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Hypothesis

Coordinate regulation of murein peptidase activity and AmpC β-lactamase synthesis in *Escherichia coli*

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In the periplasmic space of *Escherichia coli*, the (L)-*m*-A_2pm-(D)-*m*-A_2pm peptide, the lipoprotein, and the AmpC β-lactamase are controlled by growth rate. To explain this coordinate regulation, it is proposed that the AmpC protein functions as an LD-endopeptidase in addition to its known function as a β-lactamase. As LD-peptides, DD-peptides and β-lactams are structurally similar, LD-peptidases may belong to the larger family of DD-peptidases and serine β-lactamases. In contrast to *E. coli*, many related bacteria possess an inducible AmpC protein. Several gene systems necessary for AmpC induction are known to affect various aspects of peptidoglycan metabolism. It is proposed that AmpC induction occurs indirectly via a recyclable cell wall peptide.

Penicillin; Peptidoglycan; β-Lactamase; Murein peptidase; Murein lipoprotein; Transcription regulation

1. INTRODUCTION

The cell wall of *Escherichia coli* is composed of an outer membrane and a rigid exoskeleton called the murein sacculus which determines cell shape and protects the bacterium from osmotic lysis. The murein layer lies within the periplasmic space which separates the outer membrane from the inner cytoplasmic membrane. Since the murein is continuously remodelled as the bacterium grows and divides, its synthesis is tightly coordinated with the cell division cycle. Additionally, since the murein is both essential and unique to bacteria, the enzymes of murein metabolism are important targets of antibiotic action. Penicillins and related β-lactam antibiotics are selective inhibitors of the penicillin-binding proteins (PBPs) which are enzymes of murein biosynthesis (reviewed in [1]).

The PBPs construct murein from peptidoglycan precursors which are synthesized in the cytoplasm. The structure of the peptidoglycan that becomes incorporated into the murein layer of *E. coli* is shown in Fig. 1. Glycan chains of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) are linked by β-1,4-glycosidic bonds. The glycan chains are interconnected by short peptides which are attached by amide bonds to the carboxyl group of each MurNAc residue. In enterobacteria, the cell wall peptides are derived from the pentapeptide L-alanyl-D-γ-glutamyl-(L)-*meso*-diaminopimelyl-D-alanyl-D-alanine (L-ala-D-γ-glu-(L)-*m*-A_2pm-D-ala-D-ala) [1].

Soon after peptidoglycan is incorporated into the glycan network of the murein, the terminal D-ala-D-ala peptide bond is cleaved by a DD-peptidase. The enzyme catalyzes nucleophilic displacement between an active site serine hydroxyl group and the carbonyl carbon of the peptide to form an intermediate acyl–enzyme. The carbonyl carbon of the acyl–enzyme ester bond then reacts with an acceptor nucleophile which regenerates the active serine enzyme and releases the modified peptide. The DD-transpeptidases employ the ε-amino group of a neighboring *m*-A_2pm residue as acceptor to form a D-ala-(D)-*m*-A_2pm cross-link which interconnects the glycan chains of the murein sacculus (Fig. 1). Approximately one quarter of the cell wall peptides in *E. coli* are cross-linked by transpeptidation [1]. The DD-carboxypeptidases employ water as an acceptor and thereby convert the remaining pentapeptides to tetrapeptides; the D-ala-(D)-*m*-A_2pm cross-link is structurally similar to D-ala-D-ala and subject to hydrolysis by a DD-endopeptidase. These murein hydrolases are necessary for cell growth and division since they help maintain the dynamic structure of the murein sacculus (reviewed in [2]). The family of DD-peptidases are inactivated on binding penicillin.

The molecular basis of penicillin action was predicted by Tipper and Strominger in 1965; they observed that penicillin is a structural analog of the D-ala-D-ala terminus of cell wall peptides [3]. Indeed, if the reactive amide bond in the β-lactam ring of penicillin is superposed on the scissile peptide bond of acyl-D-ala-D-ala, the α-car-

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bon backbone of the peptide can adopt a conformation that is nearly isosteric with penicillin. Tipper and Strominger suggested that transpeptidation might proceed by an acyl–enzyme mechanism which conserves the D-ala-D-ala bond energy needed to form a peptide cross-link in the extracytoplasmic milieu [3]. They also proposed that penicillin may exert its antibiotic properties by acylating the active site of a DD-peptidase to form an inactive penicilloyl enzyme [3]. An important corollary was that β-lactamases, which hydrolyze the β-lactam amide bond, may be derived from the DD-peptidases [3].

It has since been demonstrated that DD-peptidases and serine β-lactamases catalyze amide bond cleavage via an acyl–enzyme mechanism which involves an active site serine residue located at the junction of two domains (reviewed in [4]). In the presence of penicillin, the DD-peptidases form a stable penicilloyl enzyme, whereas the serine β-lactamases form a penicilloyl enzyme which is hydrolytically labile. Since DD-peptidases and serine β-lactamases of class A and C exhibit similar tertiary structures, it is now believed that they evolved divergently from an ancestral enzyme [5]. However, the serine β-lactamases of class A are distinct from the class C enzymes (AmpC) [5]. Some relationships between peptidoglycan structure and the regulation of enterobacterial AmpC synthesis are difficult to interpret in terms of the DD-peptidase analogy. This article will describe these relationships and interpret them by analogy with LD- peptidases.

2. METABOLISM AND PHYSIOLOGY OF LD-PEPTIDES

As the murein matures, the (L)-*m*-A_2pm-D-ala peptide bond in cell wall tetrapeptides can be ruptured by several LD-peptidases. The LD-carboxypeptidase of *E. coli* is insensitive to penicillin although it is inhibited by nocardicin A, a monocyclic β-lactam which interacts with the enzyme non-covalently [6]. It has been proposed that D-amino acids and a related substituent in nocardicin A are important for LD-carboxypeptidase inhibition [7]. No mutants in the LD-carboxypeptidase gene are available nor has the enzyme been characterized structurally. However, it is probable that the LD-carboxypeptidase operates by an acyl–enzyme mechanism analogous to that of the DD-carboxypeptidases. The LD-carboxypeptidase produces murein tripeptides which are preferentially used as acceptors by PBP3, a DD-transpeptidase required for septation [8]. It is possible that the LD-carboxypeptidase is coordinated with septation since its expression is elevated at the time of cell division [9].

The (L)-*m*-A_2pm-D-ala peptide bond is also important for attachment of Braun's lipoprotein to the murein sacculus. Approximately one third of lipoprotein molecules in *E. coli* are covalently attached through the ε-amino group of their C-terminal lysine to the (L)-*m*-A_2pm residue of a murein tripeptide [10]. By analogy with DD-transpeptidases, the putative (L)-*m*-A_2pm:lipoprotein transpeptidase may catalyze nucleophilic displacement of the (L)-*m*-A_2pm-D-ala peptide bond to form an acyl–enzyme which employs the lipoprotein as an acceptor. In mutants of *E. coli* and *Salmonella typhimurium* with decreased levels of murein-bound lipoprotein, the outer membrane fails to invaginate with the murein layer during septation [11,12]. However, a mutant of *E. coli* that lacks the lipoprotein grows and divides normally; the abnormally leaky outer membrane of this mutant suggests the lipoprotein helps maintain the integrity of the cell wall [13].

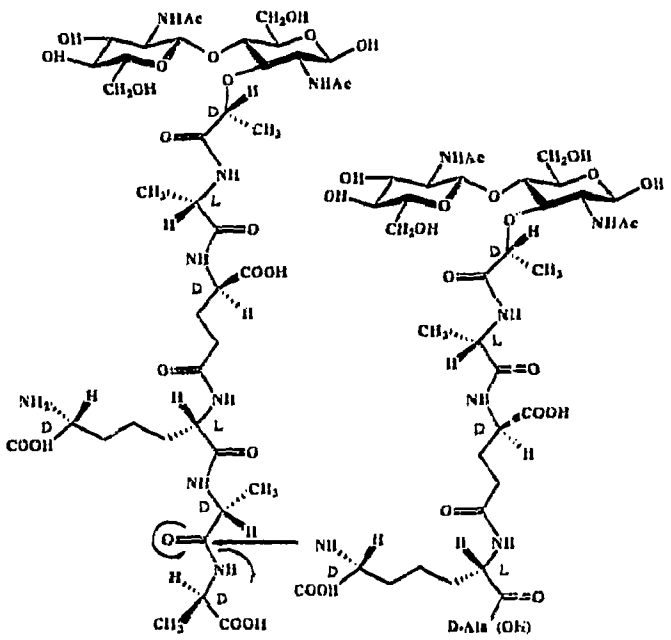

Fig. 1. Structure of the principle disaccharide pentapeptide precursor which is incorporated into the murein layer of *Escherichia coli*. DD-Transpeptidases catalyze acyl-transfer between the carbonyl carbon of the terminal D-ala-D-ala peptide and the ε-amino group of a neighboring A_2pm residue to form a peptide cross-link. DD-Carboxypeptidases transfer the acyl group to water, and a DD-endopeptidase ruptures the peptide cross-link (see text for discussion).

Recently, a novel (L)-*m*-A_2pm-(D)-*m*-A_2pm peptide that comprises a small fraction of murein cross-links was discovered in *E. coli*. It has been suggested that an LD-transpeptidase may catalyze nucleophilic displacement of the (L)-*m*-A_2pm-D-ala peptide to form an acyl–enzyme which employs the ε-amino group of a neighboring *m*-A_2pm residue as an acceptor [14]. It has also been suggested that the LD-transpeptidase and the (L)-*m*-A_2pm:lipoprotein transpeptidase may be identical since the lipoprotein is initially attached preferentially to cell wall peptides in which the *m*-A_2pm residue participates in a cross-link [15]; the (L)-*m*-A_2pm-(D)-*m*-A_2pm cross-links that carry the lipoprotein are twice as abundant as the lipoprotein-free species [16]. Additionally, the (L)-*m*-A_2pm-(D)-*m*-A_2pm peptide bond and the murein lipo-

protein are the only murein structures regulated by bacterial growth rate; their abundance decreases coordinately with increasing growth rate [17]. This may reflect the activity of an LD-endopeptidase which could modulate the availability of (L)-*m*-A_2pm-(D)-*m*-A_2pm peptides for lipoprotein attachment and liberate lipoprotein-tripeptides from the cross-linked species.

3. GROWTH RATE CONTROL OF AmpC SYNTHESIS AND MUREIN STRUCTURE

The AmpC β-lactamase of *E. coli* is specifically expressed in direct proportion with growth rate [18]. The chromosomal *ampC* gene is controlled by a promoter which lies within the coding sequence of the neighboring and overlapping fumarate reductase operon (*frd*) [19]. Transcription of the *ampC* gene is initiated through the *frd* terminator, also known as *ampA*, which functions as an *ampC* attenuator. Anti-termination is occasionally achieved when the translational apparatus couples with a ribosome-binding sequence in nascent *ampC* transcripts [20]. Since ribosome biogenesis is independently coordinated with growth rate by the Stringent Response, AmpC expression is coordinated with growth rate by the *ampA* attenuator [20]. The *ampC* gene is not induced by β-lactam compounds and, in the absence of rare mutations that lead to AmpC overproduction, is not normally an agent of β-lactam resistance [21,22].

The role of growth rate in control of *ampC* gene expression remains an enigma. However, the phenomenon of phenotypic tolerance, whereby slowly growing bacteria are more resistant to the killing action of penicillin, may partly result from structural changes in the cell wall [23]. The *ampA* attenuator provides levels of AmpC protein that are exactly inversely proportional, over similar growth rates, to levels of both the (L)-*m*-A_2pm-(D)-*m*-A_2pm peptide and the lipoprotein (compare [17] and [18]). To the same degree, cell diameter in *E. coli* is also inversely proportional to growth rate [24]. In *Salmonella typhimurium*, which lacks an *ampC* gene, no correlation between growth rate and cell diameter is evident [25]. The coordinate control of *ampC* gene expression and murein composition in *E. coli* could be explained if the AmpC protein functions as the putative LD-endopeptidase.

4. STRUCTURAL SIMILARITIES BETWEEN CELL WALL PEPTIDES

Is it possible that the AmpC protein functions both as a β-lactamase and an LD-peptidase? As demonstrated in Fig. 2, the (L)-*m*-A_2pm-(D)-ala peptide is a stuctural analog of the D-ala-D-ala peptide and of penicillin. Due to the D-configuration of the ε-carbon in *m*-A_2pm, the two endopeptide cross-links are analogous to their corresponding exopeptides. Therefore, the DD-peptides and the LD--peptides can be largely superposed on each other

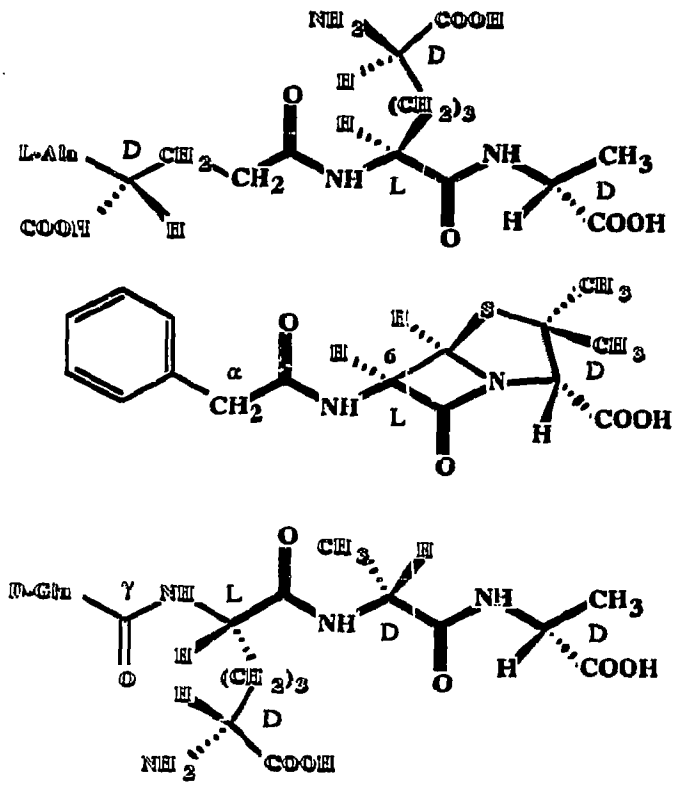

Fig. 2. Structural similarity between cell wall peptides and penicillin. Enboldened atoms in the α-carbon backbones of the LD-peptide (top) and the DD-peptide (bottom) can be superposed on the reactive amide bond in the β-lactam ring of benzyl penicillin (middle). Substituents that differ between the three amide classes are outlined (see text for discussion).

and on penicillin. This relationship suggests that DD-peptidases, LD-peptidases and serine β-lactamases catalyze the cleavage of similar amide substrates and may thereby comprise a single family of enzymes which evolved divergently from a primordial murein amidase.

Structural differences between the murein peptidases may reflect the structures of their corresponding peptide substrates and the origins of the different classes of serine β-lactamases. The position that corresponds to the α-carbon on the 6-β-aminoacyl side chain of penicillin is not substituted in the LD-peptide (Fig. 2). Generally, β-lactams that lack substituents at this position exhibit higher turnover numbers than their substituted counterparts due to hydrolysis by class C β-lactamases [26]. The major secondary structural elements of DD-peptidases and serine β-lactamases can be superposed on each other. An important structural difference, which may somehow influence substrate recognition, is the inverted orientation of helix α10 in the class C β-lactamase [5]. These relationships support the concept that the AmpC protein may recognize both β-lactams and LD-peptides as substrates.

An alternate LD-peptide hypothesis was originally proposed by Wise and Park; they demonstrated that penicillin is a structural analogue of the L-ala-D-γ-glu

peptide [27]. However, L-ala-D-γ-glu is not a substrate for any known peptidases and it is unable to adopt the conformation that mimics penicillin and D-ala-D-ala [28]. The fact that the LD-carboxypeptidase is not inactivated by penicillin [6] may either reflect a conformational difference between penicillin and (L)-*m*-A_2pm-D-ala or an ability of the LD-carboxypeptidase to hydrolyze penicillin. Pollock originally proposed that some β-lactamases may have retained an additional metabolic function which has escaped detection [29]. The overriding importance of the AmpC protein as a β-lactamase may reflect a relatively benign function of (L)-*m*-A_2pm-(D)-*m*-A_2pm peptides in cell wall metabolism. Additionally, Saz suggested a role for β-lactamases in cell wall turnover based on studies of an inducible chromosomal β-lactamase in sporeforming bacteria [30]. In many bacteria related to *E. coli*, the chromosomal *ampC* gene is inducible and relationships between *ampC* induction and murein metabolism are evident.

5. CONTROL OF THE INDUCIBLE AmpC β-LACTAMASE

In Gram-negative bacteria such as *Enterobacter cloacae*, *Citrobacter freundii* and *Pseudomonas aeruginosa*, the chromosomal *ampC* gene is induced by β-lactam antibiotics [31–33]. Induction depends on the *ampR* gene which separates *ampC* from *frd*, and thereby replaces the *ampA* attenuator of *E. coli*. The *ampRC* locus constitutes a divergently transcribed control unit wherein the AmpR protein binds specifically to the intergenic operator and represses transcription of both genes [31]. In the presence of β-lactam compounds, AmpR is somehow converted to an activator of *ampC* gene expression. AmpR belongs to the LysR family of DNA-binding transcriptional regulators, many of which are controlled by binding specific ligands [34]. It is unlikely that penicillin controls AmpR directly because the primary structure of AmpR lacks the β-lactam binding motifs that are common among the PBPs and the serine β-lactamases [31]. Additionally, induction of the *ampC* gene does not require that β-lactams penetrate the cytoplasmic membrane [35]. Therefore, a signal transducing second messenger probably mediates information from the cell envelope to the *ampC* transcriptional apparatus.

Mutations that affect control of the inducible *ampC* gene have revealed important aspects of the induction mechanism. The *ampD* mutation has been identified both in *E. coli* transformants that harbor the cloned *ampRC* locus in a recombinant plasmid and in *Enterobacter cloacae* [36,37]. The *ampRC* transformants of *E. coli* normally express the plasmid-encoded *ampC* gene in a β-lactam-inducible manner. In the presence of β-lactam antibiotics, the *ampD* mutation is selected at a high frequency in the inducible bacteria; these mutants exhibit stably derepressed AmpC expression and extreme resistance to β-lactams [36,37]. In *E. coli*, the *ampD* mutation maps to an operon that encodes an additional downstream gene named *ampE* [38,39]. The AmpE amino acid sequence shares similarities with a family of membrane-localized bacterial transport proteins, which include the oligopeptide permease (Opp), whereas AmpD is a cytoplasmic protein [39].

The non-inducible *ampC* gene of the *E. coli* chromosome is unaffected by the *ampDE* operon. However, *ampD* mutants of *E. coli* accumulate cell wall peptides in the growth medium [40]. Additionally, in the simultaneous presence of exogenous A_2pm and a functional *ampD* gene, *E. coli* increases the number of pentapeptides in the murein; this implicates *ampD* in the general regulation of murein carboxypeptidase activity [40]. In the inducible bacteria A_2pm functions as a non-specific inducer of AmpC expression [41]. These data suggest a dual role for the *ampDE* operon in control of cell wall peptide metabolism and regulation of AmpC induction.

6. INDIRECT INDUCTION OF AmpC BY PEPTIDOGLYCAN

In the final stages of peptidoglycan metabolism, *E. coli* liberates cell wall peptides from the glycan network of the murein due to the action of MurNAc-L-ala amidase [42]. Although some of the free cell wall peptides diffuse through the outer membrane porins most are recycled by the bacterium. Growing cells process the free cell wall peptides to L-ala-D-γ-glu-(L)-*m*-A_2pm (tripeptide), which is recovered in the cytoplasm as a uridine diphosphate (UDP)-activated peptidoglycan precursor (UDP-MurNAc-tripeptide) [43]. Therefore, cell wall peptides are recycled by a pathway that requires a membrane-bound tripeptide permease and a cytoplasmic UDP-MurNAc:tripeptide ligase.

Is it possible that the *ampDE* operon specifies enzymes of cell wall peptide recycling? The accumulation of cell wall peptides found in *ampD* mutants can be explained if the AmpD protein functions as a UDP-MurNAc:tripeptide ligase; by conveying tripeptides to metabolism, AmpD could determine the tripeptide concentration. This postulate fits into a model for control of the inducible *ampC* gene. If the AmpR protein is regulated by binding the tripeptide, or a tripeptide metabolite, the ligand concentration would fluctuate in response to events that alter murein metabolism. Conceivably, β-lactams could indirectly induce the AmpC protein by disrupting murein synthesis and stimulating the murein hydrolases which produce the tripeptide. Therefore, a product of murein hydrolase activity is modelled into a regulatory circuit which controls AmpC synthesis (Fig. 3).

Although the Opp protein contributes to the uptake of cell wall peptides, the primary role of Opp is in the transport of protein-derived peptides with L-amino acids and α-peptide bonds [44]. The unusual structure

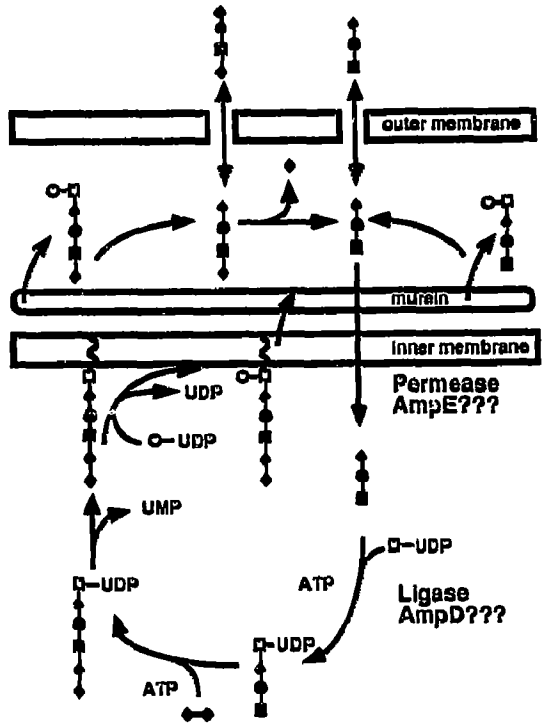

Fig. 3. Recycling of cell wall peptides in *E. coli*. Peptides are liberated from the murein and processed to the tripeptide, L-ala-D-γ-glu-(L)-*m*-A_2pm (tripeptide), which is coupled to a nucleotide-activated precursor of peptidoglycan biosynthesis. The process requires a tripeptide permease and a UDP-MurNAc:tripeptide ligase. Symbols: ○, GlcNAc; □ MurNAc; ♦ Ala; ● Glu; ■; A_2pm. (See text for discussion. Modified from [43] with permission.)

of cell wall peptides may necessitate a unique permease under conditions where Opp activity is saturated. A role for AmpE as a permease with specificity for tripeptides can explain conflicting reports that AmpE is either essential for AmpC induction [38] or dispensable [39]. In this way, the *ampDE* operon could exert dual control over cell wall peptide recycling and induction of the AmpC protein.

AmpC induction depends on several additional genes which influence the structure of the cell envelope. At the time of cell division in *E. coli*, the *pbpA* and *ftsZ* gene products are required to divert peptidoglycan synthesis from the lateral walls to the septum; temperature-sensitive mutants in each of these genes fail to induce the AmpC protein from a cloned *ampRC* plasmid at the non-permissive temperature [45,46]. The *ampG* mutation, unlinked from *ampC* and *ampDE*, exhibits a stably repressed phenotype both in *Enterobacter cloacae* and the *ampRC* transformants of *E. coli* [47,48]. The AmpG protein carries several putative transmembrane regions and is therefore most likely an integral cytoplasmic membrane protein possibly acting as a ligand transporter (Staffan Normark, personal communication). The AmpG protein may also interact directly with AmpR [48]. Whether the AmpR protein controls *ampC* induction by sensing cell wall peptides or other components of murein metabolism remains to be discerned. However, these results lend support to the concept that AmpC induction may occur indirectly via peptidoglycan metabolites.

7. UNIFYING PERSPECTIVE

The AmpR protein and the *ampA* attenuator exert unique mchanisms of control over *ampC* gene expression; how can they be rationalized in terms of the same AmpC gene product? An important relationship between the two control processes is that the Stringent Response regulates the biogenesis of ribosomes, the synthesis of peptidoglycan, and the murein-lipoprotein [49]. Additionally, the product of the *lov* gene helps coordinate the translational apparatus with the peptidoglycan synthetic machinery [50]. Therefore, control by growth rate could be exerted by mechanisms that monitor either the ribosomes or the peptidoglycan. By sensing peptidoglycan, AmpR could accommodate the AmpC protein both as an LD-peptidase and a β-lactamase through a dual response to growth rate and β-lactam inducers. Since the ribosomes are not directly sensitive to penicillin, the genetically inexpensive *ampA* attenuator may have evolved to employ the AmpC protein solely as an LD-peptidase in the absence of any selective challenge by β-lactam compounds. In this way, *ampC* gene expression is rationalized in terms of control processes which exploit a functional duality in the AmpC protein. Indeed, a growing body of evidence demonstrates coordinate control of murein structure and AmpC synthesis. We hope the LD-peptidase analogy will be of value both in discerning the physiological function of the AmpC protein and in the rational design of improved chemotherapeutic agents. In the treatment of many infectious diseases, effective inhibitors of AmpC expression and activity are urgently required.

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