The Target of Penicillin

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The Murein Sacculus of Bacterial Cell Walls Architecture and Growth

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Preface

This book is based on the papers read at the International Symposium on the Murein Sacculus of Bacterial Cell Walls (March 13-18 in West Berlin). The aim of the symposium was to present the current state of knowledge on the subject of the murein sacculus. The contributions to this book, therefore, include very different approaches, ranging from physical studies on the three-dimensional architecture of this unique biopolymer, the biological processes responsible for cellular growth to medical implications of the bacterial cell wall. We intentionally allowed equal space for poster presentations and talks, in order to cover as many experimental results as possible. It is up to the reader to interpret the papers and draw his own conclusions. We hope that this collection will provide a comprehensive up-to-date review of the many recent advances in this field. If it does this, then its success is mainly due to the excellent contributions by the participants at the symposium. For this, we would like to thank all authors.

We would like to express our gratitude to the Senat der Stadt Berlin (West), the Max-Planck-Gesellschaft, the Deutsche Forschungsgemeinschaft, and the Federation of European Microbiological Societies for sponsoring the symposium, and Bayer AG, the Bundesgesundheitsamt, the Paul Martini Stiftung and Schering AG for financial support. In the end, it was their aid which also enabled us to present this book. Thanks are also due to the publisher for publishing these proceedings so rapidly. Finally, we thank all our colleagues, especially I. Baxivanelis, A. Brauer, H. Lange, B. Nürnberg and E. Vorgel for their enthusiastic and devoted help in the organization of the symposium and/or in the realization of the proceedings.

Berlin (West) and Tübingen
May 1983

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INTRODUCTION

THE BACTERIAL CELL WALL - STRUCTURE AND FUNCTION

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Introduction

The history of our current interest in bacterial cell walls can be traced all the way back to the birth of microbiology with Antony van Leeuwenhoek's description of the microscopic morphology of bacteria and other microorganisms in 1675. Leeuwenhoek accurately recorded the characteristic shapes of bacteria and obviously anticipated that surface structures were responsible for their shape, one of the main features of the cell wall still recognized today. The cell surface envelope structures are specially important for free-living bacteria as rigid protective barriers against the adverse environments they may encounter. Early attempts, prior to the turn of the century, to experimentally establish a cell wall in bacteria and to probe its chemical nature stemmed from two types of approach as exemplified by Fischer's (1891) "physiological" experiments on plasmolysis and attempts to chemically characterize the resistant rod-shaped structures of Bacillus subtilis by Vincenzi (1887). The uncertainties of the traditional bacterial cytologists efforts to understand the chemical structure and function of the cell wall ended in the mid 1940's to early 1950's when a new era of the application of electron microscopy and biochemistry to subcellular fractionation ushered in the rapid advance of our knowledge of wall structure and function. In looking back over some of the major milestones in the devel-

The Target of Penicillin

Table I. Walls - Lysozyme - Penicillin

1. Vincenzi, 1887 - First attempt at cell wall chemistry
2. Fleming, 1922 - Lysozyme and bacteriolysis
3. Fleming, 1929 - First observations on penicillin
4. Epstein & Chain, 1940 - First chemical study of lysozyme substrate
5. Chain et al., 1940 - Isolation of penicillin
6. Duguid, 1945 - Penicillin acts on walls
7. Park, 1952 - Discovery of Park nucleotides
8. Strange, 1956 - Muramic acid

opment of this field (Table I), studies of walls, lysozyme and penicillin became intertwined and finally integrated into a new unified understanding of wall structure and biosynthesis. At the forefront of this era, the outstanding and incisive contributions of Professor Wolf Weidel and his colleagues will long be remembered. It is fitting that we pay tribute to Wolf Weidel, who in collaboration with Pelzer developed the concept of the "Murein Sacculus" of bacterial walls as "Bagshaped Macromolecules." The significance of the chemical studies of walls in the mid 1950's was placed in perspective when Park and Strominger (1) proposed the biochemical basis for the mechanisms of action of penicillin and its selective toxicity and Weidel and Primosigh (2) drew attention to the biochemical parallels between phage and penicillin lysis. The exciting developments following this period form the solid basis on which the newer and equally exciting approaches of today are emerging to give us new insights into wall structure and biochemistry.

Wall Ultrastructure

Although we are often prone to think about cell walls and envelopes of Gram-negative bacteria as the flat collapsed structures we see in electron micrographs of isolated walls,they constitute the external compartment of the cell and functionally should perhaps be regarded more as an

organelle rather than a simple rigid corset surrounding the more fragile plasma membrane. As seen in thin sections, the cell wall profile of many Gram-positive bacteria has a relatively thick, amorphous appearance. Occasional evidence of layered and fibrillar structures is seen in some. Profiles of the mycobacterial cell wall, however, are much more complex, showing distinct layering with the murein or peptidoglycan layer forming the innermost of four sheetlike structures (3). Fibrous, ropelike structures (peptidoglycolipid) although not resolved in thin-section profiles can be readily seen by negative-staining and freeze-fracture techniques and they appear to be a general ultrastructural feature of the walls of all species of mycobacteria (3). These surface components can be extracted with ethanolic-KOH revealing a residual smooth (murein) layer (3). These changes in the mycobacterial wall following extractions with ethanol-KOH or pyridine, contrast with the lack of any changes seen in the surface of the Gram-positive organism, Listeria monocytogenes following extraction of its lipopolysaccharide-like component with phenol (4). This suggests that the organization of this LPS-like macromolecule in the cell surface of L. monocytogenes differs from the arrangement of LPS in the outer membranes of Gram-negative species.

The surface topography of Gram-negative bacteria has been well documented by thin sections, showing under appropriate conditions the densely-stained profile of the peptidoglycan layer. Freeze-fracture of the envelopes of Gram-negative species also shows the complexity of their surface structures, but with the principal fracture faces passing through the plasma membrane (5).

Ultrastructural studies of isolated walls can also reveal further details of the organization of the peptidoglycan in cells as seen in recent experiments carried out with Dr.

K.-S. Kim on dividing Micrococcus agilis. The cross- wall septum possesses an annular region in the center with a fibrillar-like appearance, perhaps representing the centripetal growth and assembly of the peptidoglycan. Similar annular structures have also been previously documented in Staphylococcus aureus walls by Giesbrecht et al. (6).

Chemical Components of Bacterial Walls

Peptidoglycan or murein constitutes the major structural polymer of the walls of both Gram-positive and Gram-negative bacteria and the recently discovered pseudomurein of the Archaebacteria containing N-acetyltalosaminuronic acid instead of N-acetylmuramic acid in the glycan moiety forms the analogous structure in these organisms (7,8). In Gram-positive bacteria such as M. lysodeikticus the peptidoglycan may account for as much as 95% of the wall, the remaining 5% being accounted for by the associated teichuronic acid composed of N-acetylmannosaminuronic acid and glucose (9). Walls of Staphylococcus aureus and Bacillus subtilis possess high contents of teichoic acids and correspondingly lower contents of peptidoglycan (40-50%). In addition to teichoic acids as wall associated polymers, other bacterial species contain polysaccharide components and the mycobacteria contain an array of complex lipids and peptidoglycolipids. Some of the features of the walls of Gram-positive bacteria are summarized in Table 2. In Gram-negative bacteria the peptidoglycan accounts for a much smaller fraction of the total envelope although its chemical structure is remarkably similar throughout these organisms (11).

Biological Properties of Wall Components

The walls of Gram-positive bacteria and the envelopes of

Table 2. Chemical Components of Cell Walls of Selected Gram-positive Bacteria (modified from Rogers et al.,ref. 10)

Bacillus licheniformis	Peptidoglycan, teichoic acid, teichuronic acid
Bacillus subtilis	Peptidoglycan, teichoic acid, teichuronic acid
Micrococcus lysodeikticus	Peptidoglycan, teichuronic acid
Lactobacillus spp.	Peptidoglycan, teichoic acids, polysaccharides
Staphylococcus aureus	Peptidoglycan, teichoic acid, teichuronic acid
Mycobacteria spp	Peptidoglycan, peptidoglycolipids, trehalose dimycolate, acylglucoses (ref. 3)

Gram-negative possess a variety of macromolecular components exhibiting a remarkable array of biological activities and responses in many animal species. The pioneering work of Lederer and his colleagues leading to the elucidation of the minimum structure of peptidoglycan units (muramyldipeptides) responsible for adjuvant effects is a remarkable achievement. Moreover, as shown by Krause and Schleifer and their colleagues peptidoglycan induces formation of antibody populations of limited heterogeneity. In addition to immunostimulatory and pyrogenic effects of peptidoglycans, Krueger et al.(12) have made the fascinating discovery that muramyl peptides have somnogenic effects. It is suggested that receptors in the brain have a high affinity for muramylpeptides. A variety of wall polysaccharides, including those of M.lysodeikticus and Propionibacterium acnes are powerful immunomodulators and possess anti-tumor properties. Lipopolysaccharides of Gram-negative bacteria and of the Gram-positive L. monocytogenes have long been known to possess a great variety of immunological and biological responses in humans and animals.

Walls and Plasma Membranes

Since there can be no bacterial cell wall without the existence of the plasma membrane it is only fair to mention it as the site of some of the terminal events in peptidoglycan biosynthesis and assembly and the site of the penicillin targets, the PBP's. Their specific localization on one or both faces of the plasma membrane remains to be conclusively established.

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PART I

PRIMARY AND THREE DIMENSIONAL STRUCTURE OF MUREIN

PRIMARY STRUCTURES OF MUREIN AND PSEUDOMUREIN

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Murein is the main cell wall polymer of most eubacteria. It is a heteropolymer consisting of polysaccharide chains which are cross-linked through short peptides (Fig. 1).

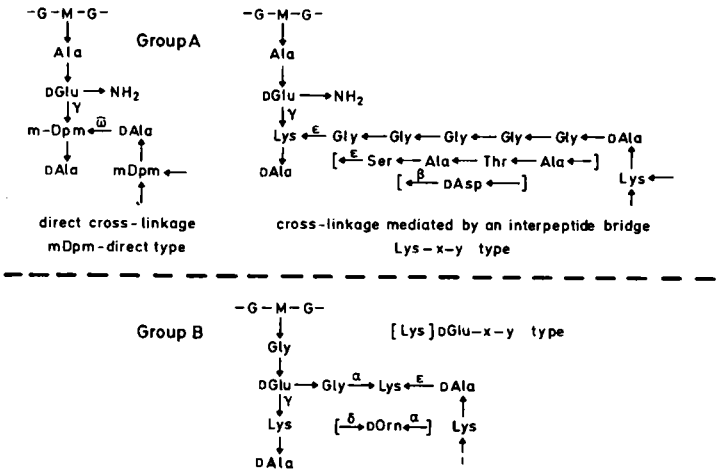


Fig. 1. Primary structures of group A and B mureins. (Designation of murein types according to Schleifer and Kandler 1972).

The Target of Penicillin

The glycan moiety of murein resembles chitin. Like chitin it is made up of alternating ß(1,4)-glycosidically linked units of N-acetylglucosamine. However, in contrast to chitin, each alternate glucosamine residue is substituted by a D-lactic acid ether at C-3. This derivative of glucosamine is called muramic acid. The carboxyl group of muramic acid is substituted by a peptide containing alternate L- and D-amino acids. Adjacent stem peptides may be cross-linked by a peptide bond between the carboxyl group of a D-alanine residue and the distal amino group of a diamino acid residue either directly or via an interpeptide bridge. This gives rise to a huge macromolecule encompassing the entire bacterial cell. The main characteristics of the murein structure can be briefly summarized as follows:

1. The glycan part is rather uniform. It exhibits only few variations, such as acetylation or phosphorylation of the muramyl 6-hydroxyl groups or the occasional absence of N-acyl or peptide substituents. Muramic acid is always of D-gluco configuration. Moreover, the muramic acid residues of some coryneform bacteria and mycobacteria may occur as N-glycolyl derivatives.

2. Depending on the mode of cross-linking, one can distinguish two main groups, named A and B. The cross-linkage of group A mureins extends, as shown in Figure 1, from the distal amino group of the diamino acid in position 3 of one stem peptide to the carboxyl group of D-alanine in position 4 of an adjacent stem peptide. Group B cross-linkage extends between the α-carboxyl group of D-glutamic acid in position 2 of one stem peptide and the D-alanine residue in position 4 of an adjacent peptide.

3. The number of amino acids occurring in various mureins is rather restricted. From 3 to 6 chemically different amino acids can be found in a particular murein. Branched and aromatic amino acids or cysteine, methionine, arginine,

histidine and proline have never been found.

4. The stem peptide always consists of alternating L- and D-amino acids. Even meso-diaminopimelic acid is integrated into this pattern and bound with its L-asymmetric center.

5. Only a restricted variation is found within the stem peptide. The greatest variation could be found within position 3. Up-to-now ten chemically different amino acids have been detected in this position, whereby meso-diaminopimelic acid and L-lysine are the most frequent ones. The α-carboxyl group of D-glutamic acid is usually amidated but in a few rare cases other substituents can also occur. Extensive investigation of the murein structure (Schleifer and Kandler, 1972) of many different bacteria leads to the following classification system (Fig. 2).

Fig. 2: Classification system of the murein

Classification	Abbreviation	Basis for classification
Groups	Roman capital letter (A,B)	Class of cross-linking
Subgroups	Arabic figures (1 - 5)	Kind of interpeptide bridge or lack of it
Variations	Greek letter (α - δ)	Amino acid present in position 3 of the stem peptide
	Prime (')	Replacement of Ala in position 1 of the stem peptide of group A peptidoglycans by Gly
Types	Depending on the different amino acid sequences of the peptide moiety. In particular, the amino acid sequence of the interpeptide bridge.	

A more detailed description of the different subgroups and variations of mureins can be found in the recent review by Schleifer and Stackebrandt (in press).

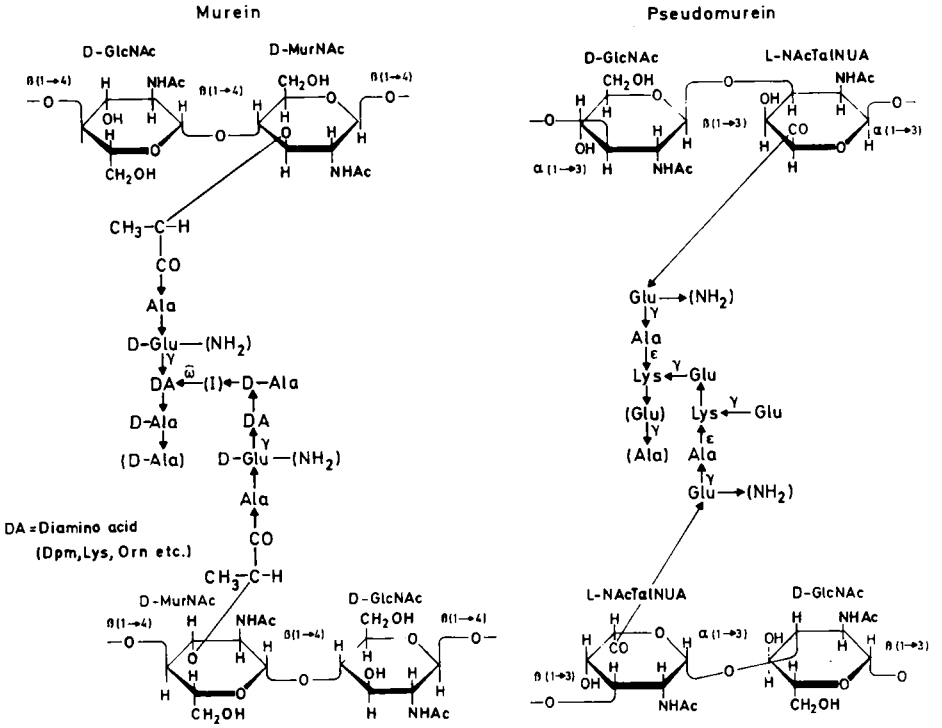
Until the middle of the seventies it was thought that murein is one of the key characters defining the Kingdom Prokaryota. There were only a few prokaryotes known such as mycoplasma or halobacteria which did not contain murein. However, in recent years the number of non-murein containing bacteria increased rapidly following the detection of the so-called archaebacteria. Comparative analysis of 16S rRNA demonstrated that archaebacteria are no more related to other bacteria (eubacteria) than they are to eukaryotic cells (Woese and Fox, 1977). One of the characteristic phenotypic features of the archaebacteria is the lack of murein in their cell walls. A survey of the various cell wall structures of archaebacteria is given in the Table.

Table: Cell wall and cell envelope structures in archaebacteria

Organism	Rigid Sacculus	Protein envelope	Polymer
Methanobacteriaceae	+	-	Pseudomurein
Methanosarcina	+	-	Heteropolysaccharide
Other Methanogens	-	+	Protein or Glycoprotein
Halobacterium	-	+	Sulfated Glycoprotein
Halococcus	+	-	Sulfated Heteropolysaccharide
Sulfolobus	-	+	Glycoprotein
Thermoproteus	-	+	Glycoprotein
Thermoplasma	-	-	none

Pseudomurein is found only in Methanobacteriales (Kandler, 1979, 1982). A comparison of the primary structures of murein and pseudomurein is shown in Figure 3.

Fig. 3: Fragments of the primary structures of murein and pseudomurein



Both are peptidoglycans which are composed of glycan strands cross-linked through short peptides. The glycan strands consist of alternating N-acetyl-D-glucosamine and N-acetyl-L-talosaminuronic acid residues which are ß-1,3 and α-1,3 glycosidically linked, respectively (König et al., in press). The amino acids of the peptide moiety exhibits in contrast to that of the murein exclusively L-configuration. In contrast to typical proteins the distal carboxyl and amino groups of glutamic acid

and lysine, respectively, are involved in peptide formations. Pseudomurein with slight modifications could be detected in some species of the order Methanobacteriales (König et al., 1982). Since carbons 3 of D-glucosamine and D-galactosamine possess the same anomeric configuration, it is not astonishing that a mutual replacement is possible. In some strains a mixture of both amino sugars is found. In the pseudomurein of Mbr. ruminantium an additional glucosamine is linked to the glycan moiety via a phosphodiester bond.

This strain also contains a pseudomurein in which at least part of the alanine is replaced by threonine. Ornithine is found as an additional component of the pseudomurein of Mbr. smithii.

A comparison of the main properties of murein and pseudomurein can be summarized as follows: In the murein the hexosamines possess the D-gluco-configuration, whereas pseudomurein contains hexosamines of the D-gluco- or D-galacto-configuration and an acid derivative of the L-talo-configuration.

The constituents of murein are ß-1,4-linked, those of pseudomurein are ß-1,3 or α-1,3 linked. A replacement of glucosamine by galactosamine in the pseudomurein does not change the structure of a 1,3-glycan. Such a replacement is not possible in the murein since carbons 4 of the two hexosamines exhibit a different anomeric configuration and a replacement of glucosamine by galactosamine would lead to a change of the secondary structure of the glycan strands. Pseudomurein is not attacked by lysozyme since the linkage is different and muramic acid is replaced by L-talosaminuronic acid.
The stem peptides of both polymers consist of a similar set of amino acids but their molar ratios and sequences are quite different. Moreover, murein contains both L- and D-amino acids whereas only L-amino acids are present in pseudomurein.

Cross-linking of the stem peptides proceeds via diamino acids in both polymers.
The resistance against proteases is mainly caused by alternating L- and D-amino acids in the murein or by unusual peptide bonds in pseudomurein. Pseudomurein is resistant to all cell wall-targeted antibiotics, such as ß-lactams, D-cycloserine or vancomycin , which interfere with reactions involving D-amino acid residues. It is, however, sensitive to antibiotics interfering with the lipid cycle of the murein synthesis such as bacitracin or nisin (Hilpert et al., 1981). This indicates that also undecaprenyl-lipid-bound intermediates are involved in the biosynthesis of pseudomurein.

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STRUCTURE OF THE PEPTIDOGLYCAN OF THE CYANOBACTERIUM SYNECHOCYSTIS PCC 6714

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Introduction

The rigid layer of Synechocystis sp. strain PCC 6714 consists of a polymer containing mannosamine (ManN), galactosamine (GalN), mannose (Man), glucose (Glc) and phosphorus in addition to the constituents of the A1-type of peptidoglycan (2-5). The presence of non-peptidoglycan components which is also known for other cyanobacteria (1) has been hampering structural studies on the Synechocystis peptidoglycan. This article describes experiments on composition, primary and secondary structure of peptidoglycan.

Results

1) Chemical composition of peptidoglycan. The rigid layer was obtained from Synechocystis sp. strain PCC 67144 by extraction of cell walls with hot sodium dodecyl sulfate (SDS). Non-peptidoglycan amino acids, amounting to 3.4 % of the dry weight of the SDS-insoluble fraction of the cell wall, were completely removed by digestion with

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pronase and trypsin, respectively, including lysine and arginine. The protease-treated fraction contained MurN, GlcN, L-Ala, D-Ala, D-Glu and meso-A_2pm in molar ratios of 0.9:1.5:1.1:0.8:1.0:1.0 (Tab. 1). In addition, ManN, GalN, Man, Glc and phosphorus were found in this fraction. Removal of this additional polymer and of non-peptidoglycan GlcN could be achieved by treatment with hydrofluoric acid in the cold (48 %, 0^{o}C, 48 h). The result suggests that a charged polysaccharide is very likely bound to the peptidoglycan via phosphodiester bonds.

The amino sugars of the peptidoglycan backbone are completely N-acetylated. Only small amounts of O-acetyl groups were detected. There is evidence that the peptide side chains bear at least a partial amidation of carboxy groups, since amide ammonia is easily set free in high amounts during the first hour of acid hydrolysis: molar ratios of D-Glu-meso-A_2pm-NH_3 = 1.0:0.7:1.5 were found. D-Ala and meso-A_2pm were the only C-terminal amino acids found on hydrazinolysis of peptidoglycan. Thus, tripeptides L-Ala-D-Glu-meso-A_2pm and tetrapeptides L-Ala-D-Glu-meso-A_2pm-D-Ala are suggested to be present as structural components of the peptidoglycan of Synechocystis.

Tab. 1: Chemical composition of peptidoglycan fractions

Component	Amt in peptidoglycan fraction (nmol per mg of fraction dry weight)		
	Pronase-digested	Trypsin-digested	Pronase-digested and hydrofluoric acid (48%, 0 C, 48 h) treated
Amino sugars			
MurN	520	514	837
GlcN	890	876	953
ManN	222	225	-[a]
GalN	15	16	-
Amino acids			
D-Ala	504	503	812
L-Ala	669	667	1077
D-Glu	604	598	959
meso-A_2pm	642	632	933
Neutral sugars			
Man	313	289	-
Glc	33	32	-
Acetyl groups			
N-Acetyl	1402	1372	1346
O-Acetyl	12	10	15
Phosphorus	150	139	-

[a] -, None

2) Isolation and characterization of peptides. Peptides were formed by the partial acid hydrolysis (4 M HCl, 100°C, 30 min) of peptidoglycan and were separated by combined low-voltage thin layer electrophoresis and thin layer chromatography. Eight peptides were isolated from fluorescamine stained cellulose plates and were characterized by amino acid analysis, gas-liquid chromatography (Fig. 1), and dinitrophenylation studies. They were identified as the dipeptides MurN-L-Ala, L-Ala-D-Glu, γ-D-Glu-meso-A_2pm, meso-A_2pm-D-Ala (mediating direct cross-linkage), meso-A_2pm-D-Ala (terminating the tetrapeptide), tripeptides MurN-L-Ala-D-Glu-meso-A_2pm, L-Ala-D-Glu-meso-A_2pm, and a tetrapeptide L-Ala-D-Glu-meso-A_2pm-D-Ala. The cross-linking dipeptide meso-A_2pm-D-Ala was also found to be sensitive upon degradation with a specific endopeptidase (from E. coli, kindly given by Dr. Höltje).
The peptide analyses show that the peptidoglycan of Synechocystis belongs to the A1γ-peptidoglycan type.

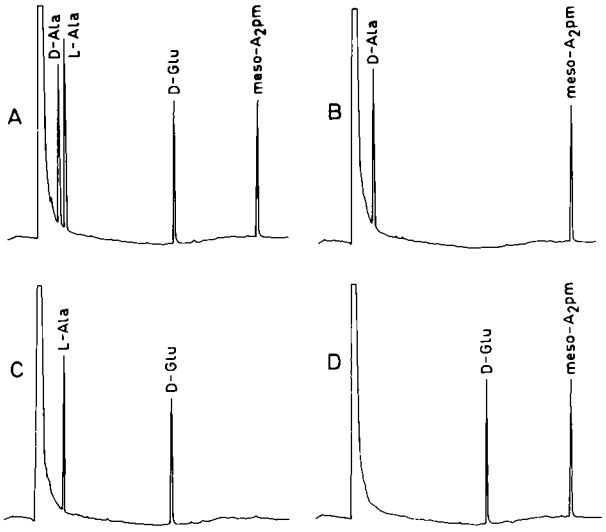

Fig. 1: Separation of amino acid isomers of major peptides by gas-liquid chromatography on a Chirasil-Val glass capillary column as N-Heptafluoro-butyryl-isobutylester derivatives.
(A) L-Ala-D-Glu-meso-A_2pm-D-Ala
(B) meso-A_2pm-D-Ala
(C) L-Ala-D-Glu
(D) D-Glu-meso-A_2pm

3) Degree of cross-linkage. The degree of cross-linkage of the Synechocystis peptidoglycan was investigated by two different methods. One is based on 1-fluoro-2,4-dinitrobenzene (FDNB)-treatment (7) of the peptidoglycan which formed mono-2,4-dinitrophenyl (DNP)-diaminopimelate as the only DNP-derivative. About 44 % of the total meso-A_2pm present in the Synechocystis peptidoglycan was accessible to the FDNB-reaction. This shows that in 56 % of the peptide side chains the ε-amino groups of meso-A_2pm were blocked as a result of cross-linkage.
Since the amount of C-terminal carboxylic end groups is related to the degree of cross-linkage, the cross-linking index was determined by infrared (IR) spectroscopy (6). The relative absorbance of the group frequency band of carboxylic acid located at about 1730 cm^{-1} in the infrared spectrum (Fig. 2) of the acid form of the Synechocystis peptidoglycan was used for the calculation of the degree of cross-linkage. The sugar ring band near 1040 cm^{-1} served as an internal standard (marker for the total amount of subunits present). From the IR-data a cross-linking index of approximately 43 % was calculated. However, this value was not corrected, because the degree of amidation is not yet known.

4) Infrared spectroscopy. The infrared spectrum of the peptidoglycan (acid form) of Synechocystis sp. strain PCC 6714 (fig. 2) showed amide A, B, I and II absorption bands centered around 3291 cm^{-1}, 3080 cm^{-1}, 1657 cm^{-1} and 1534 cm^{-1}, respectively. They are very sensitive to conformational alterations in the peptide backbone. Since only one amide I and one amide II band occurs in the infrared spectrum of the peptidoglycan, it is likely that the two -NH and two -C=O groups of the carbohydrate moiety and the five -NH and five -C=O groups of the peptide moiety contribute to the same conformation-dependent amide I (C=O stretching)

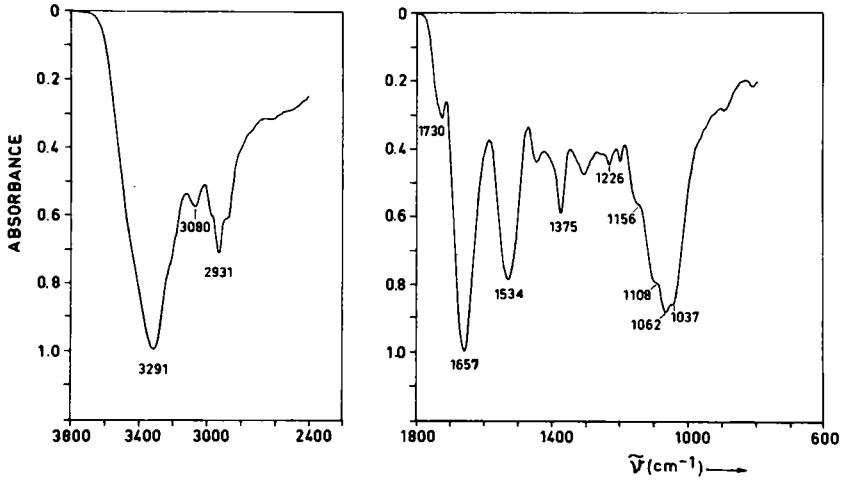

Fig. 2: Infrared spectrum of peptidoglycan recorded on a Fourier-transform/infrared spectrophotometer FT-IR MX-1E.

and amide II (N-H deformation) absorption bands. An absorption band near 1730 cm^{-1} is assigned to hydrogen-bonded >C=O vibrators of carboxylic acid groups (-COOH). The spectral region between 1200 cm^{-1} and 800 cm^{-1} is characterized by several strong absorption bands which are dominated by complex ß(1→4)-linked sugar ring modes. The analysis of band half-widths revealed no crystalline state of order of peptidoglycan.

5) X-Ray diffraction. The exposure of dried powders or dried foils of the Synechocystis rigid layer face-on to X-rays resulted in a Debye-Scherrer-like pattern with Bragg periodicities of about 0.45 nm and 0.94 nm (Fig. 3). Both rings were relatively diffuse. The 0.7 nm-periodicity known for other peptidoglycans, which occured only after cell breakage (6) was not detected in whole cells or in peptidoglycan fractions of Synechocystis. The widths of the interference rings indicate that a portion of the peptidoglycan exhibits a partially ordered although non-crystalline structure. The X-ray diagram of the Synecho-

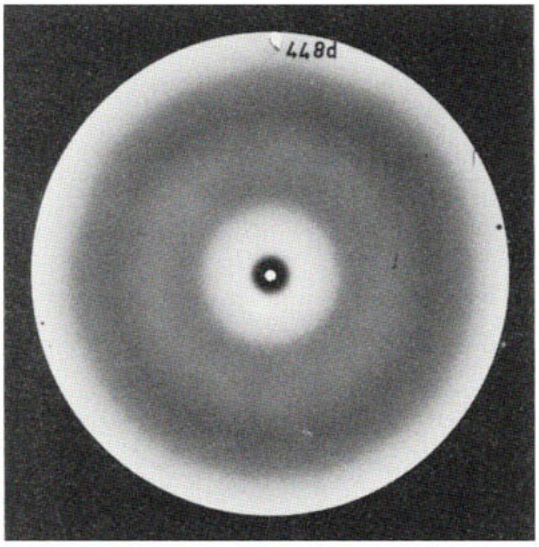

Fig. 3: X-Ray diffraction pattern obtained with Ni-filtered CuK_{α}-radiation in a Kiessig camera from powders or foils of isolated rigid layers from Synechocystis sp. strain PCC 6714.

cystis peptidoglycan foil,when viewed from the side (edge-on), showed no meridional reflection (i.e. oriented vertically to the foil plane) corresponding to a periodicity of 4.2 nm as known for S. aureus peptidoglycan (6).

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Characterization of Muropeptides Released from Murein of *Caulobacter crescentus* by Endo-N-acetylmuramidase

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Introduction

The dimorphic bacterium *Caulobacter crescentus* is an attractive prokaryotic model organism for the study of differentiation since it undergoes an obligatory cell cycle in which cell division results in two morphologically distinct cell types, i.e., a swarmer cell and a stalked cell (1). The shape of a bacterial cell is assumed to be maintained by the murein sacculus which completely encloses it and in several cases changes in cell morphology have been shown to be correlated with changes in the activity of murein hydrolases and in murein structure (2, 3, 4). The murein of *C. crescentus* has been described as a typical gram-negative Alγ type (5, 6) though more recent studies indicate a certain modification of this primary structure being involved in morphogenesis of the stalk (7). In the present study we characterized the soluble products released from the murein of *C. crescentus* CB15 by endo-N-acetylmuramidase from *Chalaropsis*. Our findings indicate that the composition of C. crescentus murein visibly differs in some respects from that of other gram-negative bacteria with a similar type of murein - Alγ.

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Results

1) Identification of pentapeptide subunits. A survey for murein hydrolyzing enzymes in C. crescentus CB15 revealed the presence of several different activities including a transglycosylase, endopeptidase and amidase. However, no detectable DD-carboxypeptidase and LD-carboxypeptidase activities were found (Markiewicz, unpublished observations). To verify the absence of carboxypeptidase activity in C. crescentus, murein sacculi labelled with [^{3}H]diaminopimelic acid, prepared essentially as described (7), were treated with endo-N-acetylmuramidase from Chalaropsis and the soluble products were separated by paper chromatography (8). The distribution of the label on the chromatograms closely resembled that for Escherichia coli. However, assay for vancomycin binding material according to de Pedro and Schwarz (9) revealed very high pentapeptide content in the three fastest migrating spots. This result was confirmed by HPLC analysis of the digestion products, followed by amino acid analysis of the separated murein subunits, which showed that approximately 50% of all monomeric subunits and 57% of all dimeric muropeptides had pentapeptide side chains. The absence of LD-carboxypeptidase activity was verified by the complete lack of disaccharide tripeptide among the murein subunits. These results clearly indicate that the synthesis of cross-linked murein in C. crescentus does not require carboxypeptidase activity. This is in contrast to Gaffkya homari in which the pretreatment of nascent murein with carboxypeptidase is necessary for the occurrence of transpeptidation, or even to E. coli where carboxypeptidase has been suggested to have a regulatory function in determining the synthesis of septum-forming or elongation murein (3). The absence of carboxypeptidase in Caulobacter may be related to the cell cycle of this bacterium and may allow for greater plasticity of the murein needed for the transformation

from one morphological type to the other.

2) Substitution of terminal alanine by glycine. Amino acid analysis of C. crescentus murein from bacteria grown in various media revealed the presence of low but reproducible amounts of glycine which were put down to contamination of the isolated sacculi by protein (5, 7). HPLC analysis of the soluble products of murein digestion demonstrated the presence of glycine in approximately half of the pentapeptide carrying subunits. Treatment of the muropeptides with carboxypeptidase from E. coli and HPLC analysis of the reaction products showed that glycine is in position 5 of the pentapeptide in both the disaccharide and bis-disaccharide subunits (to be published).

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THE ANALYSIS OF MUREIN COMPOSITION WITH HIGH-PRESSURE-LIQUID CHROMATOGRAPHY

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Introduction

The metabolism of murein in E. coli seems to involve a host of synthesizing and hydrolyzing enzymes (1) which necessarily would imply multiple recognition sites. This contrasts with the accepted model of a simple murein chemistry (2). Consequently, we reinvestigated murein composition with a new technique, high-pressure-liquid chromatography (HPLC).

Results and Discussion

Sacculi were prepared from E. coli cells as described previously, including digestion with amylase and pronase (3). The purified murein was degraded with muramidase from Chalaropsis (4). Before fractionation, the muropeptides were reduced with sodium borohydride to prevent anomerisation of the muramic acid residues. The fractionation (details see legend to Fig. 1) yielded about 60 well separated peaks as shown in Fig. 1. The composition of the peaks as indicated (Fig. 1) has been determined by amino acid analysis and by partial digestion with murein-hydrolases from E. coli (5,6). The "classical" murein subunits C3, C5, and C6 represent about 70% of the total murein. Some of the other minor components such as X and X' (3), a disaccharide tetrapeptide trimer (7), a disaccharide pentapeptide (8) and a dimer consisting of C6 and X' (9) had been

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Fig.1 SEPARATION OF E. COLI MUROPEPTIDES BY HPLC (STRAIN KN 126)

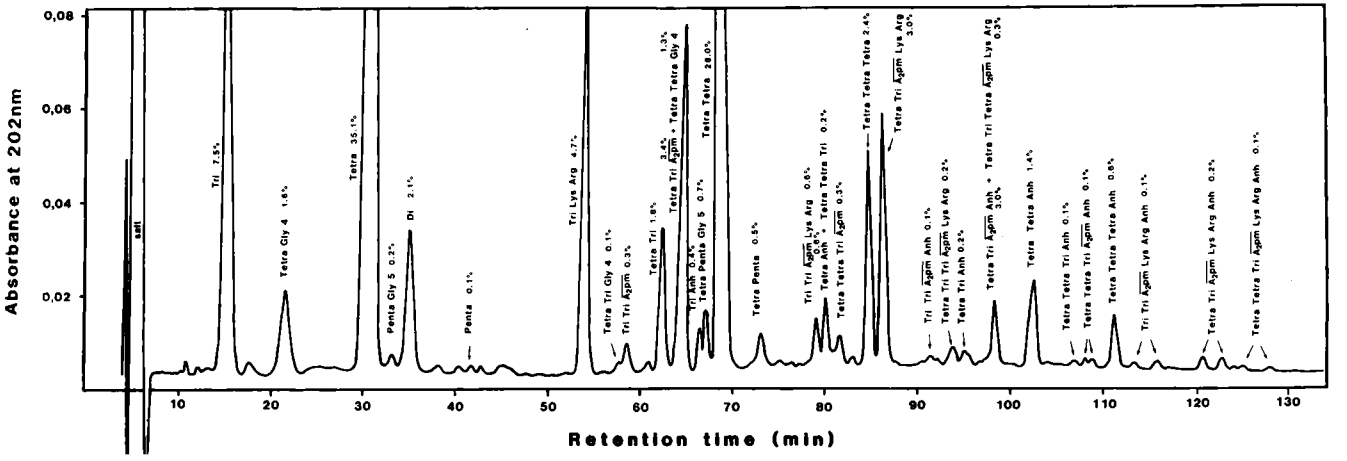

Fig. 1: Sacculi prepared (3) from E. coli KN126 (13) were digested with Chalaropsis muraminidase (4). The muropeptide solution (0.1 ml; 2 mg murein/ml) was reduced with $NaBH_4$ (0.1 ml; 10 mg/ml in 0.5 M borate buffer, pH 9.0; 30' at RT). After adjustment of pH to 4 with H_3PO_4, 50 µl of the solution were fractionated on a reverse phase column (Hibar 250x4, LiChrosorb RP18, 5 µm; Merck, Darmstadt, Germany), equilibrated with 50 mM phosphate buffer, pH 4.69; 0.0005% NaN_3. The eluted compounds (linear gradient of equilibration buffer rising to 15% methanol in the same buffer; 0.5 ml/min; 110 min run time; equipment Waters, Milford, Mass., USA) were detected at 202 nm. The composition of compounds was determined by partial enzymatic hydrolysis and amino acid analysis. Designation of compounds: Di: GlcNAc-MurNAc-L-Ala-D-Glu; Tri: GlCNAc-MurNAc-L-Ala-D-Glu-meso-A_2pm; Tetra: GlcNAc-MurNAc-L-Ala-D-Glu-mesoA_2pm-D-Ala; Penta: GlcNAc-MurNAc-L-Ala-D-Glu-mesoA_2pm-D-Ala-D-Ala; Gly 4(5): glycine in position 4(5) of the peptide chain; Anh: 1-6-anhydro muramic residue; LysArg: Lys-Arg residue at the meso A_2pm from murein-bound lipoprotein; $\overline{A_2pm}$: compound with a mesoA_2pm-mesoA_2pm crossbridge; other oligomers have the normal mesoA_2pm-D-Ala-mesoA_2pm crossbridge. The amounts of the compounds are calculated by integration of the UV-response.

described already, most of the others are novel. Their properties can be summarized as follows: In dimeric and trimeric subunits all possible combinations of different side chains exist. Not only monomeric subunits carry lipoprotein (10), as indicated by residual -lys-arg residues. Dimeric and trimeric subunits as well are linked with lipoprotein, the ratio of lipoprotein on monomers and on dimers is about 2:1. All compounds mentioned also exist in the anhydro-form (3), defining chain ends. That means, that any type of muropeptide can terminate a glycan chain in murein, crosslinked compounds, however, being preferred at both ends (11). On basis of our new data the average chain length is about 35 disaccharide subunits, which is in agreement with previous calculations (9, 11). Finally, a novel type of crossbridge, representing about 1/5 of all crosslinks, was found. Because of its retention time, its amino acid composition and its degradation by E. coli endopeptidase (5) we assume a crossbridge between two diamino pimelic acid residues (meso-2,6 diamino pimelic acid = A_2pm) as it has been found in other organisms (12).

Are the novel compounds accidental or do they fulfill structural and/or regulatory roles in murein assembly and bacterial morphogenesis? Some compounds indeed may be accidental such as subunits with a glycine replacing an alanine residue (Fig. 1). The amount of glycine in murein depends on the medium in which cells grow, which makes an essential function of this subunit quite unlikely. Other compounds, however, play essential roles in murein metabolism. As an example, pulse-labelling experiments *in vivo* strongly indicate that the novel dimer with the A_2pm-A_2pm crosslinkage is the acceptor for the attachment of lipoprotein to newly synthesized murein. Formation of this very dimer precedes the attachment of lipoprotein (Fig. 2). The kinetics of the two processes match each other. In mutants with impaired attachment of lipoprotein, the amount of the A_2pm-A_2pm dimer is clearly reduced and vice versa (data not shown).

Fig. 2 Pulse Labeling of Lipoprotein - Attachment Sites

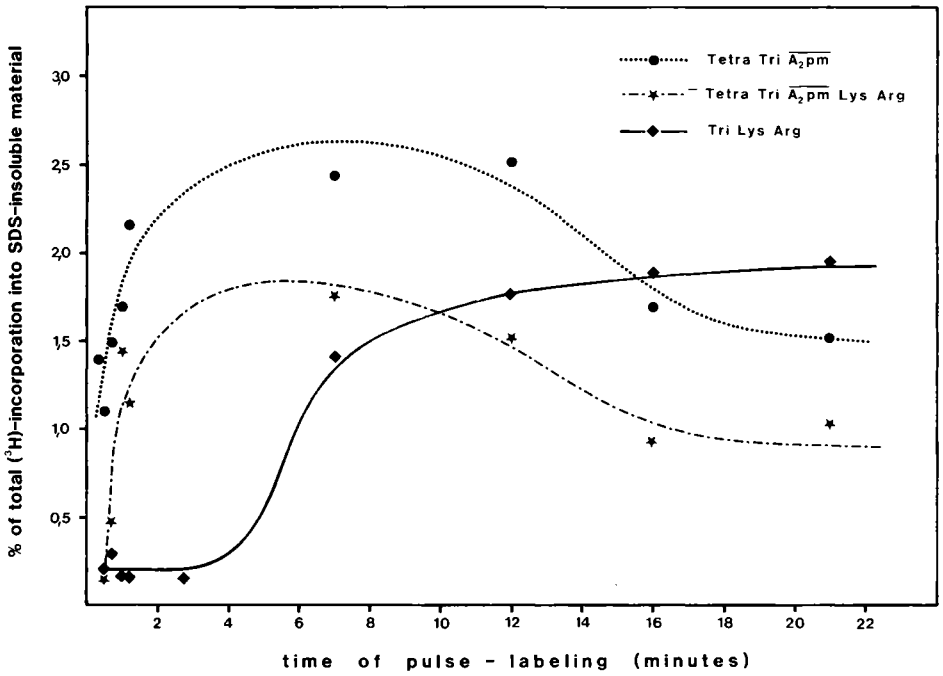

Fig. 2: E. coli W7 (14) was grown at 37°C in supplemented Penassy Medium (Difco, with 20 µg/ml lysine and 2 µg/ml A_2pm); at an OD_{578} of 0.4, 50 ml culture were filtered on a membrane filter (0.45 µM), washed twice with prewarmed, A_2pm-free medium and labelled directly on the filter (up to 60 sec/pulses) with 0.2 ml medium containing ^{3}H-A_2pm (CEA, Saclay, France; 1 mCi/ml; 50 Ci/mM). For pulses longer than 1 min, cells were resuspended in fresh medium (50 ml; 4 µCi/ml of ^{3}H-A_2pm), collected by filtration and boiled in 4% SDS (3); analysis of murein composition as described (Fig. 1). Radioactivity was measured in a liquid scintillation counter.

Pulse-labelling experiments revealed another unexpected feature of newly synthesized murein. After very short pulses (20') three murein subunits are found which are rapidly processed. After 60'-pulses their proportional amount in murein becomes negligible (Fig. 3), in aged murein they are undetectable. The composition of these early murein compounds is still unknown.

Fig. 3 PULSE LABELING OF E. COLI W7 MUREIN

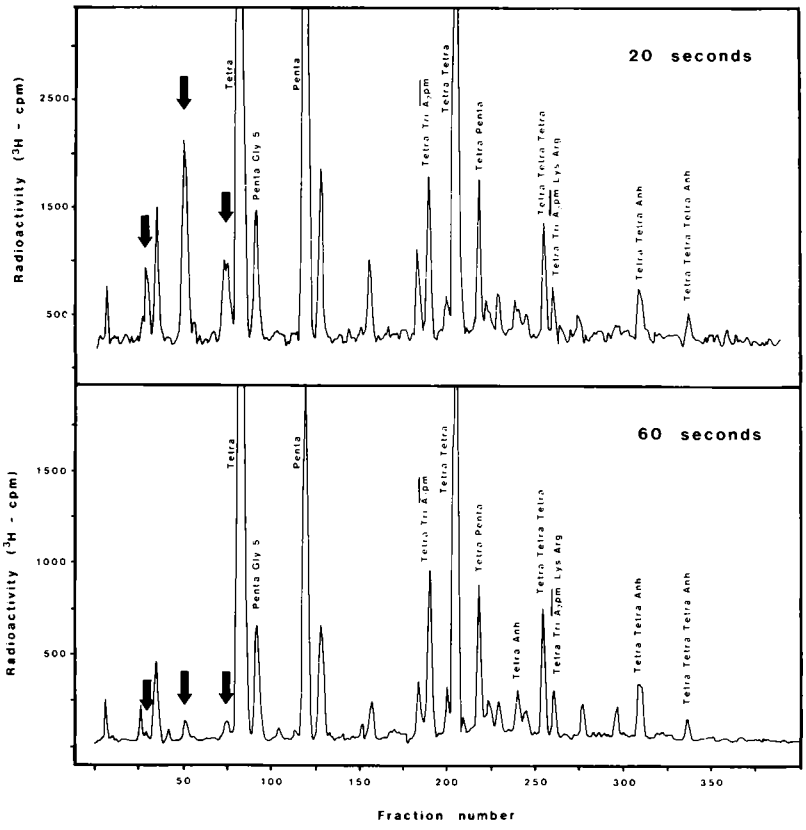

Fig. 3: Murein from E. coli W7 (14) was labelled for 20 and 60 seconds respectively and analyzed by HPLC. Arrows mark "early" compounds with a short life time. For experimental details see legends for Figs. 1 and 2.

Interesting questions arise from the existence of minor murein components. Are they signals recognized by enzymes engaged in murein metabolism? Is part of the regulation of murein biosynthesis and bacterial morphogenesis achieved by substrate modification?

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THE ANIONIC NATURE AND SOME PERMEABILITY CHARACTERISTICS OF MUREIN

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Introduction

With few exceptions all bacteria possess a wall which forms the cell's outer-most boundary and separates its vital constituents from the external milieu. This wall is of fundamental importance since it not only contributes shape and form to the cell, but it must also provide an inanimate boundary through which the cell perceives the surrounding environment and fluctuations therein. Given the stress and strain of the microbial habitat, it is conceivable that this boundary would act as a "microenvironment" which could interact with, buffer, and even modify the stressing influence before it comes in contact with the protoplast. Clearly, the high tensile strength of the wall is able to resist mechanical injury, but recent evidence suggests that it may also have a seiving function (2,13,22-24,26). The wall, to a certain extent, must influence the molecular (22-24) or ionic (2,4,7) form of what gets in and what gets out of cells. But since it takes time to physically alter the chemistry of the framework (i.e., the time for induction → transcription → translation → secretion to occur), a certain degree of accommodation to environmental factors must be built into the pattern. This would help fulfill the economy of design which we expect from all life forms capable of rapid evolution.

It is apparent that the peptidoglycan (PG) which forms the murein sacculus is of fundamental importance to wall integrity; it is the scaffolding upon which the other chemical constituents of the fabric are bonded. There must be mechanisms available to allow expansion (cell elongation) and ingrowth (septation) without effecting apparent imperfections within the strength of its meshwork.

It has been a difficult macromolecule to study. Chemical digests have allowed the identification and molar ratios of its molecular constituents to

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be established (25). Infrared spectroscopy, e^- and X-ray diffraction, and nmr, have presented information concerning the lattice constant and the degree of flexibility within the PG network (1,9,10,15-19). All of these methods have been important for our conceptual view of murein, but we must remember that these are tremendously averaging techniques. Small differences between and within sacculi would not be observed.

The Anionic Nature of Bacterial Walls

Bacterial walls are anionic, and interact with and bind aqueous metallic cations (4-7,14,20). Bacillus walls consist primarily of a PG matrix which is interwoven with linear strands of teichoic and/or teichuronic acids. When B. subtilis 168 walls (54.3% teichoic acid + 44.6% PG) were suspended in 5 mM metal solutions and washed of unbound metal, substantial quantities of metal remained associated with the walls (Table 1). A strict stoichiometric interaction between metallic cation and the available anionic sites within the wall could not account for these amounts (Fig. 1). Presumably, they are the end result of a complex series of chemical reactions initiated by the inaugural binding of cation and are based on the stability of metal in aqueous solution (5-7).

Studies with extracted teichoic polymer indicated that sites were available for metal binding (6), but chemical neutralization of carboxylate groups of PG suggested that this was the primary metal chelating agent (ref.6 and Table 1). Since accurate molar ratios of metal to wall sites was impossible with the salts used in these studies, chloropentaamine osmium III chloride, which is stable in aqueous solution, was used to clarify the issue (5). In this case, the expected stoichiometry between divalent probe and carboxylate groups was observed.

Naively, we felt that these results could be applied to all similar Gram-positive walls. Clearly, this is not the case; our studies on the metal binding capacity of B. licheniformis NCTC 6346 walls (51.7% teichoic acid + 27.4% teichuronic acid + 20.9% PG) presented an entirely different picture (4). These walls did not bind as much metal, nor was PG the principle binding agent (Table 1). In this case, teichuronic and teichoic acids, in concert, developed the major binding capacity of the wall. This points to a fundamental difference between these wall types.

The wall of Escherichia coli K-12 contains a layer of PG which is chemically analogous to our bacillus examples (8). This PG can be physically separated to purity and can therefore be compared. In general, the binding capacity of E. coli PG is lower than that of B. subtilis and B. licheniformis (Table 1).

Tenacity of Metal Binding

The binding of metallic cations to walls must be ionic, but it is surprisingly strong. Some of these ions can be replaced by other ions (20); others are more permanently bound (7,14,21). This binding withstands the test of time since walls can be recognized in ancient sediments by their inherent electron scattering ability with electron microscopy (11,12).

We have recently tested the tenacity of metal binding to B. subtilis walls by following the biogeochemical events of sediment diagenesis when metal-loaded cells are mixed with a synthetic sediment and incubated under conditions which mimic a low-temperature metamorphic horizon (3). Our initial idea was that the metal would be leached from the wall into the inorganic matrix. In actual fact, the metal remained associated with the cell and cellular material leached metal from the surroundings (Fig. 4). The organic matrix initiated and was responsible for highly distinctive minerals during diagenesis (Fig. 5). Since the PG of the bacteria was responsible for most of the metal input into the system, we assume that these results are a reflection of the tenacity of the PG-metal binding.

Some Permeability Aspects of Murein

We are so familiar with the casual growth and manipulation of bacteria that we sometimes forget the complex series of events that are involved. Most certainly bacterial cells utilize nutrients and grow; they can be plasmolyzed under osmotic stress, and they can be physically stained for microscopic observation. Each of these facts implies physical passage of commodities through the wall. We must distinguish between wall types in a discussion concerning permeability aspects since Gram-positives are emphatically different from the Gram-negative variety. The simplest way to make correlation between the two types is to look at the Gram reaction itself.

The Gram reaction produces a staining response which divides the Eubacteria into two fundamental groups; those that stain Gram-positive and retain the

crystal violet/iodide complex (CV/I), and those that are Gram-negative and are decolorized by ethanol treatment. We have determined that the CV/I precipitate is due to the metathetical replacement of the small Cl^- ion of the CV salt with the bulkier I^- of Gram's Iodine solution. We have been able to replace I^- with trichloro (n^2-ethylene) platinum II (TPt^-) to produce CV/TPt which is chemically analogous to the CV/I precipitate. Since it contains Pt we have been able to follow the staining mechanism of B. subtilis and E. coli by electron microscopy.

From these experiments it has been determined that TPt (a 0.82 x 0.62 nm elipsoid) passes through both Gram-positive and -negative walls and enters the cytoplasm (Fig. 6 and 7). Indeed, crystal violet (a 1.88 x 0.65 "Y"-shaped molecule) also passes through both wall fabrics and interacts with intracellular TPt to form CV/TPt. This complex is dissolved by ethanol and is removed from E. coli to leave a colorless cell. Electron microscopy suggests that both outer and plasma membrane were disrupted by the ethanol and that the thin murein sacculus was not a retention barrier for the stain. The implication is that discontinuities ca. 2.0 nm in diameter exist in this murein. B. subtilis walls, on the other hand, did provide a barrier to the stain. This, presumably, is a reflection of greater thickness (i.e., greater seiving character) and a condensation of the PG's lattice constant due to the dehydrating quality of the ethanol.

Concluding Remarks

Successful architecture is a proper blend between structure and function. Quite clearly bacterial walls fulfill this mandate; they are ubiquitous among the vast majority of bacteria and have proven the test of time.

I have presented numerous and diverse data in this report. My intention was to offer some perspectives so that we could appreciate the diversity of function which walls must fulfill. Since murein is a major component of walls, it is fitting that a research emphasis be placed on its elucidation. But, we must not be too shallow in our vision; the complex interactions between PG and secondary polymers in Gram-positive walls, and those between PG and outer membrane in Gram-negative walls are, no doubt, all important.

Certainly we do not know all the answers, and often those we answer simply provide more questions.

If nothing else, this report provides more questions.

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Table 1. Metal Binding by Bacterial Walls

Metal	*B. subtilis*			*B. licheniformis*		*E. coli*[a]
	Native[b]	Neutralized[b] COO^-	No[c] Teichoic Acid	Native[a]	No teichoic[c] or teichuronic Acids	Murein
Na	2.697	0	1.497	0.910	0.080	0.290
K	1.944	0	0.782	0.560	0	0.058
Mg	8.226	0.520	7.683	0.400	0.024	0.035
Ca	0.399	0.380	0.012	0.590	0.096	0.038
Mn	0.801	0.732	0.656	0.662	0.004	0.052
FeIII	3.581	2.260	1.720	0.760	0.172	0.100
Ni	0.107	0.024	0.021	0.520	0	0.019
Cu	2.990	0.993	2.488	0.490	0	n.d.
AuIII	0.363	0.214	0.265	0.031	0.012	n.d.

n.d. = not determined

[a] µmol metal per mg dry weight of walls.

[b] Neutralized by the addition of glycine ethyl ester to carbodiimide activated carboxylate groups.

[c] The dry weight of these walls has been adjusted downwards to reflect the loss of mass due to the extraction. All quantities are expressed in µmol.

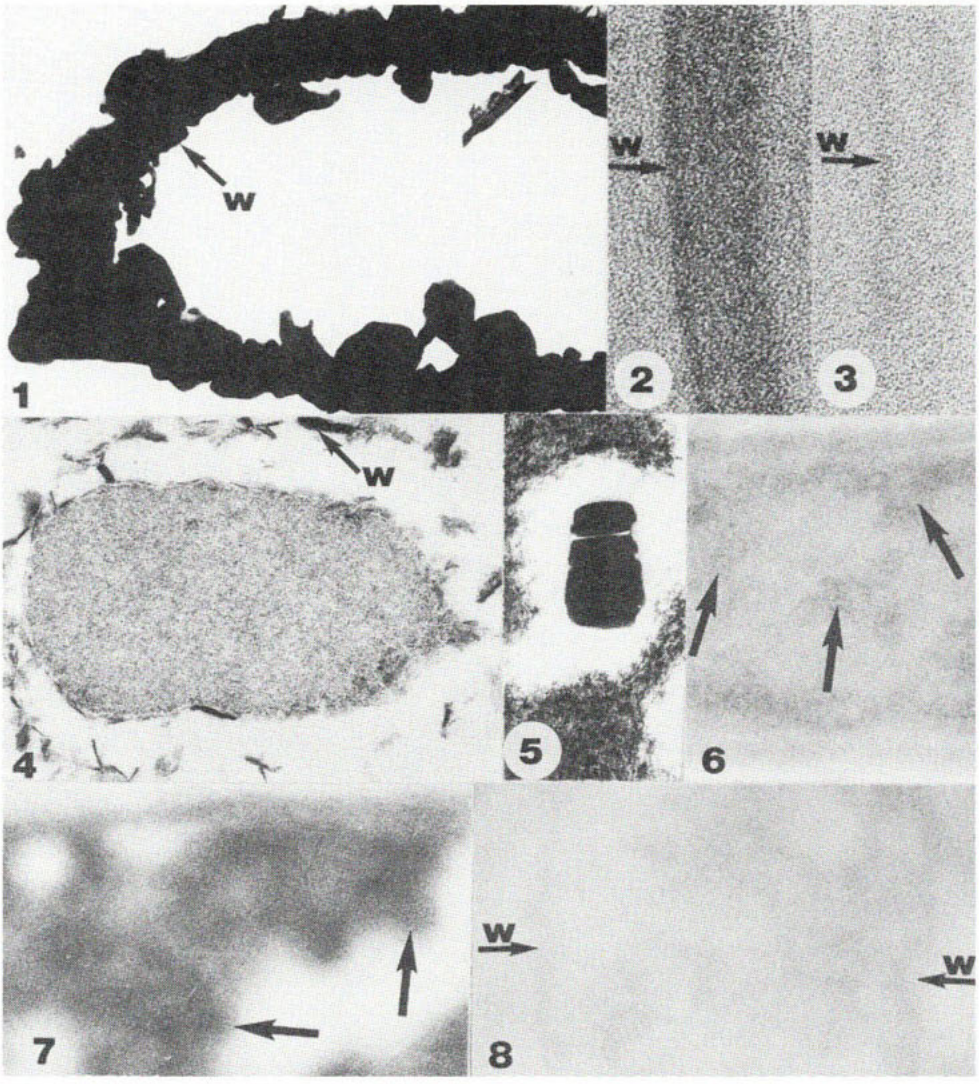

Fig. 1. Unstained B. subtilis wall coated with Au^{o}. 2. Same as 1, but In has been bound. 3. Same as 2, but the COO^{-} groups have been neutralized. Little In has been bound. 4. Diagenic degradation of U-loaded B. subtilis in quartz-magnetite matrix. Platy meta-ankoleite $(K_2(UO_2)(PO_4)2.6H_2O)$ microcrysts are all that remain of the wall. 5. Similar crystal surrounded by the organic matrix. 6. B. subtilis which has been Gram stained using TPt. 7. E. coli, but before ethanol decolorization. 8. E. coli after decolorization. Arrows = CV/TPt precipitate. W = wall.