Review Article

The murein hydrolases of *Escherichia coli*: properties, functions and impact on the course of infections *in vivo*

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Introduction

Most bacteria are stabilized by the polymeric glycopeptide murein (peptidoglycan), a network of glycan strands interlinked by short peptides (see Fig. 1). The murein completely encloses the cell, thereby forming a kind of bag-shaped exoskeleton called a sacculus (Weidel & Pelzer, 1964; Mirelman, 1979; Tipper & Wright, 1979; Rogers *et al.*, 1980). Expansion of the wall during bacterial growth and splitting of the septum for cell separation call for enzymes that cleave covalent bonds within the murein sacculus. Thus, bacterial growth depends on murein hydrolases, a class of enzymes ubiquitous among bacteria (Ghuysen, 1968). Such simple statements, however, belie the complexity and remarkable activity of these enzymes on the bacterial surface. New insight gained over the past decade has led to an emerging picture of a rather dynamic state of the bacterial sacculus in which the murein net is continuously subjected to intense hydrolytic attack, as indicated by insertion of new material, and remodelling and release of aged material. In addition to acting as pacemaker and spacemaker enzymes for cell wall growth (Weidel & Pelzer, 1964; Tomasz, 1983, 1984) some of the murein hydrolases can act as autolysins, thereby representing potential suicide enzymes.

This life and death dichotomy of function demands efficient and strict regulation of murein hydrolase activity, a highly sophisticated physiological task. The enzymes, which must be controlled at their extracytoplasmic location, are required to act coordinately to govern orderly growth and division of the cell with its specific shape preserved. Antibiotics such as penicillin induce bacteriolysis by interfering with the control of the endogenous autolytic enzymes (Tomasz & Höltje, 1977; Tomasz, 1979), indicating the major chemotherapeutic relevance of this class of enzymes. Fine tuning of

Fig. 1. Structure of the murein of *E. coli*. N-Acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) are interlinked in an alternating sequence by β-1,4-glycosidic bonds forming polysaccharide chains which are crosslinked by peptides consisting of L- and D-alanine, D-glutamic acid and m-diaminopimelic acid (m-A₂pm) as indicated. Murein hydrolases cleave specific bonds: ↓, lytic transglycosylase; ◊, β-N-acetylglucosaminidase; ↑, N-acetylmuramyl-L-alanine amidase; ▴, D,D-endopeptidase; Y, D,D-carboxypeptidase; ↓, L,D-carboxypeptidase.

individual enzyme activities is also required to modulate the chemical composition of the sacculus in response to different growth conditions or during aging of the newly inserted components (de Pedro & Schwarz, 1981; Pisabarro *et al.*, 1985; Glauner & Höltje, 1990). It is indeed justified to call these enzymes 'smart enzymes' (Koch, 1990).

This review will present the current knowledge of the biochemistry, function and regulation of the murein hydrolases of *Escherichia coli* and present hypotheses for the likely roles of these enzymes in cellular physiology and the as yet emerging evidence for their importance as determinants of the course of infectious diseases.

The system of murein hydrolases

In *E. coli* nine different enzymes have been described that specifically cleave covalent bonds in high molecular mass murein or in the murein subunits, in the mucopeptides (Høltje & Schwarz, 1985). Their site of action is indicated in Fig. 1 and their properties are summarized in Table 1. Those enzymes which break down the network structure of high molecular mass murein cause bacteriolysis. *Sensu stricto* only two muramidases (Slt and Mlt) and two endopeptidases (PBP 4 and MepA) may be considered autolytic in *E. coli*.

Glucosidases

Although there was a very early claim that *E. coli* contains lysozyme (Pelzer, 1963*a, b*), it was found later that only muramidases that form 1,6-anhydromuramic acid are present (see below). No other muramidases have been proven to exist in *E. coli*.

Lytic transglycosylases. A lysozyme-like enzyme cleaving the β -1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine is present in *E. coli* (Høltje *et al.*, 1975). Interestingly, this enzyme catalyses quite a sophisticated reaction (see Fig. 2): cleavage of the β -1,4-glycosidic bond is followed by an intramolecular transglycosylation reaction transferring the *O*-muramyl residue onto its own C-6 hydroxyl group. Thereby, a 1,6-anhydromuramic acid is formed. The enzyme has been named intramolecular transglycosylase. To avoid confusion with the biosynthetic transglycosylases which polymerize the murein precursors to yield murein (van Heijenoort *et al.*, 1978) it is advisable to refer to the muramidase in *E. coli* as the *lytic* transglycosylase. Almost at the same time as the *E. coli* enzyme was identified, an enzyme catalysing an identical lytic transglycosylase reaction was shown to be the phage λ gene R product (Taylor *et al.*, 1975; Bienkowska-Szewczyk *et al.*, 1981). Later, the existence of a 1,6-anhydromuramic-acid-producing muramidase in *Neisseria gonorrhoeae* was reported (Sinha & Rosenthal, 1980).

Although highly interesting as a reaction mechanism, the physiological significance of the intramolecular transferase reaction has not yet been explained. Since part of the chemical energy of the β -1,4-glycosidic bond that is cleaved is conserved in the newly synthesized 1,6-anhydro bond, it has been proposed that this energy may be used to catalyse rearrangements (repairs) in the murein sacculus. Experimental proof for this reasonable hypothesis is, however, lacking.

Two distinct lytic transglycosylases have been purified from different fractions of the cell: a soluble enzyme from the cytoplasm (Høltje *et al.*, 1975; Kusser &

Fig. 2. Reaction catalysed by the lytic transglycosylases.

Schwarz, 1980) and a membrane-bound enzyme from the envelope (Mett *et al.*, 1980). The two enzymes are encoded by different genes and show no immunological cross-reactivity (Keck *et al.*, 1985). The soluble lytic transglycosylase has a molecular weight of 65 kDa, a pH optimum of 4.5 and a pI of 5.4. The gene (*slt*) has been cloned and mapped at 97 min (Betzner & Keck, 1989). Mutants with deletions in this area of the chromosome have existed for quite some time (Roeder & Somerville, 1979), and testing for Slt by Western blot analysis, showed, as expected, a total deficiency of the enzyme (A. Betzner, unpublished results). Morphology and growth rate of the mutants correspond with those of the parent strain. Thus, the *slt* deletion mutant reveals no obvious phenotype. Recently, deletions in the *slt* gene have been constructed via marker exchange by sequential recombination events (M. Templin, unpublished results). The enzyme, which can be overexpressed 30-fold without

Table 1. *Properties of murein hydrolases of E. coli*

Enzyme	Gene	Locus	Size (kDa)	pH optimum	Substrate	Reference
<i>Glucosidases</i>						
Lytic transglycosylases						
Soluble	<i>slt</i>	99.7	65	4.5	Murein	Höltje <i>et al.</i> (1975) Betzner & Keck (1989)
Membrane bound	<i>mlt</i>	?	35	5.0	Murein	Mett <i>et al.</i> (1980)
β -N-Acetylglucosaminidase		25-27	36	7.7	Muropeptides	Yem & Wu (1976a)
<i>Peptidases</i>						
N-Acetylmuramyl-L-alanine amidase	<i>amiA</i>	51	39	8.3	Muropeptides	Tomioka <i>et al.</i> (1983)
D,D-Endopeptidases						
Membrane bound (PBP 4)						
(D,D-carboxypeptidase 1B)	<i>dacB</i>	68	49	8.0	Murein	Matsuhashi <i>et al.</i> (1977) Korat & Keck (1988)
Soluble						
(penicillin-insensitive)	<i>mepA</i>	50.4	30	6.0	Murein	Keck & Schwarz (1979) Tomioka & Matsuhashi (1978)
D,D-Carboxypeptidase 1A (PBP 5)	<i>dacA</i>	13.7	42	8.5	Muropeptides	Amanuma & Strominger (1980)
D,D-Carboxypeptidase 1A (PBP 6)	<i>dacC</i>	19	40	8.5	Muropeptides	Amanuma & Strominger (1980); D. Edwards, pers. comm.
L,D-Carboxypeptidase	?	?	86	8.0	Muropeptides	Beck & Park (1977)

harming the overproducing cell (Betzner & Keck, 1989), has been crystallized (Rozeboom *et al.*, 1990).

The membrane-bound lytic transglycosylase (Mlt) has a molecular mass of 35 kDa, a pH optimum of 5.0 and a pI of 6.9. The enzyme is effectively released from the membrane by 1% Triton X-100 and 1 M-KCl in 10 mM-Tris/maleate buffer, pH 6.5 (Mett *et al.*, 1980). Its activity depends totally on the presence of a detergent such as Triton X-100.

β -N-Acetylglucosaminidase. A β -N-acetylglucosaminidase is also present in *E. coli* (Maass *et al.*, 1964). It has been purified and has a molecular mass of 36 kDa (Yem & Wu, 1976a). The pH optimum is about 7.7 and the K_m for the substrate *p*-nitrophenyl- β -D-2-acetamido-2-deoxyglycopyranoside is 0.43 mM. It does not accept high molecular mass murein (i.e. isolated sacculi) as a substrate but hydrolyses soluble murein fragments such as muramidase reaction products. This enzyme, therefore, is not an autolytic enzyme as such but may be involved in muropeptide recycling (see below). Mutants in this enzyme still showing residual activity grow and divide properly (Yem & Wu, 1976b; Hrebenda, 1979).

Peptidases

The appearance of a high percentage of *m*-diaminopimelic acid (A_2pm)-D-Ala dipeptides in the periplasm (Goodell & Schwarz, 1985) as a result of murein turnover (see below) indicates that a peptidase cleaving the D-Glu-*m*- A_2pm peptide bond exists. No further characterization or purification of this hypothetical enzyme activity has been reported.

N-Acetylmuramyl-L-alanine amidase. The amide bond between the carboxyl group of the lactyl moiety of muramic acid and the α -amino group of the first amino acid of the stem peptide, L-alanine, is cleaved by an amidase. *N*-Acetylmuramyl-L-alanine amidases are found widely among bacteria and are also present in mammalian sera (Valinger *et al.*, 1982). The enzyme isolated from *E. coli* (van Heijenoort & van Heijenoort, 1971; van Heijenoort *et al.*, 1975) does not accept whole sacculi as a substrate but acts on muropeptides, a finding similar to that for the β -N-acetylglucosaminidase. The enzyme has a molecular mass of 39 kDa and a pH optimum of about 8.3. With *N*-acetylmuramyltripeptide as a substrate, a K_m of 5×10^{-4} M was estimated. The amidase is sensitive to Zn^{2+} ions which, at a concentration of 10 mM, cause 75% inhibition of enzyme activity. Muramic acid, *N*-acetylmuramic acid and *N*-acetylglucosamine exert marked inhibition at concentrations around 10 mM. The gene (*amiA*) encoding the enzyme has been mapped at 51 min on the chromosome (Tomioka *et al.*, 1983).

D,D-Endopeptidases. The crucial bond for the mechanical stability of the murein net is the D,D-peptide bond formed by crosslinking the carboxyl group of the D-alanine residue of one peptide side chain to the ϵ -amino group of the D-centre of the diaminopimelic acid residue of a neighbouring muramyl-peptide. The formation of this crossbridge is the most important target of β -lactam antibiotics, which inhibit the D,D-transpeptidases (Izaki *et al.*, 1968; Blumberg & Strominger, 1974; Frere & Joris, 1985). Enzymes capable of catalysing the reverse reaction are called endopeptidases and have been found in many bacterial species. In *E. coli*, two unrelated endopeptidases are present, both catalysing the same reaction, splitting the D,D-Ala- A_2pm crossbridges in the murein sacculus.

One of the endopeptidases is penicillin-sensitive (Hartmann *et al.*, 1972) and is identical with penicillin-binding protein (PBP) 4 (Iwaya & Strominger, 1977). The enzyme has a molecular mass of 49 kDa and a pH optimum of 8.0. It is, however, a membrane-bound protein and must be solubilized in the presence of a detergent (e.g. Triton X-100) for purification. The enzymic activity of the solubilized, purified protein may not reflect the enzymic specificity of the protein in its native environment, in the membrane. As a matter of fact, temperature-sensitive mutants in PBP 4 do not show the expected increase in the degree of crosslinkage at the restrictive temperature (de Pedro *et al.*, 1980). This was taken as evidence for transpeptidase activity of the enzyme *in situ*. However, overproduction of PBP 4, the product of the *dacB* gene cloned on a low-copy-number plasmid, results in a decrease in crosslinkage and the relative amount of pentapeptide moieties of the murein (Korat & Keck, 1988). The enzyme, therefore, seems to function *in vivo* as an endopeptidase and a carboxypeptidase.

A penicillin-insensitive D,D-endopeptidase has been described independently by workers in two laboratories (Tomioka & Matsushashi, 1978; Keck & Schwarz, 1979). The specificity of the enzyme was deduced from its ability to cleave the dimeric muropeptide bis(disaccharidetetrapeptide) into the monomeric disaccharidetetrapeptides. The purified enzyme has a molecular mass of 30 kDa and a pH optimum of 6.0 in 10 mM-Tris/maleate buffer. The pI is 6.8. The enzyme is inhibited by single-stranded DNA and synthetic polynucleotides. The gene (*mepA*) mapping at 50.4 min has been cloned by Keck *et al.* (1990) and a 5–6-fold overproduction of the enzyme appeared to cause no alterations of growth. A mutant deficient in *mepA* unfortunately showed residual endopeptidase activity of about 10–20% (Iida *et al.*, 1983).

Because of the recent finding of a L,D crosslinking peptide bond in the case of *m*- A_2pm -*m*- A_2pm crossbridges which are present in minor amounts in the

murein of *E. coli* (Glauner *et al.*, 1988) it is possible that a specific L,D-endopeptidase exists.

D,D-Carboxypeptidases. The carboxypeptidases that cleave the D-Ala-D-Ala peptide bond of the muramyl-peptide side-chain are penicillin-binding proteins. The enzymic reaction catalysed by these enzymes corresponds to the first step of the two-step reaction performed by transpeptidases (Ghuysen, 1977; Yocum *et al.*, 1980). Whereas transpeptidases transfer the murein moiety from an enzyme-substrate intermediate onto the free ϵ -amino group of the dibasic amino acid (which in *E. coli* is diaminopimelic acid) forming a crossbridge between two peptide side chains, the carboxypeptidase-substrate intermediate reacts with water releasing the substrate hydrolytically shortened by one D-alanine. In *E. coli* two different D,D-carboxypeptidases have been found, which are identical with the PBPs 5 and 6 (Spratt & Strominger, 1976). As discussed above, another enzyme, PBP 4, which seems to function mainly as an endopeptidase, shows carboxypeptidase activity as well; it is referred to as carboxypeptidase IB. PBPs 5 (*dacA*) and 6 (*dacC*) have been named carboxypeptidase IA (Tamura *et al.*, 1976). Deletion mutants in each of the two carboxypeptidases as well as a double mutant have been constructed, which show no obvious defects in growth and morphology (Matsushashi *et al.*, 1978; Broome-Smith, 1985). However, overproduction of PBP 5 causes the formation of osmotically stable spherical cells (Markiewicz *et al.*, 1982).

The best characterized D,D-carboxypeptidase is the PBP 5 which has a molecular mass of 42 kDa. The pure enzyme is inhibited strongly by extremely low concentrations of penicillin; a K_i value of 10^{-8} M was determined for penicillin G. The K_m for the substrate UDP-MurNAc-pentapeptide was 0.4×10^{-3} M at 30 °C (Amanuma & Strominger, 1980).

PBP 6 is not well characterized except for its molecular mass of 40 kDa (Spratt & Strominger, 1976). Interestingly, the level of this enzyme in stationary phase cells is 2–10-fold higher than in exponentially growing cells (Buchanan & Sowell, 1982).

L,D-Carboxypeptidases. An L,D-carboxypeptidase that cleaves the L,D-peptide bond between *m*-A₂pm and D-Ala is also known in *E. coli*. The enzyme has not been purified to homogeneity, but a molecular mass of 86 kDa has been ascribed to the protein which migrates on SDS-PAGE as a monomer of 43 kDa (Beck & Park, 1977). (Preliminary experiments by A. Ursinus, H. Steinhaus & J.-V. Höltje, however, seem to indicate a molecular mass of 32 kDa on SDS-PAGE.) The pH optimum is 8.0. The enzyme is easily released from cells by Tris/EDTA treatment suggesting a periplasmic localization. In contrast to the D,D-carboxypeptidases, this enzyme is not

inhibited by penicillin G. It is, however, sensitive to nocardicin A (Criegee & Hammes, 1983). Mutants in this enzyme are not available yet.

Subcellular localization

The site of action of murein hydrolases is placed beyond the permeability barrier of the cell at the murein sacculus. Since, in *E. coli*, the sacculus is sandwiched in between the cytoplasmic and outer membrane, murein metabolism takes place in a defined cellular compartment, the periplasm. In those cases for which the amino acid sequences of the enzymes are known (PBPs 5 and 6, MepA, Slt), appropriate leader sequences for protein export have been demonstrated (Pratt *et al.*, 1986; Broome-Smith *et al.*, 1988; Keck *et al.*, 1990). PBPs 5 and 6 are anchored to the membrane through short sequences at the carboxyl end (Pratt *et al.*, 1986; Jackson & Pratt, 1987). A genetically engineered water-soluble derivative of PBP 5 has been shown to be fully active (Ferreira *et al.*, 1988). It has been proposed that the membrane-bound lytic transglycosylase (Mlt) is associated with both the inner and outer membrane but is active only when residing in the outer membrane (Höltje & Keck, 1988).

Some details of the distribution of the soluble lytic transglycosylase have been obtained recently (B. Walderich & J.-V. Höltje, unpublished data). Immuno-gold labelling showed the enzyme to be bound to the murein itself. Interestingly, transformants overproducing the lytic transglycosylase from a plasmid coding for the *slt* gene accumulate excess enzyme in the cytoplasm. This suggests that the amount of enzyme bound to murein is regulated by controlled transport through the membrane. It also explains the finding that an almost 30-fold overproduction of this autolytic enzyme does not harm the cells (Betzner & Keck, 1989). The binding of the enzyme to the murein sacculus is extremely tight since even after boiling in 4% SDS the enzyme is still bound to sacculi as shown by immuno-labelling. This seems to be the reason why this otherwise soluble enzyme is not released by the osmotic shock procedure (Höltje *et al.*, 1975) that specifically discharges other periplasmic proteins.

The soluble murein hydrolases involved in murein turnover, including the amidase and the penicillin-insensitive endopeptidase, reside in the periplasmic space. The endopeptidase is released by osmotic shock (Iida *et al.*, 1983). The amidase, however, could not be detected in the shock fluid (Parquet *et al.*, 1983) indicating that the amidase may be bound to a cell wall substructure, possibly trapped in the outer-membrane-murein complex. The L,D-carboxypeptidase can be released by osmotic shock or by Tris/EDTA treatment of the cells (Beck & Park, 1976). As mentioned above, a still

hypothetical D-glutamyl-*m*-diaminopimelic acid peptidase seems also to be present in the periplasmic space.

Regulation of murein hydrolase activity

It is important to note that, to a great extent, murein metabolism involves enzymic reactions between immobilized enzymes and the insoluble high molecular mass murein sacculus. Consequently, control of cell shape may be achieved by a defined distribution in space of active enzymes with the existing wall serving as a matrix. It would not be surprising if the regulation of the membrane-bound enzymes differed significantly from that of the soluble periplasmic enzymes.

Regulation of murein hydrolases must allow for continuous activity of enzymes needed for cell wall maturation, turnover and controlled hydrolysis sufficient to support cell growth and separation of daughter cells. On the other hand it must prevent hydrolysis of stress-bearing murein so as to avoid autolysis. Regulation of autolysin activity is believed to occur most commonly at the post-translational level. This is suggested by the finding that the activities of most hydrolases (except for the membrane-bound endopeptidase) increase exponentially in synchronized cultures (Hakenbeck & Messer, 1977). Since triggering of wall hydrolysis does not require the synthesis of new enzyme (Kitano & Tomasz, 1979), murein hydrolases may well be continuously present on the cell surface. Thus, it would appear reasonable to suppose that although murein hydrolases are in position to act, their activities are carefully constrained. Besides activation/inhibition by cofactors (see below) possible post-translational regulatory mechanisms include: specific activation of the enzyme by substrate modification (Goodell & Tomasz, 1980; Tuomanen *et al.*, 1988*a*), topological restriction of enzyme distribution in the cell wall in general (Hartmann *et al.*, 1974; Goodell & Schwarz, 1977), or even specific localization in either the inner or outer membrane (Hakenbeck *et al.*, 1974; Høltje & Keck, 1988) or on the murein sacculus itself (B. Walderich & J.-V. Høltje, unpublished results); and control at the site of export (B. Walderich & J.-V. Høltje, unpublished data). Whatever the mechanism of export of the autolytic enzymes, it does not appear to be highly sensitive to disruption of the proton-motive force since uncoupling agents do not interfere with autolysis unless used at high concentrations (Leduc *et al.*, 1982).

For membrane-bound hydrolases such as the penicillin-sensitive endopeptidase (PBP 4), it has been suggested that only the newly synthesized subset of PBPs initiates autolysis of *E. coli*, while the 'biosynthetically old' enzymes are functionally senescent (Tuomanen, 1986*a*). Multiple growth zones occur in *E. coli* (Burman

et al., 1983; Woldringh *et al.*, 1987) and it appears that, at least for membrane-bound hydrolases, location within a growth zone promotes enzyme activation. It has been suggested that membrane-bound hydrolases are neutralized at least in part by being kept out of reach of the target cell wall component except in the growth zone (Tuomanen & Tomasz, 1990). The barrier which seems to exist between enzyme and substrate can be abolished *in vitro* simply by incubating the cells at 0 °C in the presence of a wide range of agents including trichloroacetic acid, sucrose, EDTA and NaCl (see below; Hartmann *et al.*, 1974).

In contrast, such topographical restrictions are not likely to affect the activities of the periplasmic hydrolases such as the soluble endopeptidase and amidase. With new evidence suggesting a multi-layered arrangement for murein even in Gram-negative species (Schwarz & Glauner, 1988; Prats & de Pedro, 1989; Høltje & Glauner, 1990), it is feasible that the soluble murein hydrolases adsorb exclusively to the outermost surface layers of the murein matrix, leaving the stress-bearing layers of the wall (which lie in the middle of the matrix) relatively protected. Thus, the constitutive production of soluble hydrolases may require only simple on-site regulation to prevent dangerous bond breakage in the wall. This is consistent with four findings. First, the periplasmic endopeptidase gene *mepA* possesses only a weak promoter with no obvious consensus DNA binding site for known regulatory proteins (Keck *et al.*, 1990). Second, overproduction of the soluble lytic transglycosylase does not change significantly the periplasmic concentration of this enzyme (B. Walderich & J.-V. Høltje unpublished results) and lysis does not occur (Betzner & Keck, 1989). Third, soluble murein hydrolases are functionally prominent in the processes of cell wall maturation (de Pedro & Schwarz, 1981; Burman & Park, 1983; Glauner & Høltje, 1990; Høltje & Glauner, 1990), murein turnover and muropeptide recycling (Goodell & Higgins, 1987; Goodell & Schwarz, 1983, 1985). Fourth, a variety of inhibitors and cofactors exist that may modulate the activity of these enzymes.

Vanderwinkel and co-workers (Vanderwinkel *et al.*, 1981, 1987; Vanderwinkel & de Vlieghere, 1985) have demonstrated that phospholipids are multisite, nonallosteric inhibitors of the *E. coli* *N*-acetylmuramyl-L-alanine amidase and that their effect is reversible by cations and polyamines. Coenzyme A-glutathione disulphide inhibits purified endopeptidase and transglycosylase from *E. coli* and mutants deficient in this compound readily autolyse (Høltje & Ulla Schwarz, 1983). Other molecules capable of affecting autolysis include exogenous magnesium and DNA which inhibit autolysis (McQuillen, 1958; Keck & Schwarz, 1979; Kusser & Schwarz, 1980; Leduc *et al.*, 1982; Tomioka & Matsushashi, 1978) and

Dowex or Versene (EDTA) which induce autolysis (Repaske, 1958).

The most closely regulated murein hydrolase appears to be the membrane-bound endopeptidase (PBP 4). Its activity peaks just before cell division (Hakenbeck & Messer, 1977), coincident with the onset of autolysis in synchronously growing cells (Hoffmann *et al.*, 1972; del Portillo *et al.*, 1989). This is consistent with the finding that the rate-limiting enzyme in autolysis is also likely to be the membrane-bound endopeptidase (PBP 4) (Tuomanen & Cozens, 1987). The apparent cell cycle dependence of both the activity of the membrane-bound endopeptidase and the onset of autolysis, and the relative lack of autolysis of *hipA* mutants which are believed to spend a longer period of time in nonlytic parts of the cell cycle (Moyed & Bertrand, 1983), may indicate a linkage between the replication of DNA and the activity of hydrolases necessary to achieve cell division (Hoffmann *et al.*, 1972; del Portillo *et al.*, 1987). Although the subject of much controversy, recent strong evidence suggests that DNA replication and cell division are not linked, but rather are driven by two parallel and independent clocks which may both be initiated by the same signal, such as a single calcium flux (Bernander & Nordström, 1990; Norris *et al.*, 1988).

Aside from activation of hydrolases during specific events in the cell cycle, other global regulators also affect murein hydrolase activity but these effects are not apparent during normal growth. Rather, these effects are manifested only during stress-induced deregulation of hydrolase activity. The stringent response, possibly also the heat shock response and the phenotypic modulation to avirulence are all examples of modulation of hydrolase activity in response to environmental signals and are discussed in the section headed 'Autolysis by endogenous murein hydrolases'.

Proposed physiological functions

The normal growth of β -lactam-tolerant mutants, which apparently lack certain murein hydrolases completely (Sanchez-Puelles *et al.*, 1986), indicates that at least some hydrolases are dispensable for growth under laboratory conditions (Tomasz, 1983). As indicated by the survival of deletion mutants, Slt belongs to this class of enzymes (Roeder & Somerville, 1979; M. Templin & J.-V. Höltje, unpublished results). The question then arises as to what the biological relevance of these dispensable murein hydrolases is. Since spontaneous cellular lysis occurs in some Gram-positive bacteria when the cultures enter the stationary phase of growth (Kaufman & Bauer, 1958), it may be that (partial) culture lysis provides the nutrients for growth, and hence extended viability, of those cells

that survive. In addition, the opportunity for genetic transformation of the survivors by the DNA released during cellular lysis might confer some advantage.

More importantly, other murein hydrolases seem to be involved in such diverse processes as cell elongation and division (Schwarz *et al.*, 1969), murein turnover (Goodell & Schwarz, 1985), muropeptide recycling (Goodell 1985), autolysis (Kitano *et al.*, 1986; Betzner *et al.*, 1990), spore formation, transformation and flagella extrusion (see Rogers *et al.*, 1980). Some of these functions must clearly be vital for the cell. Experimental proof for the participation of murein hydrolases in most of these processes is still far from convincing. Only in a few cases have suitable deletion or temperature-sensitive mutants in these enzymes been isolated. Even then, the physiological function of the enzymes could not be determined. *E. coli* therefore appears to be supplied with a set of enzymes that mutually can replace each other so that isolation of double or triple mutants will be necessary to reveal their functions. The cloning of an increasing number of these enzymes will greatly facilitate the future isolation (construction) of such multiple mutants.

It seems obvious from the molecular design of the murein network that enzymes capable of cleaving covalent bonds in the murein are absolutely necessary to allow enlargement of the sacculus by the insertion of additional murein subunits. Such a spacemaker function for bacterial growth (Weidel & Pelzer, 1964; Tomasz, 1983) has yet to be shown experimentally. However, in all models currently discussed, the mechanism of wall expansion depends crucially on the action of murein hydrolases (Burman & Park, 1984, Höltje & Glauner, 1990; Koch, 1990).

The expected function of hydrolytic enzymes in cell division, that is specifically in the splitting of the septum has been shown most convincingly in *Streptococcus pneumoniae* (Tomasz, 1968; Tomasz & Waks, 1975). Electron micrographs of *E. coli* treated with low concentrations of penicillin G suggest the localized action of murein hydrolases in the division site (see Fig. 3) (Schwarz *et al.*, 1969). It was found that certain chain-forming mutants of *E. coli* (*envA*) show reduced amidase activity. Electron microscopy revealed that in these mutants the septum was only partially split (Wolf-Watz & Normark, 1976). However, mutants in the amidase (*ami*) grow and septate properly (Tomioka *et al.*, 1983; Klencke, 1980). Unfortunately, these mutants show a residual activity of about 15–30% which prohibits any clear conclusion about the function of the amidase in the growth of *E. coli*.

Recently, mutants (deletion or insertion mutations) in the gene for the soluble lytic transglycosylase were found to grow and divide normally, at least under laboratory

Fig. 3. Localized action of murein hydrolases after penicillin treatment of *E. coli*. Sacculi were isolated from *E. coli* grown for 90 min in the presence of penicillin G (100 units ml⁻¹). For details see Schwarz *et al.* (1969). (Electron micrograph by H. Frank.)

conditions (Roeder & Somerville, 1979; A. Betzner, unpublished results; M. Templin, unpublished results). Any involvement of lytic transglycosylases in murein enlargement and/or cell division will be demonstrable only when double mutants in the soluble (Slt) and membrane-bound (Mlt) enzyme are available. Since a complete deletion of lytic transglycosylase activity is likely to be lethal for the cell, isolation of a temperature-sensitive mutation in the *mlt* gene should be looked for in the *slt* deletion mutant.

Some of the murein hydrolases are involved in murein turnover and recycling. Similar to findings in Gram-positive bacteria, during growth of *E. coli* about 50% of the murein is released continuously from the sacculus each generation (Goodell & Schwarz, 1985). Analysis of the turnover products indicates a multistep process: first, disaccharidetetra- and tripeptides are cleaved off. Although not shown directly, it seems likely that the lytic transglycosylases which function as exo-enzymes (Beachey *et al.*, 1981) degrade the glycan strands by releasing anhydrodisaccharidepeptides. These products are then subject to further degradation by other murein hydrolases. By amidase action the peptidyl moieties are removed. Possibly the disaccharide is further cleaved by β -*N*-acetylglucosaminidase. The released tripeptides are reused for murein synthesis after being actively transported back into the cytoplasm via the oligopeptide

permeases (Opp) (Goodell, 1985; Goodell & Higgins, 1987).

Autolysis by endogenous murein hydrolases

Autolysis is the result of deregulation of endogenous murein hydrolases which can sever the stress-bearing layers of the murein sacculus. Murein from *E. coli* undergoing antibiotic-induced autolysis bears structural evidence of the two hydrolytic activities which represent the autolytic system of the cell: a degradative (lytic) transglycosylase and an endopeptidase (Kitano *et al.*, 1986). The major role played by these enzymes in autolysis is further indicated by the autolysis-resistance of endopeptidase-transglycosylase deficient mutants (Kitano & Tomasz, 1980; Harkness & Ishiguro, 1983), the induction of lysis in cells overproducing the soluble lytic transglycosylase (Betzner *et al.*, 1990) and the detection of endopeptidase-induced cleavage of murein crosslinks early in the course of autolysis (Burman *et al.*, 1983).

Autolysis is the typical response of cells subjected to certain nonspecific chemical treatments or specific inhibition of murein synthesis, either by antibiotics or deprivation of cell wall constituents such as diaminopimelic acid (McQuillen, 1958). However, the nature of the autolytic process in these two cases differs. For example, uncontrolled activation of murein hydrolases occurs when cells are submitted to a combination of osmotic up- and downshocks (Leduc & van Heijenoort, 1980) or when they are exposed to agents as diverse as trichloroacetic acid, EDTA, sucrose or NaCl (Hartmann *et al.*, 1974), which presumably affect an inhibitory membrane environment. It has been suggested that there is some sort of barrier that hinders uncontrolled murein hydrolysis by the wall-bound enzyme (Hartmann *et al.*, 1974). This is supported by the observation that insertion of the lysis protein of phage MS2 (a low molecular mass, hydrophobic protein) into the membranes not only causes the formation of membrane adhesion sites but also interferes with the control of the lytic enzymes in the *E. coli* host, thereby inducing cellular lysis (Höltje *et al.*, 1988; Walderich *et al.*, 1988; Walderich & Höltje, 1989). Disturbances of autolytic control by alteration of the membrane environment have also been reported for *envC* mutants (Karibian *et al.*, 1981) and in *E. coli* treated with chaotropic agents such as ethanol (Ingram, 1981). This suggests that lipids may bind to autolytic enzymes and inhibit their activity (see above), perhaps in a manner analogous to the inhibition of autolysins by lipoteichoic acids in Gram-positive bacteria (Höltje & Tomasz, 1975; Cleveland *et al.*, 1975; Briese & Hakenbeck, 1985). Wall degradation by chemical treatments is prevented only by the alteration of the composition of the

murein so as to render it a poor enzyme substrate. Such alteration of murein occurs during the phenotypic tolerance developing late in nutrient deprivation (Goodell & Tomasz, 1980; Tuomanen *et al.*, 1988a).

In contrast to autolysis triggered by harsh chemical treatments, autolysis induced by specific inhibition of murein synthesis is a more controlled event initiated under topographic restriction and subject to modulation by other metabolic processes. Topographic restriction of the initiation of autolysis is indicated by the facts that acylation of only the biosynthetically new cell wall synthetic enzymes induce autolysis (Tuomanen, 1986) and only enzyme physically within the growth zone can be deregulated in this manner (Tuomanen & Tomasz, 1990). It appears that the rules governing autolysis are similar for a wide variety of antibiotics which inhibit murein synthesis, irrespective of the specific site at which they block the multistep murein biosynthetic pathway (Tomasz, 1979). Thus, except in the case of the events associated with autolysis induced by harsh chemical treatments (see above), deregulation of murein hydrolases during autolysis can be thought of as a disturbance beginning in the growth zone. Although this is a feature most readily demonstrated in Gram-positive bacteria (Mitchell & Moyle, 1957; Shockman *et al.*, 1967; Tomasz *et al.*, 1975; Tuomanen & Tomasz, 1990) it can also be seen in *E. coli* (see Fig. 3; Schwarz *et al.*, 1969).

Regulators of many cellular metabolic events are linked to murein hydrolases. For example, modulation of antibiotic-induced autolysis occurs at the level of the PBPs. Inhibition of the activities of PBPs 1a and 1b induces rapid bacteriolysis (Tamaki *et al.*, 1977; Kitano & Tomasz, 1979). However, autolysis does not occur if PBP 3 is inhibited together with PBP 1a (Tuomanen *et al.*, 1986c) suggesting that PBP 3 may be linked to a negative regulatory loop for autolysis. PBP 2 has been suggested as a positive effector of autolysis (del Portillo *et al.*, 1989) perhaps through a link to the ribosomal synthetic machinery mediated by the *lov* gene product (Boulouc *et al.*, 1989).

The most striking examples of powerful negative regulation of autolysis occur during bacterial responses to stress: the stringent response to nutrient deprivation and possibly the heat-shock response. The stringent response to nutrient deprivation which depends on the *relA* locus prevents autolysis in *relA*⁺ but not *relA* strains (Goodell & Tomasz, 1980; Kusser & Ishiguro, 1985; Tuomanen & Tomasz, 1986; Betzner *et al.*, 1990). This phenomenon has been termed phenotypic tolerance and is the most universal mechanism for inhibition of autolysis amongst all bacteria (Tuomanen *et al.*, 1986b). The mechanism of this response is unknown, but recent evidence suggests that repression of autolysis during

growth arrest may involve two stages (Pisabarro *et al.*, 1990). In addition to the repression of autolytic activity, growth arrest is accompanied by a gradual change in the cell wall structure to a less hydrolysable form (Goodell & Tomasz, 1980; Harkness *et al.*, 1981; Pisabarro *et al.*, 1985). This results in the eventual appearance of resistance of nongrowing cells to even the harsh effects of detergents. Thus, changes in both murein hydrolases and cell wall substrate conspire to prevent autolysis in nongrowing bacteria.

The heat-shock response genes are apparently also involved in the control of the autolytic system of *E. coli*. Mutants in any one of the five genes *dnaK*, *dnaJ*, *groEL*, *groES* and *grpE*, which form the heat-shock regulon, are more sensitive to autolysis induced by expression of the cloned lysis protein of phage ϕ X174 (Young *et al.*, 1989) which seems to trigger the endogenous murein hydrolases of the host cell (Lubitz *et al.*, 1984). Overproduction of these heat-shock proteins confers complete protection against lysis by cephalosporins but not imipenem (K. Jones, personal communication). Sensitivity to lysis was restored by a mutation in any one of the five genes, indicating that all are required for the lysis-resistant phenotype.

Another generally observed suppression of the lytic effects of β -lactams depends on growth at low pH (Lopez *et al.*, 1976). Growth of *E. coli* at pH 5 protects the bacteria against lysis, although the typical morphological alterations of cell shape such as bulges and filaments do develop indicating that the antibiotics indeed interact with their targets (Goodell *et al.*, 1976). The basis for this phenomenon is still not understood.

In general, bacteriolysis due to inhibition of murein synthesis occurs at a rate strictly proportional to the growth rate (Tuomanen *et al.*, 1986a). Three exceptions to this rule have been identified. First, penicillin-induced lysis of amino acid starved, non-growing *relA*⁺ cells is triggered by relaxation of the cells with protein synthesis inhibitors such as chloramphenicol (Kusser & Ishiguro, 1985). Second, rapid bacteriolysis can also be observed when ribosome inhibitors, which cause relaxation of the cells, are added to Slt-overproducing cells, irrespective of the presence or absence of a functional *relA* gene (Betzner *et al.*, 1990). Third, penem and carbapenem antibiotics appear to be able to induce autolysis in nongrowing *relA*⁺ *E. coli* (Tuomanen & Tomasz, 1986). The mechanism of this unique ability appears to relate to two properties of these antibiotics. First, penems which lyse nongrowing bacteria share the ability to inhibit PBP 7 of *E. coli* (Tuomanen & Schwartz, 1987). Since PBP 7 is required to synthesize the autolysis-resistant murein characteristic of nongrowing bacteria (Tuomanen *et al.*, 1988a), its inhibition ensures that hydrolysable murein continues to be incorporated into the growth zone even

during growth arrest. Second, penems appear to trigger a set of autolysins that is different from that triggered by penicillin in both growing and nongrowing bacteria (Cozens *et al.* 1989). In particular, imipenem-induced autolysis proceeds independently of the activity of the rate-limiting endopeptidase. It is proposed that this alternative 'penem' set of autolysins is still exported to the murein layer and remains active in nongrowing cells.

The examples of stress-induced global regulators of autolysis described above demonstrate the existence of strong negative controls on hydrolase deregulation. However, there exists one example of activation of autolytic capacity by a global transcriptional regulator. In the Gram-negative bacterium *Bordetella pertussis*, the capacity for autolysis is regulated by the sensor/transducer system encoded by the *bvg* locus (Tuomanen *et al.*, 1990). In response to environmental stimuli, the *bvg* gene product coordinately represses the expression of virulence determinants. Only such avirulent cells degrade cell wall in response to antibiotic treatment suggesting that the *bvg* gene product derepresses murein hydrolases. This demonstrates that autolysis can be activated at the transcriptional level in response to environmental cues.

The role of murein hydrolases in infectious diseases

Since many autolysins appear to be non-essential enzymes (see above) the selection pressures which perpetuate these suicide hydrolases remains an enigma. It has been suggested that autolysins may exert their selective advantage only in the *in vivo* environment (Handwerger & Tomasz, 1985). A prediction of this hypothesis is that regulation of autolysin activity might be modified in clinical isolates and two recent observations support this prediction. Repression of autolysis accompanies the phase shift from avirulence to virulence in *B. pertussis* (Tuomanen *et al.*, 1990). Second, the ability to repress autolysis in response to antibiotics, a property termed tolerance (Tomasz *et al.*, 1970), has arisen in a large number of β -lactam-resistant clinical isolates (Handwerger & Tomasz, 1985). Furthermore, a large proportion of sensitive clinical isolates are also lysis defective; the mechanism of this trait is not elimination of autolysin production but rather repression of autolysin activity (Tuomanen *et al.*, 1988b).

It is clear that by virtue of their capacity to release highly bioactive cell wall subcomponents, autolysins make a major impact on the course of infection *in vivo*. This has been most clearly demonstrated in animal models of meningitis in which strains which autolyse produce an intense inflammatory response and those that do not produce a cryptic infection complicated by a relapsing course (Tuomanen *et al.*, 1988b). Not only is

the total autolytic capacity of a strain an important determinant of pathogenicity, but the particular hydrolases triggered *in vivo* also determine events during infection. For instance, lysis of *E. coli* by ampicillin causes greatly enhanced inflammation while lysis by the different set of hydrolases triggered by imipenem is associated with only mild inflammation in the meningitis model (M. Tauber & E. I. Tuomanen, unpublished data). By determining which murein degradation products are released into the host environment, murein hydrolases play a major role in shaping the time course and the signs and symptoms of infection. Activation of the lytic transglycosylases, for example, liberates 1,6-anhydromuramyl peptides which induce sleep (Krueger *et al.*, 1982), are cytotoxic to ciliated epithelial cells (Melly *et al.*, 1984; Cookson *et al.*, 1989) and induce arthritis (Fleming *et al.*, 1986) and meningitis (Tuomanen *et al.*, 1986d). However, more extensive degradation of the muropeptides causes loss of activity. As the biological activities attributable to murein fragments continue to increase in number (Adam *et al.*, 1981; Seidl & Schleifer, 1986; Lederer, 1986; Karnovsky, 1986), the precise roles of the murein hydrolases which produce them will achieve major significance in the study of the molecular mechanisms of infectious and immunologically mediated diseases. Our understanding of the induction and repression of autolysis has major implications for our ability to successfully control tissue damage during many inflammatory states (Tomasz & Saukkonen, 1990; Tuomanen *et al.*, 1986d).

Outlook

During the early days of bacterial cell wall research murein hydrolases were mostly of interest as suitable tools to degrade murein into muropeptides that could be analysed chemically (Ghuysen, 1968). Nowadays, murein hydrolases find use in the isolation on a preparative scale of specific muropeptides which indeed display a number of fascinating properties (Adam *et al.*, 1981; Seidl & Schleifer, 1986) including such diverse activities as slow-wave sleep promotion (Martin *et al.*, 1984; Krueger *et al.*, 1982, 1986) and immunomodulation (Lederer, 1986). With some of the enzymes cloned on expression vectors that allow considerable overproduction, the enzymes can easily be purified for the purpose of muropeptide production from isolated murein sacculi (Høltje & Schwarz, 1989).

The most important aspect of murein hydrolases, however, is that their autolytic capacity renders bacteria a kind of 'time bomb'. The integrity of a bacterium depends absolutely on the precise control of its autolytic system. It must be considered a real challenge to

chemotherapy to find the trigger(s) for the 'delayed-action cap', the cellular control circuits for the endogenous autolysins. Inhibition of murein synthesis seems to be an indirect way to abolish cellular control of these autolytic enzymes. The mechanism of action of certain phage lysis proteins (MS2, ϕ X174) seems to follow another route in triggering bacterial lysis by the activation of murein hydrolases (Walderich & Höltje, 1989). As discussed above detailed information on the control and regulation of these enzymes is still unfortunately lacking.

The regulatory systems of the autolytic enzymes are of interest not only for chemotherapy but also for a general understanding of the morphogenesis of bacteria (Schwarz & Leutgeb, 1971; Höltje & Schwarz, 1985; Nanninga, 1988). Murein hydrolases must be considered an important subset of proteins of essential bacterial morphogenes (Donachie *et al.*, 1984). The maintenance of the genetically determined specific shape of bacteria (for example the typical rod-shape of *E. coli*) is likely to be realized by a finely tuned and topologically well-organized cooperation of quite a number of enzymes forming and hydrolysing covalent bonds in the murein net.

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