³H- and ¹⁴C-labelled compounds was observed during HPLC fractionation of this galactosylated material (results not shown), although glycan strands longer than 4 disaccharide units, when substituted with galactose, eluted in between the galactose-free glycan strands in co-chromatography (Fig. 1). This finding

indicates that all glycan strands longer than 4 disaccharide units had been modified by galactose.

To show also that the shorter glycans which, under standard elution conditions do not separate into galactose-free and galactosylated oligosaccharides (see Fig. 1), had been fully galactosylated, specific conditions to separate di- and trisaccharides by HPLC were established. These conditions were used to further fractionate the first peak obtained from a standard chromatography of double-labelled glycan strands (Fig. 1), which was expected to contain the galactosylated disaccharide. Whereas anhydro-disaccharide and Gal-anhydro-disaccharide could be separated under these conditions, the ³H- and ¹⁴C-label of the double-labelled material did not split up into two peaks (results not shown), indicating that the disaccharides also had been modified to Gal(β1-4)GlcNAc(β1-4)MurNAc. Consequently, a quantification of the double-labelled glycans on the basis of the [¹⁴C]Gal label resulted in an identical pattern of glycan strand distribution when compared with [³H]GlcNAc-labelled material (Fig. 2). This result proves the reliability of the galactosylation procedure.

Muropeptide composition of minicells

Minicells produced by *E. coli* P678-54 (Adler *et al.*, 1967) were isolated by repeated Percoll density gradient centrifugation until only about one normal rod-shaped cell was present per 10⁵ minicells as judged by electron micro-

Table 1. Muropeptide composition of minicells and rod-shaped whole cells

Muropeptide	Relative amount (percentage of total murein)	
	Whole cells	Minicells
Tri	7.36 ± 0.28	7.23 ± 1.03
Tetra(Gly ₄)	2.78 ± 0.50	2.31 ± 0.07
Tetra	26.06 ± 1.05	23.39 ± 0.40
Penta(Gly ₅)	0.56 ± 0.08	0.33 ± 0.09
Di	1.53 ± 0.15	0.69 ± 0.04
Penta	0.12 ± 0.01	0.35 ± 0.21
Tri-Lys-Arg	5.19 ± 1.11	5.88 ± 0.97
Tri(Anh)	0.34 ± 0.05	0.32 ± 0.06
Tetra-Tri(Gly ₄)	0.20 ± 0.01	0.59 ± 0.13
Tri-Tri(A ₂ pm)	0.65 ± 0.09	1.00 ± 0.07
Tetra-Tri(A ₂ pm)	2.26 ± 0.28	3.85 ± 0.20
Tetra-Tetra(Gly ₄)	2.35 ± 0.31	1.88 ± 0.09
Tetra-Tri	4.99 ± 0.77	3.08 ± 0.03
Tetra-Penta(Gly ₅)	0.52 ± 0.10	0.45 ± 0.10
Tetra-Tetra	21.63 ± 0.81	20.35 ± 0.06
Tetra(Anh)	0.46 ± 0.07	0.37 ± 0.05
Tetra-Penta	0.37 ± 0.01	0.75 ± 0.08
Tri-Tri-Lys-Arg(A ₂ pm)	0.71 ± 0.02	1.28 ± 0.23
Tetra-Tetra-Tri(A ₂ pm)	0.54 ± 0.03	0.88 ± 0.30
Tetra-Tetra-Tri	0.58 ± 0.04	0.40 ± 0.01
Tetra-Tri-Lys-Arg	4.12 ± 1.19	4.25 ± 1.26
Tetra-Tetra-Tetra	3.06 ± 0.30	2.79 ± 0.38
Tetra-Tetra-Penta	0.02 ± 0.01	0.07 ± 0.03
Tetra-Tri(A ₂ pm, Anh I)	0.18 ± 0.06	0.44 ± 0.09
Tetra-Tri(A ₂ pm, Anh II)	0.18 ± 0.08	0.20 ± 0.11
Tetra-Tri-Tri-Lys-Arg(A ₂ pm)	0.19 ± 0.05	0.32 ± 0.16
Tetra-Tri(Anh I)	0.63 ± 0.06	0.67 ± 0.17
Tetra-Tri(Anh II)	0.32 ± 0.05	0.32 ± 0.08
Tetra-Tetra-Tetra-Tetra	0.14 ± 0.03	0.12 ± 0.03
Tetra-Tetra-Tri-Lys-Arg	0.70 ± 0.05	0.60 ± 0.15
Tetra-Tetra(Anh I)	0.69 ± 0.20	1.21 ± 0.21
Tetra-Tetra(Anh II)	1.04 ± 0.11	1.13 ± 0.15
Tetra-Tetra-Tri(A ₂ pm, Anh)	0.16 ± 0.05	0.26 ± 0.09
Tetra-Tetra-Tri(Anh)	0.16 ± 0.03	0.19 ± 0.03
Tetra-Tetra-Tetra(Anh)	0.82 ± 0.12	0.80 ± 0.14
Tetra-Tetra-Tetra-Tetra(Anh)	0.09 ± 0.02	0.11 ± 0.03
Tetra-Tri-Lys-Arg(Anh I)	0.31 ± 0.01	0.45 ± 0.17
Tetra-Tri-Lys-Arg(Anh II)	0.24 ± 0.05	0.48 ± 0.11
Tetra-Tetra-Tri-Lys-Arg(Anh I)	0.09 ± 0.01	0.12 ± 0.04
Tetra-Tetra-Tri-Lys-Arg(Anh II)	0.10 ± 0.01	0.12 ± 0.05
Tetra-Tetra-Tri-Lys-Arg(Anh III)	0.10 ± 0.03	0.11 ± 0.03

scopic examination of the preparation. A control experiment in which cells of *E. coli* MC4100 were subjected to the very same procedure (which, incidentally, represents an osmotic stress for the cells) proved to cause no detectable changes in the structure of the murein.

The muropeptide composition of minicells and rod-shaped whole cells was compared. The averages of three independent analyses are compiled in Table 1. The major

structural parameters of murein and those muropeptides showing significant differences are listed in Table 2. Two changes in the composition of minicell murein are most evident: an increase in the relative amount of LD-A₂pm-A₂pm crosslinkages by about 66% and a decrease in the dipeptide residues by nearly 55%. The average chain length of the glycan strands calculated on the basis of the anhydro-muropeptide components (Höltje *et al.*, 1975) showed a decrease from 27.8 to 23.5 disaccharide units.

Table 2. Mucopeptides showing significant differences when comparing minicells with rod-shaped whole cells

Mucopeptide	Relative amount (percentage of total murein)		Δ (%)
	Whole cells	Minicells	
Monomers	47.63	45.19	-5.1
Di	1.53	0.69	-54.9
Dimers	45.04	47.08	+4.5
Tetra-Tetra	21.63	20.35	-5.9
Tetra-Tri(A ₂ pm)	2.26	3.85	+70.4
Tetra-Tri	4.99	3.08	-38.3
Trimers	7.08	7.49	+5.8
Tetra-Tetra-Tri(A ₂ pm)	0.54	0.88	+62.9
Tetra-Tetra-Tri	0.58	0.40	-31.0
Tetramers	0.25	0.25	± 0.0
Crosslinkage	27.43	28.71	± 4.7
Ala-A ₂ pm	24.66	24.30	-1.5
A ₂ pm-A ₂ pm	2.62	4.35	+66.0
Average chain length	27.8	23.5	-15.5

This latter finding of a shift to shorter glycan strands in minicells was analysed in more detail by a determination of the complete profile of the different lengths of the glycan strands.

Length distribution of the glycan strands in the murein of minicells

The glycan strands can be released in their native lengths from the murein network by human serum amidase (Mollner & Braun, 1984; Harz *et al.*, 1990). The polysaccharides can then be separated from the peptide moieties by ion exchange chromatography and finally be fractionated by reversed-phase HPLC according to size, that is degree of polymerization. To introduce a radioactive label, the isolated glycan strands were galactosylated using UDP-[³H]Gal as described in detail in Methods prior to HPLC-separation. The fractionation profiles of the glycan strands from minicells and whole cells are shown in Fig. 3.

The cells of the minicell producing culture were quite irregular with respect to shape, consisting of normally sized rods and elongated rods arising from cells that have divided asymmetrically during minicell formation. Therefore, we also determined the length distribution of the glycan strands from murein of the *E. coli* K12 strain MC4100 (Fig. 3). Comparison of the minicell murein with either of the two murein preparations, from whole cells of the minicell producer or from *E. coli* MC4100, revealed a shift of the length distribution to shorter glycan strands in the case of the minicells.

Since only the GlcNAc terminus of the strands is labelled

by this method, the radioactivity reflects the number of molecules of the different size classes and not the amount of glycan. A meaningful comparison of the murein of minicells with the murein of whole cells, however, calls for identical amounts of murein to be compared. Therefore, the amount of murein represented by each size class of glycan strands was calculated on the basis of their known lengths (molecular masses).

Furthermore, in order to compare specifically the spherical murein of polar caps with cylindrical murein synthesized during cell elongation, the murein material of minicells, which are believed to consist of polar caps only, had to be subtracted from the murein of whole cells, which consist of both polar caps and cylindrical murein. Assuming an identical thickness of the murein for both the cylindrical and the polar part of the sacculus (Wientjes *et al.*, 1991), the length distribution of the cylindrical murein can be calculated on the basis of the known proportions of polar caps and cylinder. Detailed surface measurements of isolated murein sacculi have shown that the polar caps of a rod-shaped sacculus represent about 2/9 of the total surface (Woldringh *et al.*, 1987). Although the ratio of polar to cylindrical surface can vary according to strain and growth conditions, we used this value for an approximate calculation. Accordingly, the amounts of material for each chain length were multiplied in the case of minicell murein by a factor of 2 and in the case of whole cell murein by a factor of 9. The resulting distribution profiles were then subtracted from each other to obtain the distribution of the relative amounts of the different chain lengths for cylindrical murein. As shown in Fig. 4, the relative amount of glycan strands in the range 1–10 disaccharide units is significantly increased in minicell murein; in other words, a larger portion of the total murein of polar caps is made of shorter glycan strands as compared with cylindrical murein. In particular, the shortest glycan strand possible, the disaccharide, was increased by a factor of almost five.

PBP pattern

Changes in the relative amounts of the PBPs in minicells of *E. coli* have been reported (Buchanan, 1981). Unfortunately, in that publication the pattern of the PBPs in the unfractionated initial culture, which was supposed to represent the control, was notably different from the well-established pattern (Spratt, 1975), having a greatly reduced amount of PBP3. Therefore, in this study we analysed the PBP pattern in both the whole cell preparation and the minicells. As shown in Fig. 5, the pattern of PBPs differed substantially. Most prominent was the dramatic reduction in the amounts of PBP4 and PBP6 and the increase in the amount of PBP8 in the membranes of minicells. The identity of PBP8 was proven by co-chromatography with partially purified PBP8 (P. Bindels & J.-V. Hölte, unpublished results). Our results are in conflict with those published by Buchanan (1981), which showed principally an altered PBP1B in minicells of *E. coli* X925. However, since the control in that report showed an abnormal PBP pattern also, as mentioned above, the

Fig. 3. Length distribution of the glycan strands from murein of minicells (■) and whole cells of *E. coli* P678-54 (□) as well as *E. coli* MC4100 (▨). The murein glycan strands were prepared, galactosylated with [14 C]Gal and separated by reversed-phase HPLC on ODS Nucleosil as described in Methods.

Fig. 4. Glycan composition of cylindrical murein and spherical murein. The relative length distributions of glycan strands in identical amounts of minicell murein and of cylindrical murein of whole cells are shown. The composition of cylindrical murein was obtained by subtracting the values for the polar cap murein as represented by the minicell murein from the total murein of the whole cells. ■, Spherical murein; □, cylindrical murein.

Fig. 5. PBP pattern of whole cells and minicells of *E. coli* P678-54. PBPs were labelled with ^{125}I -labelled ampicillin, solubilized with Sarkosyl and separated by SDS-10% (w/v) polyacrylamide gel electrophoresis. Approximately the same amounts of protein (30 μg) were applied to each lane. The PBPs (numbered 1 to 8) were visualized by autoradiography. Major changes between the two preparations are marked by arrowheads. Lane A, whole cells; lane B, minicells.

results may have been affected somehow by the method employed.

DISCUSSION

The question of the existence of a relationship between the chemical structure of the murein and the shape of the resulting murein sacculus has been addressed in several publications from the early days of murein research up to now (Weidel & Pelzer, 1964; Schwarz & Leutgeb, 1971; Henning *et al.*, 1972; Driehuis & Wouters, 1987; de Jonge *et al.*, 1989). The rod-shaped sacculus of *E. coli* may be considered to consist of a cylindrical middle part and two hemispherical poles, and these two different structures have been compared with each other in order to answer the above question. This has been done by pulse-labelling the murein of synchronously growing cultures (de Jonge *et al.*, 1989) or by analysing cells with a changed morphology due either to a change in the growth rate in chemostat culture (Driehuis & Wouters, 1987) or to mutations in the PBPs (Glauner, 1986; Schwarz & Glauner, 1988). Treatments with antibiotics that specifically inhibit certain PBPs have also been used to cause changes in the morphology of the cells (Pisabarro *et al.*, 1986). Filamentous or elongated cells mostly consist of

cylindrical murein whereas round or short cells preferentially consist of spherical murein.

Although the composition of murein could be shown to depend on the growth rate, it seems not to be affected by the resulting different shapes of the cells (Driehuis & Wouters, 1987). Furthermore, a detailed analysis of the murein synthesized during the cell cycle of *E. coli* revealed different modes of insertion of murein precursors but no alteration in the mucopeptide composition of newly synthesized murein (de Jonge *et al.*, 1989). It has been suggested therefore that the murein of the rod-shaped sacculus is chemically identical throughout and remains constant during the cell cycle.

However, interpretation of the results obtained from synchronously growing cultures (de Jonge *et al.*, 1989) has been criticized recently (Cooper, 1990). According to this opposing view, the results actually indicate a change in the murein structure, namely an increase in the degree of crosslinkage during septum formation. A minor increase in crosslinkage has indeed been detected when pulse-labelled murein of filaments was compared with pulse-labelling murein of filaments that were resuming septation (Romeis *et al.*, 1991). Again, a small increase in total crosslinkage of septal murein is reported in this paper, comparing minicells with rod-shaped cells.

To obtain information on the individual lengths of the glycan strands of which the murein sacculus is made, we used a novel approach. Recently, a method has been established to release the glycan strands from the murein network in their native lengths by amidase treatment (Harz *et al.*, 1990). This is followed by fractionation of the glycans according to length by reversed-phase HPLC. Since minicells can only be obtained in rather low yields, a procedure was utilized that allows labelling of murein or solubilized glycan strands after their isolation. Galactosylation of the glucosamine ends of the glycan strands is shown here to represent a reliable procedure.

It is important to note that this method yielded the same length distribution of the glycan strands as was obtained when the natural 1,6-anhydromuramic acid marker for the muramic acid termini of the glycan strands was used instead (Höltje *et al.*, 1975; Glauner *et al.*, 1988). The finding that there are as many 1,6-anhydroMurNAc ends as GlcNAc ends in the murein rules out any speculation about the existence of reducing muramic acid ends.

The length distribution of the glycan strands of the murein from minicells was shifted to shortened lengths when compared with the rod-shaped whole cells. In particular, the shortest possible strand, the disaccharide, was increased most dramatically. Furthermore, the mucopeptide composition of the murein of minicells also revealed some additional changes which have not been observed in earlier reports that used either synchronous cultures or morphological mutants (Glauner, 1986; Schwarz & Glauner, 1988; Driehuis & Wouters, 1987; de Jonge *et al.*, 1989). Significant increases in the relative amounts of LD-A₂pm-A₂pm crosslinkage as well as in the dipeptide moieties were detected in the murein of minicells.

It seems difficult to answer the question as to whether these differences in composition between minicells and whole cells actually do reflect the difference in shape of the murein sacculus. Although we cannot rule out this possibility, we think it is unlikely for the following reasons. Besides their spherical shape, the physiology of minicells also is changed as compared with that of whole cells (Frazer & Curtiss, 1974). Minicells continue to synthesize murein (Reeve, 1977), but at a greatly reduced rate (Markiewicz & Höltje, 1992). Most importantly, not only the protein composition of the membranes of minicells (Goodell & Schwarz, 1974; Buchanan, 1981) but also the pattern of PBPs is changed, as shown in this report. Therefore, the murein composition may be changed because of the altered metabolism of the DNA-less minicells rather than because they are made of two hemispherical polar caps. Furthermore, the observed changes in the structure of the murein sacculus have also all been seen under growth conditions completely unrelated to a specific spherical shape of the sacculus. The increase in disaccharide strands is reminiscent of a quite similar change that has been detected during the course of antibiotic-induced autolysis of *E. coli* (Kohlrausch & Höltje, 1991). In addition, analysis of the length distribution of the glycan strands in murein that had been pulse-labelled at different time points in a synchronously growing culture did not show a significant change in length distribution (T. Romeis, N. Nanninga & J.-V. Höltje, unpublished results). The decrease in dipeptide moieties may indeed be due to the absence of an elongation process in minicells and thus be specific for septum formation, since it has been shown that in synchronously growing cells of *Proteus mirabilis* dipeptide-substituted mucopeptides are formed preferentially during longitudinal extension of the sacculus (Gmeiner & Kroll, 1981). However, in contrast to this finding, dipeptide moieties were found to be enriched in short, almost spherical cells of *E. coli* from the stationary growth phase (Glauner, 1986). The most prominent change in the composition of minicell murein, the increase in LD-A₂pm-A₂pm crosslinkages, is known to occur during aging of murein (Glauner & Höltje, 1990), as a consequence of a decrease in the growth rate (Driehuis & Wouters, 1987) and when autolysis is threatening the cell. In the presence of penicillin (Kohlrausch & Höltje, 1991) and after a rise in the activity of endogenous murein hydrolases (Walderich *et al.*, 1988; Korat *et al.*, 1991), this type of crosslinkage is increased. Therefore, the formation of LD-A₂pm-A₂pm crosslinkages was interpreted to represent a kind of salvage mechanism to stabilize the murein in lysis-prone cells (Glauner *et al.*, 1988). Thus, by comparing the composition of the murein of minicells with that of whole cells, conclusive evidence for a specific correlation between the shape of the murein and its chemistry could not be obtained.

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