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REVIEW

Interplay between Peptidoglycan Biology and Virulence in Gram-Negative Pathogens

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SUMMARY The clinical and epidemiological threat of the growing antimicrobial resistance in Gram-negative pathogens, particularly for β-lactams, the most frequently used and relevant antibiotics, urges research to find new therapeutic weapons to combat the infections caused by these microorganisms. An essential previous step in the development of these therapeutic solutions is to identify their potential targets in the biology of the pathogen. This is precisely what we sought to do in this review specifically regarding the barely exploited field analyzing the interplay among the biology of the peptidoglycan and related processes, such as β-lactamase regulation and virulence. Hence,

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here we gather, analyze, and integrate the knowledge derived from published works that provide information on the topic, starting with those dealing with the historically neglected essential role of the Gram-negative peptidoglycan in virulence, including structural, biogenesis, remodeling, and recycling aspects, in addition to proinflammatory and other interactions with the host. We also review the complex link between intrinsic β-lactamase production and peptidoglycan metabolism, as well as the biological costs potentially associated with the expression of horizontally acquired β-lactamases. Finally, we analyze the existing evidence from multiple perspectives to provide useful clues for identifying targets enabling the future development of therapeutic options attacking the peptidoglycan-virulence interconnection as a key weak point of the Gram-negative pathogens to be used, if not to kill the bacteria, to mitigate their capacity to produce severe infections.

KEYWORDS peptidoglycan, murein, recycling, pathogenesis, β-lactamase regulation, lysozyme, inflammation, NOD receptors, bacterial secretion system, flagellum, penicillin binding proteins, peptidoglycan recognition proteins

INTRODUCTION

During the last few decades, the problem of antimicrobial resistance in nosocomial pathogens has continued to grow due to several factors, such as the development of new medical treatments usually entailing immunosuppression and/or invasive procedures, all of which lead to increased life expectancies but also to longer periods of hospital admission and an obvious increase in the probability of acquiring opportunistic infections (1, 2). Moreover, the historically erroneous politics of antibiotic use in several geographical areas, as well as the progressive appearance and horizontal dissemination of resistance determinants on plasmids and/or transposons, have decisively contributed to worsening this scenario. The worldwide emergence and spread of the so-called successful high-risk clones, mainly in species such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, or certain *Enterobacteriaceae*, which are highlighted thanks to their multiresistance patterns and excellent capacity for inter- and intrahospital dissemination, add even more severity to the situation (3, 4).

Nevertheless, the issue of antibiotic resistance is not negligible in community-acquired or common infectious diseases, given that the strains as well as the horizontally transmitted determinants are often selected and maintained thanks to the presence of antibiotics in the food chain, besides the above-mentioned wrong antibiotic prescription, misuse, and self-medication practices (5–9). Obviously, all the cited circumstances are even more serious in the case of the β-lactams, since they are the most used antibiotics in clinical practice (10–12), and in the case of the species of interest for this review, Gram-negative pathogens. This is due to the high degree of intrinsic β-lactam resistance and the outstanding capacity for additional resistance development in several classic representatives of nosocomial species, such as the above-mentioned *P. aeruginosa*, *A. baumannii*, and some *Enterobacteriaceae*, but also in emerging ones, such as the members of the *Burkholderia cepacia* complex (BCC), *Stenotrophomonas maltophilia*, or *Achromobacter xylosoxidans*, among others (13).

Therefore, in this worrying conjuncture, new therapeutic solutions are urgently needed if not to kill the microorganism at least to make the infections less harmful for the patient. This would entirely agree with the concept of antivirulence therapies, which are envisaged as excellent and still barely exploited remaining weapons in the described panorama of antibiotic arsenal reduction (14) to be used along with the novel approaches oriented toward the blockade and/or reversion of the pathways leading to β-lactam resistance (15, 16). Nevertheless, the essential previous step for the development of new therapies is the finding of targets, ergo, weak points to be attacked in the biology of the pathogen. In this sense, the general connection between resistance and virulence is a classically interesting interplay to be approached in order to find therapeutic targets, since it is generally accepted that antibiotic resistance often entails a biologic cost that usually dampens virulence, a circumstance which we could

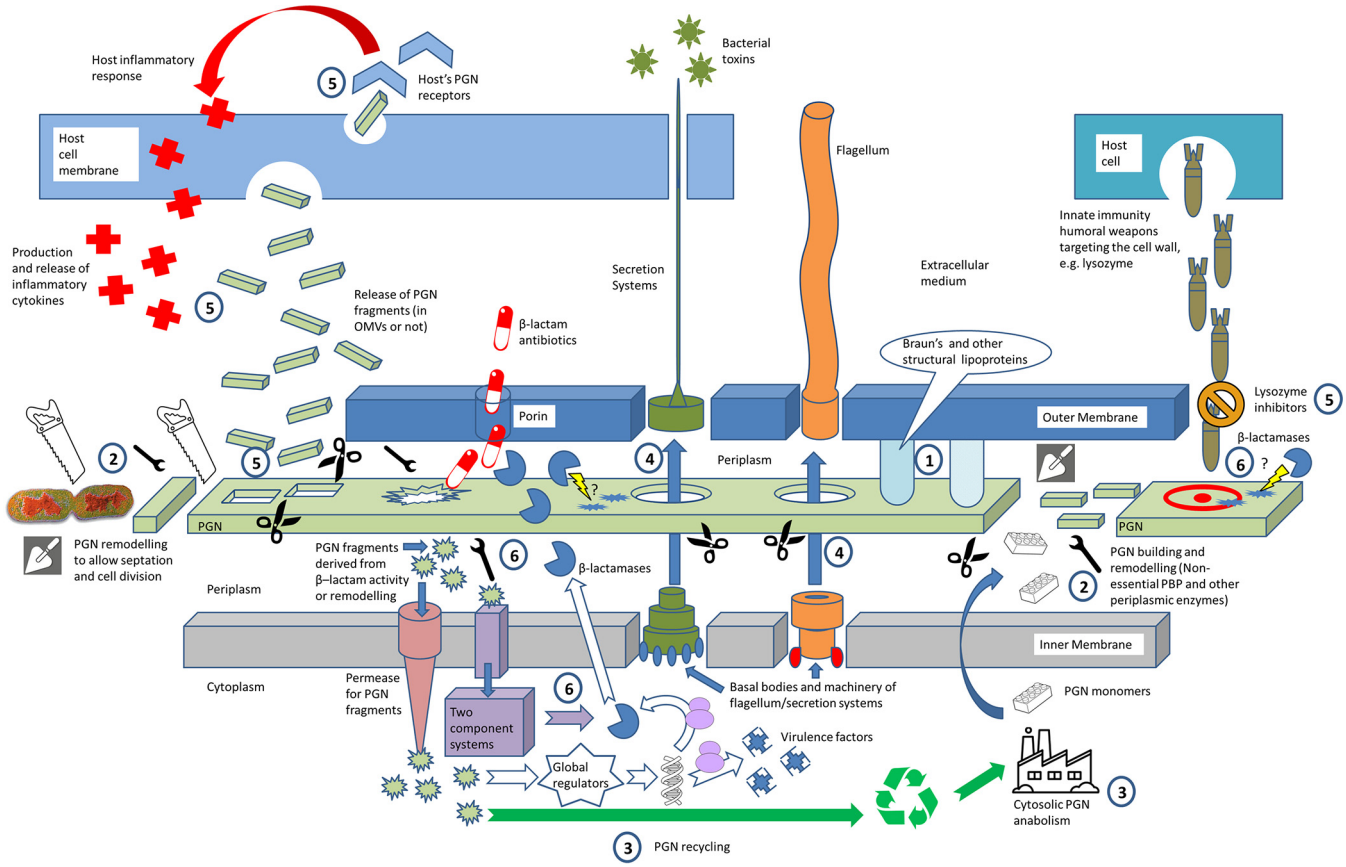

FIG 1 Overview of the peptidoglycan (PGN)-related components and processes from Gram-negative pathogens proved to influence bacterial fitness/virulence and pathogenesis in the host. The numbers indicate the different contexts in which these targets can be found, and they are directly related to the sections of this review: 1, structural targets; 2, building and remodeling of PGN in the periplasm, affecting morphogenesis, cell division, and turnover of PGN fragments; 3, biogenesis and recycling of PGN (cytosol); 4, opening of gaps in the PGN layer to allow the insertion of secretion systems and/or flagella; 5, release of PGN fragments, interaction with host receptors, and the elicited response; 6, interplay among β-lactamase regulation, PGN metabolism, and fitness/virulence.

take advantage of (17). In this work, we only collaterally address this already reviewed general interplay but add another specific ingredient, introducing as the starring actor the biology of the peptidoglycan (PGN) of Gram-negative bacteria, which also has β-lactam resistance implications, since the regulation of β-lactamases is often intimately linked to PGN recycling (18, 19). Traditionally, and in contrast to the PGN of Gram-positive bacteria, the PGN of Gram-negative bacteria has usually been neglected as a key element in pathogenesis. Obviously, this circumstance is explained by the greater width of the PGN of Gram-negative bacteria and the lack of an outer membrane in Gram-negative bacteria, with studies classically highlighting the Gram-positive bacterial cell wall in terms of its importance for antibiotic resistance, survival against immune system weapons, and virulence (20–22). Nevertheless, there is a notable amount of published evidence (much of it very recent) that identifies several targets in the Gram-negative PGN biology (related or not to the regulation or effects of β-lactamase production) and that provides clues enabling the future development of therapeutic options attacking the above-mentioned PGN-virulence/fitness interplay. As can be observed in Fig. 1, we have organized these targets into different sections to ease the reader's task: (i) purely structural-architectural targets, (ii) periplasmic enzymes involved in PGN building and remodeling/recycling (nonessential penicillin binding proteins [PBPs] and others), (iii) cytoplasmic actors involved in the biosynthesis and recycling of PGN fragments, (iv) targets related to the local modification of PGN to allow for the correct assembly of flagella and secretion systems, (v) elements involved in the interaction of PGN with the host and the elicited response (both of which consider the

mechanisms of PGN fragment release but also the host receptors and immune system elements), and (vi) the interplay among PGN metabolism, β-lactamase production regulation (including intrinsic and horizontally acquired enzymes), and fitness/virulence. Thus, as can be deduced from these lines, the PGN is an element that shows a very wide array of implications to finally influence fitness and virulence beyond its obvious architectural role, a circumstance that we wanted to reflect in the title with the concept "peptidoglycan biology." However, and additionally, it has to be noted that several of the reviewed targets could probably fall into more than one of the cited categories and could fall into even more if we think of the pleiotropic nature of some of them, and hence, these categories should be taken only as a part of a *sensu lato* organization with nonexclusive but just schematizing purposes.

DEALING WITH CELL WALL STRUCTURE: PEPTIDOGLYCAN-ASSOCIATED LIPOPROTEINS AS MAJOR VIRULENCE DETERMINANTS

The purpose of this review is obviously not to delve into the structural aspects of Gram-negative PGN, and therefore, this section only superficially assesses the architectural issue, for which several excellent reviews, including more exhaustive descriptions and analysis, may be consulted (23, 24). Briefly, the Gram-negative PGN (also called murein or sacculus) is a polymer composed of glycan chains (*N*-acetylglucosamine [NAcGlc]–*N*-acetylmuramic acid [NAcMur] disaccharides) and cross-linked peptides (initially, pentapeptides bound to the NAcMur units) that forms a mesh-like layer outside the cytoplasmic membrane. It constitutes the solid basis on which the outer membrane relies and gets punctually linked through Braun's lipoprotein (which has the only covalent bond to PGN) and other murein lipoproteins (such as the PGN-associated lipoprotein [Pal]), all of which provide shape, structure, and the capacity to counteract the osmotic pressure, as well as a certain degree of resistance to the diffusion of molecules (25–27). Therefore, the proper synthesis and maintenance of the PGN, but also a correct architecture, are essential not only for bacterial virulence but also for viability, since, for instance, defects in the structural lipoproteins (or their anchoring to PGN) usually drive the outer membrane to a loss of integrity, leading to periplasmic protein leakage, vesicle formation, blebbing, etc (25, 28). As mentioned in the introduction, the PGN of Gram-positive bacterial species has classically been bestowed with great importance in terms of resistance against the action of immune weapons or certain antibiotics, but also as a key virulence factor often related to the inflammatory response elicited in the host (20–22, 29–32). However, given the protection exerted by the outer membrane and the anchored lipopolysaccharide (LPS) and its thinner width, the PGN of Gram-negative species has not been given such a protagonistic role, or at least a role not as important as that of the Gram-positive PGN. Nevertheless, many works have studied the Gram-negative PGN from the point of view of its structure and organization, and different contradictory models regarding the architecture and orientation of glycan chains, as well as the nature of a mono- or multilayer, have been proposed (24, 33). In *Escherichia coli*, for instance, a planar layer that could occasionally include sizable regions of multilayered PGN is mostly accepted. However, some data regarding the width of PGN layers from different Gram-negative bacteria have been published and have indicated, for instance, that the PGN of *P. aeruginosa* shows less than one-half of the thickness of that of *E. coli* (23, 24, 33, 34). In any case, in this section, only the purely structural PGN targets affecting virulence are reviewed, and then all the elements related to the biosynthesis, maintenance, remodeling, and recycling of PGN are analyzed in the following sections. Therefore, some examples of the strictly architecture-related role of PGN (i.e., elements that physically build the static PGN structure) can be found, and they are displayed in Table 1. For instance, the disruption of the PGN-associated lipoprotein (Pal) of *Burkholderia cenocepacia* (belonging to the BCC) has recently been linked to a major impairment in the virulence of this species in the *Galleria mellonella* larva infection model, together with a dampened capacity for host cell attachment and a weaker elicitation of inflammatory cytokine secretion (35). In a similar context, some papers have related the defects in yersinia Braun's lipoprotein

TABLE 1 Targets related to the static PGN structure/architecture

Target	Role(s)	Species	Effect(s) of target disruption	Reference(s)
Pal	A peptidoglycan-associated lipoprotein anchoring the outer membrane to PGN	*B. cenocepacia*	Reduction of virulence in a *Galleria mellonella* model of circa 90-fold; impaired host cell attachment and reduced stimulation of proinflammatory cytokine secretion	35
Lpp1, Lpp2	Structural murein lipoproteins	*S. enterica* serovar Typhimurium	Defective in invasion of macrophages and cytotoxicity for macrophages	39
Lpp	Braun's lipoprotein	Yersiniae	Decreased proinflammatory effects and mortality rates in murine/rat models of infection, decreased survival in murine macrophages, impaired tissue dissemination capacity, and elicitation of an inflammatory response	36–38, 42
YbiS, ErfK, YcfS	L,D-Transpeptidases participating in the anchoring of Braun's lipoproteins to PGN	*S. enterica*	Changes in PGN structure leading to higher resistance to bile salts	41

to an important virulence impairment in murine models of infection, which suggested that the studied knockout (KO) mutants are potential vaccine candidates for bubonic and pneumonic plague (36–38). Similarly, in *Salmonella enterica* serovar Typhimurium, the inactivation of two murein lipoproteins caused a very significant impairment of virulence, in terms of a drastic reduction of invasion/cytotoxicity in cell culture and of mortality in murine models (39) (Table 1). And again, similarly to yersiniae, *S. enterica* serovar Typhimurium mutants with mutations in some PGN structural elements, such as Braun's lipoprotein, have recently been tested as vaccine candidates, with promising results (40). Interestingly, and still related to Braun's lipoprotein, Hernández and colleagues have shown in *Salmonella* that the effects of bile could induce the selection of some modifications leading to an increased resistance to this natural detergent (41). These changes in the PGN structure, detected during exposure to sublethal concentrations of sodium deoxycholate, consisted of a lower degree of Braun's lipoprotein binding to PGN and reduced levels of muropeptide cross-linking by L-(*meso*)-diaminopimelyl-D-(*meso*)-diaminopimelic acid peptide bridges. These structural changes to the PGN were shown to be similar to those observed in a triple L,D-transpeptidase mutant (YbiS, ErfK, YcfS), which also led to enhanced resistance to bile (41) (Table 1).

As can be concluded from the previous lines, given that PGN is essentially composed of sugar chains and stem peptides, it is not easy to find structural building elements, other than the above-mentioned lipoproteins, that may be considered static targets. As shown above, modification of these accessory structural proteins entails diverse consequences beyond the *sensu stricto* PGN probably related to the breakdown of outer membrane integrity, causing permeability barrier loss and an impaired performance of the anchored LPS (42). The direct consequences of these circumstances would be a decrease in the capacity to resist certain immune weapons of the host but also a decrease in adherence and even motility, although some contradictory findings do exist, like the above-mentioned increase in the resistance against bile achieved thanks to a weaker degree of linkage with Braun's lipoprotein (39, 41, 43). Still, regarding these lipoproteins, besides their structural roles, they have also been proposed to be elements contributing to pathogenesis during sepsis, since, once they are released (probably within outer membrane vesicles [OMVs]), they seem to elicit potent inflammatory responses in the host (44, 45). Therefore, they could also be considered relevant pathogen-associated molecular patterns (PAMPs), but as explained above, since they are only accessory elements to the bona fide PGN basic structure, we have not included them in the section dedicated to the interaction of PGN with host receptors (see below). In fact, the pattern recognition receptors (PRRs) for the PGN-associated lipoproteins are the Toll-like receptors (TLRs), mainly TLR2 (39, 46, 47), and not the receptors typical for the PGN fragments (namely, NOD receptors; see below).

Finally, the proteins participating in the anabolic pathways of the sugars and

peptides and those responsible for their assembly and incorporation into the sacculus also influence the final architecture of the PGN, but since they participate in different dynamic reactions and processes, they are dissected in the next sections.

THE BUILDING, REMODELING, AND RECYCLING OF THE PEPTIDOGLYCAN: INDISPENSABLE TASKS FOR BACTERIAL VIRULENCE

Besides the structural point of view displayed in the section above, in which the PGN is shown to be a kind of skeletal support for the cell, we have to take into account the fact that this bacterial structure is far away from being a static element and therefore continuously needs the participation of many enzymes for its building, maintenance, remodeling, and recycling, including such an elemental process as cell division. An excellent review of these issues and their link to β-lactamase regulation has recently been published by Dik and colleagues (19). But in our work, for the sake of clarity, given that we relate the cited topics to virulence in a review for the first time, the participating actors will be separated into three subsections: nonessential PBPs, other periplasmic enzymes, and finally, cytoplasmic enzymes participating in the above-mentioned processes.

Penicillin Binding Proteins: When Not Indispensable for Viability, Essential for Full Virulence

Following the structure of the PGN described above, the enzymes that catalyze the reactions indispensable for its building are the largely studied penicillin binding proteins (PBPs). There are mainly two reactions: the polymerization of the glycan strand (*N*-acetylmuramic acid [NAcMur]–*N*-acetylglucosamine [NAcGlc]), namely, transglycosylation, and the cross-linking between the glycan chains through the stem peptides bound to NAcMur units (transpeptidation) (48). The latter reaction involves a necessary step of terminal D-Ala scission from the preexisting pentapeptide performed by the PBP before the final cross-linkage is allowed.

A large amount of information has been published on the topic of PBPs (48–51), and the reason that some PBPs are considered essential is due to the reactions that they perform, as their knockout mutants are inviable because the polymer cannot be properly synthesized, leading to autolysis. In a few words, the PBPs have been classified as high-molecular-mass PBPs (HMM-PBPs) and low-molecular-mass PBPs (LMM-PBPs). The C-terminal domain of HMM-PBPs typically shows transpeptidase activity, whereas the N-terminal domain can perform glycosyltransferase activity (hence, allowing the elongation of the glycan chains; these PBPs are classified as class A PBPs) or other different functions usually related to cell morphogenesis (in this case, the proteins are classified as class B PBPs). The HMM-PBPs were classically considered essential, but this is changing, as will be shown below for *E. coli* or *P. aeruginosa*, for instance (48). Meanwhile, the LMM-PBPs (also called class C PBPs) have been considered nonessential, as they perform more specific activities and potentially secondary activities with regard to the described main function of PGN building. Some examples could be assistance with septation (i.e., septum formation, which is the concerted invagination of the cytoplasmic membrane, PGN, and outer membrane prior to cell separation), the turnover of certain fragments to initiate recycling, the scission of the terminal D-Ala from pentapeptides to block the possibility of cross-linking, and the cleavage of cross-links between stem peptides (endopeptidase activity) for remodeling (48, 50, 52). As mentioned before, the deletion of essential PBPs is not compatible with cell viability, and they have obviously been studied and are considered very good candidates for therapeutic targets. Nevertheless, given the large amount of published information on these facts, which is not specifically linked to virulence, we will not explore the topic further (50). Thus, we review the influence of the disruption of certain nonessential PBPs on fitness and virulence, and in this context, the identified targets are summarized in Table 2. The complexity of the topic must be emphasized, since most Gram-negative species differ in the number and denomination of PBPs. In *E. coli*, for instance, 12 PBPs have been described: 3 class A PBPs (PBP1a, PBP1b, and PBP1c), 2 class B PBPs (PBP2

TABLE 2 Penicillin binding proteins described to have implications on fitness/virulence

Target	Role(s)	Species	Effect(s) of target disruption	Reference
PBP3 (*ftsL*)	A transpeptidase and major protein of the divisome, the cellular division complex	*E. coli*	Growth in elongated chains of unseparated cells; probable impairment of motility	53
PBP4 (*dacB*) and PBP7 (*pbpG*)	Endopeptidases collaborating with the periplasmic *N*-acetylmuramyl-L-alanine amidases (AmiA, AmiB, and AmiC) for cell septation; unspecified auxiliary roles in maintaining regular cellular morphology and enhancing the formation of biofilms; participation during daughter cell separation after cell division, when the amidases or lytic transglycosylases are absent	*E. coli*	Aberrant morphologies with growth in elongated chains of unseparated cells; impaired formation of biofilms; probable impairment of motility	54
PBP5 (*dscA*)	A major carboxypeptidase cleaving the terminal D-Ala from pentapeptides, making them unavailable for transpeptidation, therefore participating in the fine-tuning of cross-linking in PGN, which affects morphology	*E. coli*	Increased cell diameter, aberrant contour and morphology (branching), as well as alterations in surface uniformity and overall topology of the peptidoglycan saccules; probable impairment of host cell attachment	55
PBP6 (*dacC*) and PBP6b (*dacD*)	Carboxypeptidases collaborating with the septal protein FtsZ to allow correct septation, cell division, and overall cell shape	*E. coli*	Mislocalization of cytosolic FtsZ, leading to abnormal septation events and derived cell branching	56
PBP6b (*dacD*)	A major carboxypeptidase contributing to cell morphogenesis under acidic conditions	*E. coli*	Aberrant morphologies and increased length of cells at pH 5.0	57
AmpH	A bifunctional D,D-endopeptidase and D,D-carboxypeptidase showing weak β-lactamase activity and an unknown exact role in the cell wall	*E. coli*	Uneven contours and aberrant and asymmetric constrictions between dividing cells in AmpH-AmpC double mutants	255
PBP1a (*ponA*) and PBP2 (*pbpA*)	A major transpeptidase-transglycosylase (PBP1a) and a transpeptidase involved in cell elongation (PBP2)	*P. aeruginosa*	Impairment of swarming motility in a PBP1a single mutant; impairment in *in vitro* competition experiments and biofilm formation in double mutants	52
PBP1b (*mrcB*)	A major transpeptidase-transglycosylase	*P. aeruginosa*	A decrease in biofilm formation capacity when deleted together with PBP2	52
PBP7/8 (*pbpG*)	An endopeptidase cleaving cross-bridges between stem peptides	*A. baumannii*	A decrease in survival in a rat soft tissue infection model and a rat pneumonia model; a significant increase in bacterial killing in 90% human serum *in vitro*; a greater prevalence of coccobacillary forms than wild-type forms	62
PBP1a (*mrcA*)	A transpeptidase-transglycosylase mainly involved in cell wall synthesis during elongation that works together with LpoA, a lipoprotein activator located in the outer membrane indispensable for PBP1a performance	*V. cholerae*	Growth deficiencies in minimal medium and increased susceptibility to deoxycholate and bile in a PBP1a knockout mutant (but also an LpoA knockout mutant), which also showed a decreased capacity in competition assays with the wild type both *in vitro* and in an infant mouse small intestine model, which were found only if the bacteria were used during stationary phase	63

and PBP3), and 7 LMM-PBPs. Meanwhile, in *P. aeruginosa* we find only 8 PBPs (HMM-PBPs PBP1a, -1b, -2, -3, and -3a/3x and LMM-PBPs PBP4, -5/6, and -7) and in *Neisseria meningitidis* we find 4 PBPs (2 HMM-PBPs and 2 LMM-PBPs) (48, 50), to cite some examples. Among this wide range of possibilities, it has to be highlighted that most of the information connecting the nonessential PBPs to fitness/virulence implications has been published in *E. coli*-based studies. In this regard, almost 20 years ago, a very interesting work assessing the effects of different single or combined inactivations of the PBP genes was published (53); surprisingly, mutants with almost all the PBP combinations, including mutants with up to eight deleted PBP genes, grew almost as

well as the wild-type strain, with the only exception being the mutant with the combination PBP1a-PBP1b deletion, which was not viable. Other effects were also reported, such as the essential role of PBP3 for septation and cell division, with the inactivation of this enzyme causing the filamentous growth of the mutant in long chains of unseparated cells (Table 2) (25, 53). More recently, some papers have further investigated the topic of cell division in this species, unraveling the participation of some additional actors, such as PBP4 and PBP7 (with a secondary role), besides the major periplasmic amidases AmiA, AmiB, and AmiC, which will be analyzed below (see Table 3) (54). Interestingly, PBP4 and PBP7 were also suggested to contribute to the morphogenesis and production of biofilms (54). Nevertheless, none of these works has addressed whether the alterations in morphology/septation mentioned here could have direct effects on virulence. Of course, what could be expected is an impaired motility (with obvious negative consequences for dissemination and, hence, for virulence), since flagellar movement would not be able to propel the long filaments of unseparated cells as efficiently as it does individual bacteria. Some other plausible negative effects for pathogenesis could be deduced from the aberrant morphologies (in terms of altered contours, cell branching, or increased diameters, for instance) potentially interfering with the host cell's adhesion obtained, for instance, by Nelson and Young (55) in PBP5 knockout mutants (Table 2). However, apart from these assumptions, no additional effects on virulence have been experimentally proved, not even in more recent work (56), where the LMM-PBPs of *E. coli* PBP6 and PBP6b have been related to the correct location of the septal protein FtsZ to allow proper cell division and avoid the insertion of inert PGN at unusual positions, leading to branching (56). Finally, what has recently been proposed for *E. coli* is that the redundancy in PBPs with carboxypeptidase activity (PBP4, PBP4b, PBP5, PBP6, PBP7, and AmpH) allows for the correct cell shape under a wide array of different environmental conditions; therefore, for instance, PBP6b (*dacD*) has been shown to be the most important PBP for morphogenesis under acidic growth conditions (Table 2) (57).

Moreover, a very recent work by Chen and colleagues (52) revealed that the unique PBP essential for the growth of *P. aeruginosa* is PBP3 (*ftsI*) and characterized the effects on fitness/virulence that the deletion of the rest of the PBPs could have on this species. Thus, what was observed is that *P. aeruginosa* tolerates the single deletion of the rest of the nonessential PBPs, with these single deletions not having a significant impact on fitness. Conversely, the simultaneous deletion of PBP1a (*ponA*) and PBP2 (*pbpA*) caused a significant reduction of *in vitro* competitiveness (Table 2) (52). The same deletion combination or that of PBP2 plus PBP1b (*mrcB*) also drove a significant reduction of biofilm formation, suggesting that PBP2 disruption could directly or indirectly affect the virulence phenotype mainly if it was combined with the loss of PBP1a or PBP1b (Table 2). This was supported by the attention-calling increase of circa 30% in pyocyanin secretion when PBP2 was disrupted, potentially caused by the stress derived from the above-mentioned gene deletion. Interestingly, the inactivation of PBP1a alone severely impaired the swarming motility of the mutant, although the basis for this circumstance is not yet clear. A direct involvement of the protein in this kind of movement or, alternatively, a collateral effect derived from the presumptive stress caused by the PGN alteration that the deletion of PBP1a would entail is a plausible explanation (58). In this regard, although in the past motility has been seen to be a very important feature of *P. aeruginosa* virulence (59), paradoxically, these effects were not translated into significant differences when testing the single PBP knockout mutants in a *Caenorhabditis elegans* model (52) (Table 2). In this context, regarding PBPs, it should be taken into account that the deletion of a target could potentially entail not only consequences for the derived virulence phenotype but also some polymorphisms, an issue that has barely been studied to date. In this sense, and still in *P. aeruginosa*, it has been described that some amino acid changes in PBP3 (R504H, A539T, V465A, or F533L, all of which are in the specific binding site for meropenem) lead to resistance, but their potential consequences on fitness/virulence have never been assessed (60). Therefore, analysis of these kinds of polymorphisms usually leading to increased resistance

(another example could be changes in AmpC amino acids [61]) for their potential negative consequences on fitness/virulence is a new horizon that is worth further exploration for future therapy development.

Meanwhile, in *A. baumannii* it has been shown that PBP7/8 decisively contributes to resistance to complement-mediated bactericidal activity, although the basis for this circumstance is still unknown. Therefore, the deletion of this protein was translated into dramatic reductions in virulence in soft tissue and rat lung infection models and in survival after incubation with human serum and, finally, was related to an increased prevalence of coccoidal forms, suggesting an effect of PBP7/8 on morphogenesis (62) (Table 2). Besides, in *Vibrio cholerae*, PBP1a has been shown to be very important for pathogenesis, since its deletion led to a reduced competition capacity both *in vitro* and in mouse models, as well as increased susceptibility to detergents, but only when using bacteria in stationary phase. Hence, this effect of PBP1a deletion, which apparently affects virulence, can be bypassed if cells are in exponential growth. Interestingly, given that this protein works together with lipoprotein LpoA, located in the outer membrane, the deletion of the latter caused the same phenotypes (Table 2) (63).

Thus, to conclude this section, although few virulence-specific conclusions regarding the nonessential PBPs have been published, many inferences can be drawn, if not because of a direct dampening of pathogenesis factors at least because of abnormalities in cell shape or motility, which are obviously also important for full virulence performance.

Periplasmic Actors Other than Penicillin Binding Proteins

Besides the above-mentioned role of the nonessential PBPs in the process of sacculus building, other enzymatic actors that have different functions and that are located in the periplasm have been shown to have fitness/virulence implications when disrupted. In this regard, the types of activities that these actors perform can be subdivided, as displayed in Table 3: cell septation and division-related events, modifications of mature PGN to allow cell shaping, and finally, the PGN-degrading enzymes related to remodeling to allow an increase in cell size or the release of fragments to be internalized into the cytosol for recycling.

Periplasmic elements involved in septation and cell division. With regard to PGN enzymes related to cell septation, the role of the periplasmic *N*-acetylmuramyl-L-alanine amidases (cleaving the bond between the NAcMur units and the stem peptides and, hence, disconnecting the links among glycan chains) in the process, mainly in *E. coli*, is well-known (64, 65). In this regard, the AmiA, AmiB, and AmiC enzymes have been proposed to be essential for correct continued PGN synthesis during cell division, assisting with the formation of a proper septum and, hence, avoiding filamentation, which has also been found in *Salmonella* (64, 65). With regard to other species, it has been shown that AmiA of *Helicobacter pylori* is essential for the separation of daughter cells. Therefore, its inactivation leads to the formation of long chains of unseparated cells and a consequent obvious impairment of motility and colonization capacity in the mouse stomach model (66) (Table 3). AmiB of *P. aeruginosa* is also involved in cell separation and has been proven to be essential for bacterial viability, since its deletion entailed negative consequences for outer membrane integrity and impermeability levels (67). Meanwhile, in the genus *Burkholderia*, it has been shown that the inactivation of the periplasmic AmiC amidase causes the loss of daughter cell separation capacity, obviously entailing a severe impairment for motility, which was translated into the impossibility of causing infection in the rat model or colonizing the insect gut (68, 69). Similar results for AmiC of the phytopathogen *Xanthomonas campestris* have been reported, together with negative effects on type III secretion system (T3SS) performance, obviously entailing an impairment of virulence (70). Nevertheless, at present it remains to be seen whether this impairment of the T3SS function could be related to defects in the insertion of its machinery through the PGN layer (caused by the AmiC absence), as will be displayed later for other proteins.

Besides the cited amidases, very recently the implication that a specialized enzyme

TABLE 3 Periplasmic elements other than penicillin binding proteins related to PGN biology

Target	Role(s)	Species	Effect(s) of target disruption	Reference(s)
Targets related to cell septation/division				
AmiA, AmiB, AmiC	Periplasmic *N*-acetylmuramyl-L-alanine amidases that cleave stem peptides from glycan chains on PGN	*E. coli*	Formation of abnormal septa, causing growth in long chains of unseparated cells	64, 65
YebA, EnvC, and NlpD	LytM factors participating through their peptidase activity in cell elongation and division	*H. influenzae*	Alterations in division with irregular cell architecture and massive membrane blebbing for YebA and NlpD knockout mutants; a reduction in the amount of periplasmic proteins and an impaired capacity to adhere to epithelial cells, to form biofilms, and to resist the bactericidal power of serum with EnvC deletion	76
AmiC	A periplasmic *N*-acetyl-muramyl-L-alanine amidase that cleaves stem peptides from glycan chains on PGN	*Burkholderia* spp.	In daughter cells, an inability to separate, growing in filaments and losing motility, all of which leads to an inability to survive in the rat model and colonize the insect gut	68, 69
AmiC	A periplasmic *N*-acetylmuramyl-L-alanine amidase that cleaves the peptide side chains linked to the glycan strands on PGN and whose activity is potentiated by the presence of the regulator NlpD	*X. campestris*	Impaired daughter cell separation, aberrant cell and colony morphology, and impaired T3SS performance	70
AmiA	A periplasmic *N*-acetylmuramoyl-L-alanyl amidase essential for daughter cell separation	*H. pylori*	Appearance of long chains of unseparated cells and impaired motility; a reduced capacity for colonization of the stomach in a mouse model	66
AmiB	A periplasmic *N*-acetylmuramyl–L-alanine amidase involved in stem peptide cleavage from PGN chains	*P. aeruginosa*	Filamentous growth with a marked deficiency in the invagination of the inner membrane and increased permeability of the outer membrane	67
$PBP3_{SAL}$	A specialized PGN synthase enabling formation of the division septum and promoting cell division in the acidic intraphagosomal environment	*S. enterica* serovar Typhimurium	Induction of $PBP3_{SAL}$ during infection favoring higher bacterial loads in murine models of infection when a wild type was compared with a knockout mutant	71
AmiA, AmiC, and SufI and the pathways for their correct expression (Cpx system) and export to the periplasm (Tat)	Periplasmic *N*-acetylmuramoyl-L-alanyl amidases that cleave stem peptides from glycan chains on PGN (AmiA and AmiC) and a divisomal transpeptidase (SufI)	*S.* Typhimurium (also *E. coli*)	For AmiA and AmiC, impairment in septation and separation of daughter cells (filamentation) and virulence attenuation in a BALB/c mouse infection model; for SufI, increased sensitivity to detergents and cationic antimicrobial peptides and impaired motility	72–75
Modification of PGN related to morphogenesis				
PgdA	An *N*-deacetylase for PGN modification	*H. pylori*	Decreased resistance of PGN to lytic activity of lysozyme; double mutant (PgdA-PatA) impaired for mouse colonization	81
PatA	A putative *O*-acetyltransferase for PGN modification	*H. pylori*	Decreased resistance of the PGN to lytic activity of lysozyme; double mutant (PgdA-PatA) impaired for mouse colonization	81
Csd1 to Csd3 and CcmA	Periplasmic endopeptidase homologues essential to allow a decrease in PGN cross-linking levels	*H. pylori*	In single mutants, a curved instead of a helical shape, which was related to a decrease in colonization capacity in mouse stomach model competitions	80
Ape1	An *O*-acetylpeptidoglycan esterase responsible for de-*O*-acetylation of PGN	*C. jejuni*	Changes in PGN biochemistry; defects in virulence-associated features, including motility, biofilm formation, sodium deoxycholate resistance, adhesion, invasion, intracellular survival, induction of IL-8 release, and impairment of chick colonization	79
Pgp1	A D,L-carboxypeptidase involved in maintenance of cell shape, cleaving monomeric tripeptides to dipeptides	*C. jejuni*	Loss of helical shape; increase in the level of stimulation of NOD-1 receptors and derived induction of IL-8 release; decreased motility and biofilm formation; deficient for chick colonization	77
Pgp2	An L,D-carboxypeptidase involved in maintenance of cell shape, converting PGN tetrapeptides into tripeptides, which in turn are substrates for Pgp1	*C. jejuni*	Loss of helical shape; defective in motility on semisolid agar and biofilm formation; reduced fitness in chick colonization model	78
LtgA and LtgD	Nonessential, nonredundant lytic transglycosylases	*N. gonorrhoeae*	Decreased envelope integrity, leading to increased susceptibility to lysozyme and neutrophil killing	82

(Continued on next page)

TABLE 3 (Continued)

Target	Role(s)	Species	Effect(s) of target disruption	Reference(s)
PGN-degrading enzymes related to sacculus remodeling to allow elongation and recycling				
MltB	A membrane-bound lytic murein transglycosylase participating in PGN degradation	*N. meningitidis*	Inability to cause systemic infection in an infant rat model	84
MtgA	A biosynthetic transglycosylase	*Brucella* spp.	Upregulation during infection; in a knockout mutant, lower virulence in a mouse infection model	85, 86
MltE	A membrane-bound lytic transglycosylase	*Erwinia amylovora*	Upregulation during infection; in a knockout mutant, reduced virulence and growth in a pear model	85, 87
90_A18ORF1	A soluble lytic murein transglycosylase	*Haemophilus influenzae*	Upregulation of the 90_A18ORF1 gene during infection (a KO mutant is not available)	85, 88
Ipx10.11	A lytic murein transglycosylase	*Pseudomonas syringae* pv. tomato	Upregulation during infection; in a knockout mutant, impaired virulence in an *Arabidopsis thaliana* infection model	85, 89
Ddc	A D-alanine–D-alanine carboxypeptidase	*A. baumannii*	Hypersusceptibility to serum and polymyxin B; defective in intramacrophage survival; a drastically reduced capacity for survival in a mouse bloodstream infection model	83
ShyA, ShyB, and ShyC	Periplasmic proteins containing M23 family peptidase domains; for ShyA, a D,D-endopeptidase preferentially cleaving cross-links between tetrapeptides and located in the lateral cell wall; for ShyC, preferential location in the septum	*V. cholerae*	In a ShyA and ShyB double mutant, a significant growth deficiency; in a *shyC* mutant with depletion of ShyA, dramatic impairment of cell elongation rates and a significant increase in cell width, which would presumably affect attachment to host tissue	90

takes part in the intracellular division of *S. enterica* serovar Typhimurium and therefore is probably not negligible for pathogenesis in the host has been proposed (71). More specifically, this enzyme (namely, $PBP3_{SAL}$) is 63% identical to PBP3 and, in contrast to the latter, is not essential (a lack of PBP3 renders cells unable to grow at neutral pH) but exerts a positive effect on septation and cell replication in acidic environments, such as those inside phagosomes. In fact, $PBP3_{SAL}$ expression has been shown to be induced during infection in the murine model, which has been related to the higher bacterial loads of the wild-type strain than of $PBP3_{SAL}$ knockout mutants (71). Still in *Salmonella* (but also in *E. coli*), the coordinate roles of AmiA, AmiC, and SufI (also named FtsP) have been shown to be very important for full virulence. These three proteins have been demonstrated to be essential for septation, with AmiC and SufI being located in the divisome, the complex of at least 15 proteins that allows the formation of the septum. Interestingly, the defects not only in these proteins but also in the pathways that they use to be sufficiently expressed (through the envelope stress-sensing two-component system CpxA-CpxR) and/or exported to the periplasm (through the twin arginine translocation [Tat] system) have been shown to be essential for full virulence, entailing the multiple effects from the deletion of genes encoding these proteins, including defects in motility and filamentation, an increase in susceptibility to detergents, etc. (Table 3) (72–75). Nevertheless, the implication of these pathways in a wide variety of processes, probably not directly related to septation or divisome formation, also must be taken into account as the cause of the cited virulence impairments.

Finally, in *Haemophilus influenzae*, three lysostaphin-like metalloproteases, also called LytM factors, YebA, EnvC, and NlpD, have been studied in depth (76). These factors, widely described in Gram-negative bacteria, participate in cell division by modulating the cleavage and remodeling of PGN, and in the specific case of *H. influenzae*, they have been reported to affect the outer membrane composition when disrupted, with negative consequences, such as altered cell structure, a loss of periplasmic proteins, and a reduced capacity for cell adhesion, survival against serum, and biofilm formation (Table 3) (76).

Periplasmic elements involved in morphogenesis. With regard to periplasmic enzymes related to cell morphogenesis, there are some recent works about two

enzymes (namely, the carboxypeptidases Pgp1 and Pgp2) in *Campylobacter jejuni* shown to be essential for correct cell shaping. In fact, the loss of the typical helical shape of this species has been linked to the loss of virulence in a chick infection model, besides additional features linked to pathogenesis reduction (Table 3) (77, 78). In the same species, it has been shown that an increase in susceptibility to bile, together with a decrease in many other parameters leading to a less virulent outcome in the chick infection model, arises as a result of abnormally high levels of PGN O-acetylation (caused by inactivation of the Ape1 esterase) (see Table 3 for further details) (79). Similarly, with regard to morphogenesis, it has also been shown that a helical shape is essential for full virulence in another digestive tract pathogen, *Helicobacter pylori*, since the disruption of different endopeptidases (Csd1, Csd2, Csd3, and CcmA; Table 3), which cause relaxation of the PGN cross-linking level, leads to a curved instead of a helical cell morphology, finally causing a decreased capacity for mouse stomach colonization (although no great effects on motility or resistance to stomach environment stresses were observed) (80). Still, in *H. pylori* and also with regard to resistance to external aggressions, the deletion of two PGN-modifying enzymes (the *N*-deacetylase PgdA and the *O*-acetyltransferase PatA) has been shown to increase the susceptibility to lysozyme, probably because the loss of these enzymes entails the inability to modify the PGN in order to avoid enzymatic recognition by lysozyme (81). More specifically, their combined inactivation led to a PGN circa 5 times more susceptible to lysozyme-mediated *in vitro* degradation than the wild-type purified PGN. The modification of PGN to avoid the action of certain immune proteins has been largely described in Gram-positive bacteria (mainly thanks to changes in the chemical properties/structure of the PGN molecules theoretically able to bind the active site of the protein), but through this work it was shown to be also used as a very notable strategy by certain Gram-negative species (81). In fact, the loss of the cited enzymes rendered strains with a very significant reduction in the capacity for stomach colonization in a murine model (81) (Table 3).

The decrease in resistance to lysozyme as a consequence of alterations in enzymes involved in PGN biology has also been observed in other species, such as *Neisseria gonorrhoeae* (Table 3). More specifically, the lytic transglycosylases LtgA and LtgD from the gonococcus were demonstrated to contribute to the maintenance of envelope integrity, limiting exposure to the lysozyme and other antimicrobial proteins from neutrophil granules (82). Besides these and the previously mentioned data regarding PGN modifications affecting the activity of some host elements targeting the cell wall (lysozyme, bile, etc.), the large topic of the Gram-negative PGN interaction with the host (including receptors, inflammatory implications, and the specific mechanisms that some bacteria display to inhibit the activity of lysozyme) will be more widely approached below.

Periplasmic peptidoglycan-degrading enzymes involved in remodeling and recycling. Finally, dealing with enzymes related to PGN degradation for remodeling (mainly to allow elongation) and/or recycling purposes, some examples in different species can be cited. Although in some cases the exact role of the involved elements has not yet been elucidated, the consequences for fitness/virulence have been described (Table 3). In this context, in *A. baumannii*, for instance, the D-Ala–D-Ala-carboxypeptidase-encoding gene *ddc* has been identified by Subashchandrabose and colleagues (83) to be one of those essential for bloodstream infection development. More specifically, the disruption of this target has been shown to cause hypersensitivity to complement and antimicrobial peptides as well as impaired survival within murine macrophages (83). Moreover, in *N. meningitidis* the periplasmic lytic transglycosylase MltB, allegedly contributing to PGN turnover, was shown to be indispensable for the development of systemic infection in an infant rat model, presumably because of certain alterations to the cell wall composition or even because of the elicited metabolic disorders caused by its inactivation (84). Still in this genus, but specifically in the gonococcus, the nonessential lytic transglycosylases LtgA and LtgD, besides having a crucial role in proinflammatory PGN fragment release to the extracellular medium (the

TABLE 4 Cytosolic targets involved in PGN biosynthesis and recycling

Target	Role(s)	Species	Effect(s) of target disruption	Reference(s)
LdcA	An L,D-carboxypeptidase cleaving the terminal D-alanine from tetrapeptides proceeding from cell wall turnover, releasing tripeptides which enter the recycling pathway to finally provide UDP-NAcMur-pentapeptides	*E. coli*	A dramatic decrease in the degree of stem peptide cross-linking, leading to weakening of the cell wall and to bacteriolysis in the stationary phase of growth in liquid culture	96
UppP	An undecaprenyl pyrophosphate phosphatase essential for synthesis of undecaprenyl phosphate	*Burkholderia* spp.	Increased susceptibility to lysozyme, hypotonic and hypertonic shock, and centrifugal force; decreased capacity to colonize the stinkbug gut	95
Mpl-1	A UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-*meso*-diaminopimelate ligase involved in PGN recycling	*N. meningitidis*	An inability to cause systemic infection in an infant rat model	84
AmpD	An *N*-acetylmuramyl-L-alanine amidase involved in PGN recycling	*N. meningitidis*	An inability to cause systemic infection in an infant rat model; hyperexpression during infection in wild-type strains	84
MurA	An enolpyruvyl transferase acting in the cytosolic transformation of UDP-NAcGlc to UDP-NAcMur	*P. aeruginosa*	Impaired growth in minimal medium, changes in colony morphology, dampened capacity for lung colonization in a murine model of intratracheal infection, and decreased survival against macrophage-mediated killing	101
MurD	A UDP-*N*-acetylmuramyl L-alanyl-D-glutamate-diaminopimelate ligase allowing the cytosolic building of the stem peptide in the PGN precursor UDP-NAcMur pentapeptide	*P. aeruginosa*	Impaired growth in minimal medium, changes in colony morphology, dampened capacity for lung colonization in a murine model of intratracheal infection, and decreased survival against macrophage-mediated killing; decreased pathogenesis in a plant infection model	101
MurF	A UDP-*N*-acetylmuramyl L-alanyl-D-glutamyl-diaminopimelate-D-alanyl-D-alanine ligase allowing the cytosolic building of the stem peptide in the PGN precursor UDP-NAcMur pentapeptide	*P. aeruginosa*	Impaired growth in minimal medium, changes in colony morphology, abnormally elongated cells, and decreased survival against macrophage-mediated killing; decreased pathogenesis in a plant infection model	101
DapA	A dihydrodipicolinate synthase responsible for the synthesis of *m*-DAP and lysine	*Serratia marcescens*	Upregulation during infection; in KO mutants, greater sensitivity to hypotonic shock, an aberrant irregular elliptical shape, and disruption of the cell wall structure, as well as increased cytotoxicity and hemolysin production and a reduced capacity for attachment to surfaces	85, 100

issue is reviewed below), have been shown to be very important for envelope integrity, since their disruption leads to increased susceptibility to lysozyme and neutrophil killing (82). Other lytic transglycosylases with implications for fitness/virulence are the MltE (*Erwinia* spp.), 90_A18ORF1 (*H. influenzae*), and Ipx10.11 (*Pseudomonas syringae*) elements, although very little information regarding the molecular basis has been published (Table 3) (85–89).

Finally, in *V. cholerae*, some interesting targets presumably related to cell wall remodeling to allow growth have been identified, namely, the periplasmic proteins ShyA, ShyB, and ShyC, all of which show M23 family peptidase domains (90). In their work, Dörr and colleagues reported several findings, such as the nature of ShyA as a D,D-endopeptidase with preferred activity cleaving cross-links between tetrapeptides in a key step to allow cell elongation (90). Moreover, although none of these three proteins was shown to be essential *per se*, the double deletion of ShyA and ShyB caused severe growth deficiencies, and the depletion of ShyA in a ShyC mutant rendered cells with increased widths and significant defects in elongation rates (the double ShyA and ShyC KO mutant was not viable). Finally, the locations of ShyA and ShyC were shown to be different: the first is in the lateral cell wall, whereas the latter is preferentially in the septum, although its specific role during septation has never been studied (Table 3) (90).

Cytosolic Enzymes Working on Peptidoglycan Biosynthesis and Recycling

This section reviews the role of cytoplasmic enzymes involved in the pathways of biogenesis of the PGN monomers (NAcGlc-NAcMur-pentapeptides) that have been shown to also influence fitness/virulence. These are displayed in Table 4. Obviously, this is not a review of the anabolic pathways leading to PGN unit synthesis/recycling, but to learn more about the issue, there are interesting reviews (19, 91–94). In a few words, the cytosolic biogenesis pathways of the elements constituting the PGN could be

separated into reactions allowing the synthesis of the sugars (NAcMur and NAcGlc); the synthesis of the amino acids and the stem peptide itself (initially a pentapeptide), thanks to the sequential incorporation of L-alanine (L-Ala), D-glutamic acid (D-Glu), *meso*-diaminopimelic acid (*m*-DAP), and the D-alanine–D-alanine (D-Ala–D-Ala) dimer; and of course, the reactions of the linkages among these components. However, we should also consider the events that allow the transit of the PGN monomers (disaccharide-pentapeptides) across the inner membrane, specifically, through the lipid I and II cycles and flippase action, which release the PGN monomers into the periplasm to be incorporated into the nascent PGN. Moreover, the elements that participate in cytosolic recycling processes and that allow for the reuse of some of the cited compounds (disaccharide peptides, peptides, and monosaccharides), once they are released from the PGN, instead of their *de novo* synthesis, are also assessed here (19, 93).

To start with, although UppP is not a strictly cytosolic protein but an inner membrane phosphatase that allows the synthesis of undecaprenyl phosphate (the basis for the lipid I and II structures), which, in turn, allows the export of the PGN monomers to the periplasm, UppP could be included in this section since it allows the final steps of anabolism before the new monomers are added to the PGN. It has been reported that defects in this phosphatase in the genus *Burkholderia* are responsible for increased susceptibilities to lysozyme and other PGN stresses, such as hyper- and hypo-osmotic shocks and centrifugal force, rendering strains with impaired virulence and an impaired capacity to infect the stinkbug gut (Table 4) (95). In a step before the above-mentioned lipid I and II formation, in *E. coli* the LdcA protein was first identified to be essential for the transformation of stem tetrapeptides into tripeptides, once they have entered the cytosol (Table 4) (96). The tripeptides can then be used for recycling, allowing the final addition of the terminal D-Ala–D-Ala (thanks to MurF) to finally constitute the UDP-NAcMur-pentapeptides to be incorporated into the PGN. Nevertheless, when LdcA is deleted, the precursors formed are the UDP-NAcMur tetrapeptides, which are incorporated into PGN but which do not allow transpeptidation, since the scission of the terminal D-Ala of the pentapeptides is required for this process (13). Therefore, LdcA deletion results in a low degree of cross-linked stem peptides, which finally leads to the weakening of the PGN structure and to bacteriolysis in the stationary growth phase in liquid culture (96, 97).

In *N. meningitidis*, in its turn, Mpl-1 and AmpD, two cytoplasmic enzymes involved in PGN recycling, were shown to be indispensable for the development of systemic infection in an infant rat model, although the exact molecular basis for the virulence attenuation linked to the inactivation of Mpl-1 or AmpD was not ascertained (Table 4) (84). More specifically, Mpl-1 allows the ligation of the above-mentioned recycled tripeptides (L-Ala–D-Glu–*m*-DAP) to UDP-NAcMur, whereas AmpD cleaves the bond between anhydro-NAcMur and stem peptides, once these fragments are internalized into the cytosol after release from the PGN (93). Since the role of the AmpD amidases (in this review, those from *P. aeruginosa*, *Neisseria* spp., *Ralstonia solanacearum*, or *S. enterica* serovar Typhimurium, for instance, are cited [84, 85, 98, 99]), apart from recycling, is intimately linked to β-lactamase regulation, its influence on fitness/virulence will be analyzed later in the section approaching the topic of the intrinsic β-lactamases (see Table 7).

Some other examples of cytosolic targets, such as DapA from *Serratia marcescens*, involved in the synthesis of *m*-DAP, were previously gathered by Cloud-Hansen et al. (85). DapA was reported to intervene in swarming motility and envelope architecture, and its inactivation led to an altered cell wall structure and increased susceptibility to osmotic shock but, paradoxically, also to increased hemolysin production and cytotoxicity (85, 100), although the basis for these observations remains elusive.

Some other enzymes, such as those encoded by *P. aeruginosa* genes *murA*, *murD*, and *murF*, act during the last cytosolic steps of synthesis of the PGN precursor UDP-NAcMur-pentapeptide (101) (Table 4). More specifically, MurA intervenes in UDP-NAcGlc transformation into UDP-NAcMur, whereas MurD and MurF intervene in the ligation of D-Glu to UDP-NAcMur-L-Ala and the final D-Ala–D-Ala, respectively, to con-

stitute the UDP-NAcMur-pentapeptide (102). They have very recently been described to dampen bacterial fitness and virulence when disrupted, probably because their inactivation entails disorders in PGN metabolism and a derived stress, although the exact effects have not been studied. In any case, what has been proved is that mutants with a single knockout of the cited genes are significantly impaired in their growth in minimal medium and display changes in colony morphology, and in the specific case of the *murF* mutant, abnormally elongated cells are observed by microscopy. In addition, all the cited mutants are more susceptible to macrophage-mediated killing and dampened in terms of growth in the lungs of intratracheally infected mice, intriguingly, with the exception of the *murF* mutant, which shows wild-type behavior. Also paradoxically, the *murA* mutant was the only one not impaired in terms of the pathogenesis elicited in a plant model (101) (Table 4).

THE PEPTIDOGLYCAN, A BARRIER TO BE LOCALLY MODIFIED IN ORDER TO ALLOW THE FULL PERFORMANCE OF ESSENTIAL VIRULENCE FACTORS

Again, from a partially structural point of view, the PGN has to be considered a barrier to be opened in order to allow the correct assembly, anchoring, and performance of structures that are considered very important for virulence, such as the flagella and secretion systems (which are indispensable for motility and the delivery of toxins, respectively) (59, 103). Therefore, the enzymes responsible for opening local gaps in the PGN linked to the insertion of these elements have been described in different Gram-negative species as targets to dampen motility and toxicity, also highlighting the strict control under which they usually work; since these enzymes have autolytic activities, they could lead to cell lysis if they work in a dysregulated manner (103–105). Table 5 displays different examples of PGN enzymes essential for the correct assembly of secretion systems and flagella, together with the species and the data regarding the specific role and the effects of inactivation of each target.

Secretion Systems

To cite some examples regarding the secretion systems, it must be stated that most information is related to the insertion and assembly of the systems that directly inject the effector proteins into the cytoplasm of the host cells: the type III, type IV, and type VI secretion systems (T3SS, T4SS, and T6SS, respectively). However, for the release of toxins to the extracellular medium without injection, the information on enzymes responsible for opening windows in the PGN to insert the type II secretion system (T2SS) machinery is much scarcer (and nonexistent for the type I and V secretion systems, to our knowledge) and is linked to only a few species, such as *Aeromonas hydrophila*. In this species, it has been shown that the assembly of the secretion channel ExeD (which is also called secretin and which is essential to enable the rest of the T2SS apparatus [106]) in the outer membrane is dependent on the inner membrane ExeAB complex (107–109) (Table 5). More specifically, it has been proposed that ExeA shows a PGN-binding region that could open pores in the PGN to allow the transport and assembly of ExeD (107–109). But interestingly and very recently, it has been shown that alterations in the degree of PGN cross-linking, achieved through the hyperexpression of the D,D-carboxypeptidase PBP5, allow the assembly of ExeD and, therefore, of T2SS in an ExeAB-independent manner (110).

In the case of T3SS, the linked PGN-degrading enzymes have been studied in-depth in *Escherichia coli* (mainly in enterohemorrhagic strains, with the EtgA enzyme being identified as a key actor in the process) and in *Pseudomonas* and some closely related genera, such as *Xanthomonas* (111–116) (Table 5).

In the case of T4SS and T6SS, which are involved not only in eukaryotic cell killing but also in the injection of toxins to eliminate bacterial competence, the species with which the most studies have been performed are quite diverse and heterogeneous: *H. pylori*, *Agrobacterium tumefaciens*, *Neisseria gonorrhoeae*, *Acinetobacter* spp., enteroaggregative *E. coli*, and even *Bordetella pertussis*, in whose pertussis toxin secretion apparatus (which belongs to T4SS and which comprises the products of the nine *ptl*

TABLE 5 Targets related to the proper assembly and performance of essential virulence factors across the peptidoglycan

Target	Role(s)	Species	Effect(s) of target disruption	Reference(s)
Secretion systems				
ExeAB	PGN binding and remodeling to allow secretin ExeD insertion and T2SS multimerization, assembly, and performance	*A. hydrophila*	Loss of aerolysin secretion and of lipase activity in culture supernatants	107–109
HpaH	A lytic transglycosylase required for T3SS assembly	*X. campestris*	Attenuated for pathogenicity in a plant model	116
HrpH, HopP1, and HopAJ1	Lytic transglycosylases coregulated by the T3SS to allow the translocation of effector proteins into plant cells	*P. syringae*	Attenuated for pathogenicity in a tobacco plant model	115
L0045	A putative lytic transglycosylase critical for T3SS	Enterohemorrhagic *E. coli*	T3SS impairment	114
EtgA	A lytic transglycosylase required for T3SS assembly	Enterohemorrhagic *E. coli*	Attenuated for T3SS activity and red blood cell lysis	112, 113
MltE	A lytic transglycosylase required for T6SS assembly	Enteroaggregative *E. coli*	Loss of T6SS function and, consequently, cytotoxicity impairment	119
VirB operon	A transglycosylase with activity that allows the formation of the T pilus, a subassembly of the *vir* T4SS	*A. tumefaciens*	Loss of tumorigenesis in a *Kalanchoe daigremontiana* plant model	117
AtlA	A transglycosylase with activity that allows the assembly of T4SS	*N. gonorrhoeae*	Loss of allolysis in *E. coli*	118
TagX	A L,D-endopeptidase required for T6SS assembly	*Acinetobacter* spp.	Loss of T6SS function and, therefore, cytotoxicity impairment; a decrease in bacterial killing capacity in *E. coli* coculture assays	120
PtlE	A peptidoglycanase responsible for local PGN degradation during Ptl secretion complex assembly	*B. pertussis*	Decreased release of pertussis toxin, leading to reduced effects (such as focal adhesion loss) on CHO cells	121
Flagellum				
FlgJ	A β-*N*-acetylglucosaminidase required for degradation of PGN to allow assembly of flagella	*S. enterica* and other beta- and gammaproteobacteria	Nonmotile phenotype	122
Sltf	A lytic transglycosylase interacting with FlgJ to allow the penetration of the nascent flagellar structure across the PGN	*Rhodobacter sphaeroides*	Nonmotile phenotype	123, 124
MltD	A lytic transglycosylase contributing to maturation of PGN for the proper anchoring and functionality of the flagellar motor	*H. pylori*	Nonmotile phenotype	125

[pertussis toxin liberation] genes) a peptidoglycanase (PGNase) activity linked to PtlE has been described (117–121).

Either way, what seems to be a general trend among the different space-making autolysins, which is the name given to these enzymes in a very interesting review on the topic (103), is their obvious PGN-degrading activity: they usually display the same enzymatic profile, breaking the bonds between the NAcMur and the NAcGlc disaccharide units of the glycan chains, in which case they are considered lytic muramidases or transglycosylases, although some exceptions do exist, such as the L,D-endopeptidase activity attributed to TagX of *Acinetobacter* spp. (120). Another general trend is the impairment of the toxicity and virulence of the different knockout mutants studied in these works, as is obvious for a dampened system of injection of effector proteins in the host cells (Table 5).

Flagella

In the case of enzymes that are indispensable for allowing the correct assembly of the flagellar structure through the PGN and, hence, that are essential for correct

motility, given that the bodies of the described secretion systems and flagellar apparatus are similar, it could be expected that the profiles of the PGN-degrading enzymes involved would also be alike and would thus be considered typical lytic transglycosylases (122–124). Nevertheless, it has been shown that FlgJ, widely found in beta- and gammaproteobacteria, shows *β-N*-acetylglucosaminidase activity instead (122), although the final role may be the same, similar to what was previously found for the glucosaminidase Auto of the Gram-positive bacterium *Listeria monocytogenes*, shown to be indispensable for virulence and motility (29, 125). In the case of MltD from *H. pylori*, it has been shown that its activity is indispensable not only for correct assembly but also for the appropriate localization of the flagellar motor protein MotB to the bacterial pole to allow motility (Table 5) (125).

The motility itself has an obvious utility for bacteria, as it allows the cells to move in order to expand infection, and hence, a flagellum that cannot be correctly inserted into the PGN would dampen motility and, consequently, virulence (59). Nevertheless, it is important to state that flagella are more than simple motility motors, as they have been described to contribute to adherence to surfaces, to help with differentiation into biofilms, and to assist with the secretion of certain effector molecules, but also to allow further penetration through tissue structures or to activate phagocytosis to gain entry into eukaryotic cells (59, 126). Moreover, and in an opposite sense, it has been described that the loss of flagellar motility is usually selected in *P. aeruginosa* during chronic infection in cystic fibrosis patients, since it seems to entail an impairment of phagocytosis, an issue which adds complexity to the role of flagella during pathogenesis (127). Therefore, proper flagellar anchoring and performance can have wider implications for virulence than solely motility, which makes this topic a very promising antivirulence target worthy of study from a multidisciplinary point of view.

INTERACTION OF PEPTIDOGLYCAN WITH THE HOST

Release of Peptidoglycan Fragments as an Inflammation-Mediated Pathogenesis Mechanism

As opposed to what happens with Gram-positive bacteria, given the presence of the outer membrane and the lipopolysaccharide wrapping the Gram-negative PGN, it was classically thought to have a low degree of interaction with the host receptors (which will be reviewed below), at least when the bacteria are intact. Nevertheless, in a few Gram-negative bacteria, some fragments of their PGN have been typically described to be somehow actively delivered to the extracellular medium to exert diverse biological functions, generally entailing a strong inflammatory response. The targets related to this phenomenon are displayed in Table 6. Indeed, the role of *B. pertussis* tracheal cytotoxin (TCT) as a virulence factor heavily relies on its proinflammatory power: TCT is a muramyl peptide (specifically, the 921-Da fragment *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl–D-alanine) derived from PGN, thanks to the activity of still uncharacterized periplasmic lytic enzymes, which causes damage to the respiratory epithelia and extrusion of ciliated cells through the generation of increased levels of nitric oxide and the induction of a massive inflammatory response (128–130). Similar features have been described for PGN-derived fragments of *N. gonorrhoeae* and *N. meningitidis* (85, 131, 132). More specifically, these PGN fragments are referred to as PGN-derived cytotoxin (PGCT), which is a monomer identical to the TCT cited above. In *N. gonorrhoeae*, the main enzymes responsible for the generation of the PGCT were initially identified to be the lytic transglycosylases LtgA and LtgD (133, 134). Interestingly, what has also been shown for *N. gonorrhoeae* is that, in comparison with other nonpathogenic species of the genus (such as *Neisseria sicca* and *Neisseria mucosa*), the gonococcus shows a poorly efficient PGN recycling machinery (probably because of a less functional AmpG permease, the door to the cytosol for the PGN fragments to be recycled). This circumstance has been associated with the higher degree of PGN fragment release (and, hence, of PGCT) in *N. gonorrhoeae* than in other species from the genus. In fact, the blockade of cytosolic doors for PGN fragments through the inactivation of AmpG (but also of the Opp-MppA

TABLE 6 Targets related to host detection and response against PGN

Target	Role(s)	Species	Effect(s) of target disruption	Reference(s)
Targets involved in PGN fragment release				
AtlA and LtgA	Lytic transglycosylases allowing the release of the peptidoglycan-derived cytotoxin	*N. gonorrhoeae*	Reduction in monomeric released PGN	133
LtgD	A lytic transglycosylase contributing, together with AtlA and LtgA, to the release of the peptidoglycan-derived cytotoxin	*N. gonorrhoeae*	Absence of PGN monomer release and, instead, active release of large soluble fragments in an LtgA-LtgD double mutant	134
LdcA	A periplasmic serine protease L,D-carboxypeptidase cleaving the tetrapeptide that therefore provides tripeptide stems and that is also capable of breaking specific peptide cross-bridges (endopeptidase activity)	*N. gonorrhoeae*	Elimination of NOD1 and NOD2 activation by soluble PGN products from *N. gonorrhoeae* when LdcA is disrupted, presumably leading to a significant decrease in the inflammatory response	138
Slt	A periplasmic lytic transglycosylase that is necessary for the release of PGN fragments and whose expression is induced *in vivo* during infection	*H. pylori*	Reduction of the PGN fragment release and, consequently, of IL-8 delivery by the host	142, 143, 145
SltY	A periplasmic lytic transglycosylase homologue of *H. pylori* Slt	*S. flexneri*	Effects presumably similar to those derived from *H. pylori* Slt inactivation	146
MppA	A periplasmic binding protein allowing the entrance of oligopeptides into the cytosol	*S. flexneri*	Strong attenuation of a knockout mutant in nasal and intravenous infections in mice; increased PGN fragment release to the extracellular medium; increased NOD-1-mediated activation of the NF-κB route	148
AmpG	A permease specific for PGN fragments containing the disaccharide *N*-acetylmuramic acid–*N*-acetylglucosamine	*S. flexneri*	Increased PGN fragment release to the extracellular medium; increased NOD-1-mediated activation of the NF-κB route	148
AmpG	A permease specific for PGN fragments containing the disaccharide *N*-acetylmuramic acid–*N*-acetylglucosamine	*V. fischeri*	A circa 100-fold increase in net PGN monomer release	137
LtgA, LtgD, and LtgY	Lytic transglycosylases	*V. fischeri*	Very poor accumulation of PGN monomers in culture supernatants; increased susceptibility of the symbiont host squid *Euprymna scolopes* to superinfection	137
Host PGN receptors				
NOD1	Detection of PGN fragments with a terminal *m*-DAP, promoting the secretion of inflammatory cytokines in response	*Homo sapiens*	Contradictory results, depending on the work; in some studies, a key role of the receptor in the innate immune defense against Gram-negative bacteria and the contrary finding in others	174–176, 179, 180–183
NOD2	Detection of MDP, promoting the secretion of inflammatory cytokines in response	*Homo sapiens*	Contradictory results depending on the work; in some studies, a key role of the receptor in the innate immune defense against Gram-negative bacteria and the contrary finding in others	173–175, 180–184
NLRP1	Detection of MDP and activation of the inflammasome in response	*Homo sapiens*	Pyroptosis, acute lung injury, and morbidity in a mouse model with activation of NLRP	185
Hexokinase-NLRP3	Detection of *N*-acetylglucosamine and activation of an inflammasome in response	*Homo sapiens*	Expression of IL-1β and IL-18, generation of reactive oxygen species, pyroptosis, and acute lung injury with activation of NLRP3 in a mouse model	189–191, 195, 196
PGRP2	A peptidoglycan recognition protein with amidase activity; controversial reports regarding its alleged bactericidal capacity and inflammation regulatory capacity after PGN detection	*Homo sapiens*	In some studies with PGRP2 deletion in mice, a better outcome for the host after infection (linked to a decrease in the inflammatory response); protection against infection in other studies	170, 245–248
Bacterial lysozyme inhibitors				
Ivy	An inhibitor of vertebrate lysozyme (c-type lysozyme inhibitor)	*E. coli*	Increased *in vitro* susceptibility to lysozyme plus lactoferrin and saliva	207, 208
MliC	A membrane-bound inhibitor of c-type lysozyme	*E. coli*	Increased susceptibility to lysozyme and serum and decreased virulence in chicken infection model	205, 209

(Continued on next page)

TABLE 6 (Continued)

Target	Role(s)	Species	Effect(s) of target disruption	Reference(s)
Ivy	An inhibitor of vertebrate lysozyme (c-type lysozyme inhibitor)	*Edwardsiella tarda*	Impaired tissue dissemination, resistance to serum, replication within macrophages, and overall virulence in a turbot (*Scophthalmus maximus*) model	211
MliC	A membrane-bound inhibitor of c-type lysozyme	*Edwardsiella tarda*	Impaired tissue dissemination, resistance to serum, replication within macrophages, and overall virulence in a turbot (*Scophthalmus maximus*) model	212
Ivy	An inhibitor of vertebrate lysozyme (c-type lysozyme inhibitor)	*Yersinia pestis*	Hypersusceptibility to lysozyme and polymorphonuclear phagocytes	210
MliC	A membrane-bound inhibitor of c-type lysozyme	*S. enterica* serovar Typhi	A reduction in intramacrophage survival	204
PliC	A periplasmic inhibitor of c-type lysozyme	*S. enterica* serovar Enteritidis	Hypersusceptibility to lysozyme plus lactoferrin	204
PliG	A periplasmic lysozyme inhibitor of g-type lysozyme	*E. coli*	Hypersusceptibility to g-type lysozyme	219, 220
PliI	A periplasmic inhibitor of i-type lysozyme	*Aeromonas hydrophila*	Decreased tolerance against *Tapes japonica* shell lysozyme	218
LipA and LipB	Potential c-type lysozyme inhibitors with structural similarities to MliC/PliC proteins	*Moraxella catarrhalis*	Hypersusceptibility to human lysozyme plus apolactoferrin	222

system, specific for oligopeptides) has been shown to increase the release of PGN fragments to the extracellular medium in *E. coli* or *Vibrio fischeri*, for instance (132, 135–137). Besides, the periplasmic L,D-carboxypeptidase LdcA, also of *N. gonorrhoeae*, has recently been shown to play a key role in the generation of fragments detectable by human NOD1 and NOD2 receptors (our intracellular specific PGN receptors, which will be reviewed below) and the derived responses (138) (Table 6).

Moreover, the range of Gram-negative species in which the release of PGN fragments is considered an active mechanism to damage the host and, hence, in which the cited PGN fragments could also be considered bona fide toxins is getting amplified and includes, for instance, *Haemophilus influenzae*, in which some PGN fragments and specific modifications to them have been shown to induce different degrees of inflammation and brain edema in animal models of meningitis (139–141). Still in this context, in *H. pylori* the delivery of PGN fragments into host cells via OMVs and via the *cag* type IV secretion system has been proven, leading to host tissue self-injury mediated by an excessive inflammatory response (142, 143). In fact, it has been proposed that *H. pylori* does not display a significantly productive PGN recycling pathway (which resembles that in the already mentioned case of gonococcus), which supports the presence of major PGN degradation fragments readily available in the periplasm and, consequently, in the extracellular medium, being detected by our specialized receptors to elicit the release of inflammatory cytokines, such as interleukin-8 (IL-8). Linked to this, it has been demonstrated that the lytic transglycosylase Slt is necessary for IL-8 secretion in this context and, moreover, that the production of Slt is enhanced *in vivo* in human gastric biopsy specimens, suggesting that the release of PGN fragments occurs actively and that the triggered inflammation is a desired effect for the pathogen (142, 144, 145). Finally, *Shigella flexneri* shares some of the last cited features: (i) its SltY lytic transglycosylase (a homologue of *H. pylori* Slt) is upregulated during infection (144, 146), and (ii) when PGN recycling is blocked (by inactivating either *ampG* or *mppA*), the release of PGN fragments increases, in turn leading to a higher NOD1-mediated NF-κB activation of inflammatory cytokine genes in comparison with that in the wild type. Nevertheless and intriguingly, only the mutant with a mutation in MppA was shown to be impaired for virulence in murine models (147, 148), once more showing the high level of complexity of the interplay that we review here.

The biologically active PGN fragments reviewed in this section, derived from active degradation during bacterial growth, are qualitatively and quantitatively different from those released from lysed bacteria, which, however, can also stimulate the innate immune system through our PGN-specific receptors (85). Therefore, this classic concept

of the Gram-negative PGN as a structural element with no additional biological functions is probably becoming obsolete (144). For this reason, maybe not only the cited species (*B. pertussis*, *N. meningitidis*, *N. gonorrhoeae*, *H. pylori*, or *S. flexneri*) but also others should be considered active PGN fragment deliverers; these other PGN fragment deliverers may also release PGN fragments by means of OMVs, for instance, during regular growth and not only after cell lysis (149–151). This concept could be supported by the fact that some Gram-negative species have been shown to upregulate certain genes related to PGN metabolism during infection, such as the mentioned MtgA from *Brucella* spp. or MltE of *Erwinia amylovora* (85) (Tables 3, 4, and 6).

In this sense, it has to be noted that some works have recently shown in *E. coli* but also in other more niche-specific species, such as some periodontal pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*), the association between their released OMVs and the activation of NOD1 and/or NOD2 in the host, which strongly suggests that the presence of PGN fragments within the cited vesicles potentially contributes to the inflammatory response in the infected tissues (152–154). In fact, in recent years OMVs have been recognized to be effective virulence factor delivery systems, and hence, the presence of certain PGN fragments within OMVs could contribute to this role (155–157). Moreover, to conclude and as additional evidence that Gram-negative PGN biology interacts with pathogenesis (in this case, with a close relation to OMVs), some studies have directly related PGN dynamics to the modulation of OMV production levels, which would have obvious consequences for virulence (158, 159). In short, it has been proposed that modulation of the PGN-outer membrane cross-linking level through the localized fine-tuning of PGN degradation and synthesis (therefore involving different actors, such as endopeptidases; nonessential PBPs; and the Lpp lipoprotein, which cross-links the PGN with the outer membrane) is a very tightly regulated mechanism that Gram-negative bacteria use to modulate vesiculation and contributes to a better adaptation of the bacterium to stress. For instance, the authors suggested that an inverse relationship does exist between OMV production and the PGN-Lpp cross-linking level (158, 159). Nevertheless, quantification of the potential effects derived from this PGN biology-driven OMV level modulation on virulence has not been performed yet.

Host Receptors Involved in the Detection of Gram-Negative Peptidoglycan: Potential Targets To Reduce the Self-Injury-Mediated Pathogenesis of Infections

After mentioning some relevant examples of biologically active Gram-negative PGN fragments and the enzymes involved (an excellent overview of their principal effects on the host was presented by Boneca in his work of 2005 [144]), a brief overview of their human-specific receptors seems suitable (Table 6). A very recent review on the topic, which focused only on *P. aeruginosa*, was published in 2018 and could probably be seen as a nice reference for the rest of the Gram-negative bacteria (160). In this sense, the main response after the detection of PGN by host cells is the induction of an inflammatory response. This is mainly mediated by the cytoplasmic receptors NOD1 and NOD2, although other actors may take part in Gram-negative PGN sensing, as will be mentioned below. NOD1 recognizes PGN fragments with a terminal *meso*-diaminopimelic acid (*m*-DAP), such as NAcGlc–NAcMur–L-Ala–D-isoGlu–*m*-DAP (GM-triDAP) or NAcMur–L-Ala–D-isoGlu–*m*-DAP (M-triDAP), specific for Gram-negative bacteria. NOD2 is a more general sensor, as it can recognize muramyl dipeptide (MDP), which is common to Gram-positive bacteria. NOD2 was classically thought not to recognize muropeptides containing *m*-DAP, although it has recently been shown to be able to bind these fragments even better if *m*-DAP is not at a terminal position (161–163). In any case, the recognition of their PGN ligands by these professional receptors triggers the activation of the innate immune response, mainly through the NF-κB pathway, but also that of the AP-1 transcriptional regulator, enhancing the expression of inflammatory cytokines, such as IL-1β, IL-6, IL-8, and tumor necrosis factor alpha (161, 164, 165). Interestingly, in this context, it has been described that modifications in the PGN composition trigger different levels of

activation of NOD receptors, eliciting differential levels of inflammation, and that this strategy is used by *P. aeruginosa* during chronic infection in cystic fibrosis patients to lower immune detection and, consequently, ameliorate chronic persistence (166). Related to this, in the *in vitro* context it has recently been described that the amidation of *m*-DAP from the stem peptides in the Gram-negative PGN structure would lead to the evasion of detection through NOD1 (167). Nevertheless, to our knowledge, no studies of the potential existence of this mechanism in natural strains exist, and it could be worth performing them in order to gain knowledge of unknown strategies that bacteria can use to avoid immunity mainly in the chronic infection context.

Still related to these topics, it is generally accepted that an excessive level of triggered inflammation leads to self-injury in the host tissues, constituting the pathogenic basis linked to PGN detection classically attributed to Gram-positive bacteria but increasingly to certain Gram-negative bacteria. In fact, different models of PGN-induced inflammatory pathologies (arthritis, uveitis, etc.) have been developed (168–170), and NOD1 and/or NOD2 alterations have been linked to chronic inflammatory diseases, such as asthma, Crohn's disease, or Blau syndrome, among others (161, 165, 168, 171).

Regarding the real impact of these receptors on the innate defense against Gram-negative bacteria, some contradictory results have been published. For instance, NOD2 has been shown to be dispensable for the innate macrophage response against *Yersinia enterocolitica* or for the control of *Coxiella burnetii* infection (172, 173), as have both NOD1 and NOD2 for the proper resolution of *Brucella abortus* infection in a mouse model (174). In the same sense, the inactivation of NOD1 or NOD2 did not increase the susceptibility of mice to polymicrobial sepsis (175).

On the contrary, in several other models of infection, these receptors have been shown to take part in the innate response against various Gram-negative species: for instance, NOD1 against *P. aeruginosa* (176), *H. pylori* (177), *Shigella flexneri* (147), *Campylobacter jejuni* (178), or *Legionella pneumophila* (179). In a model of infection with *Legionella pneumophila*, NOD1 knockout mice were especially impaired in bacterial clearance, neutrophil recruitment, or inflammatory cytokine production, for instance (179). Moreover, NOD1 and NOD2 together have repeatedly been shown to play an important role in the defense against other Gram-negative pathogens, such as *N. gonorrhoeae* (180), *S. enterica* serovar Typhimurium (181, 182), and *Chlamydophila pneumoniae* (183), and in the general neutrophil response (184).

Besides NOD1 and NOD2, some additional cytosolic receptors have been suggested to take part in Gram-negative PGN sensing, although the amount of information on the topic and evidence of real biological significance are still scarce. For instance, NLRP1 is allegedly capable of sensing certain PGN fragments, such as the MDP (common to both Gram-positive and Gram-negative species) and of promoting inflammatory cytokine secretion (mainly IL-1β and IL-18) in response (through inflammasome activation). It has been connected with the pathology of acute lung infections, always related to over-inflammatory self-injuries (185–189). Moreover, in a recent work, Wolf and colleagues (189) have described a very novel mechanism of PGN fragment sensing: the inhibition of our enzyme hexokinase by the NAcGlc proceeding from bacterial PGN causes its dissociation from mitochondrial outer membranes, which in turn activates the NLRP3 inflammasome, all of which lead to the expression of IL-1β and IL-18 (189, 190). In addition, the activation of NLRP1 or NLRP3 inflammasomes has been linked not only to inflammatory cytokine secretion but also to pyroptosis (a programmed cell death mode intimately linked to inflammatory processes) of host cells, therefore contributing to pathogenesis during infection (160, 185, 190, 191–193). In fact, in some other works, as happens with NOD receptors, the involvement of NLRPs and inflammasomes has been linked to certain chronic inflammatory diseases (194). However, as stated before, the real impact of Gram-negative PGN detection through NLRP1 and/or NLRP3 on the pathology of infection has yet not been truly quantified. However, what has recently been shown in several works is that NLRP1 and/or NLRP3 activation (mediated or not by PGN sensing, a circumstance not analyzed enough for the moment) decisively contributes to inflammation and pyroptosis-mediated acute tissue injury in different

models (190, 191, 195, 196) (Table 6). Therefore, these works support the idea of self-injury caused by an overinflammatory response to PGN detection to be one of the bases for the pathogenesis of infection, proposing the tuning of the inflammatory response as a strategy for future treatments (197). In fact, in an organism not classically considered to be an active releaser of PGN fragments, such as *P. aeruginosa*, the detection of these delivered signals has been linked to the virulence of the infection (involving NOD and probably NLRP receptors), once more supporting the idea of the Gram-negative PGN having a considerable and probably neglected importance during infectious disease pathogenesis, with, in this case, importance specifically being related to the detection of and response by the host (176, 186).

Lysozyme Inhibitors: Targets To Increase the Activity of the Innate Immune System

Besides the information regarding the detection of PGN by the host and the elicited response that has been provided, another kind of interaction with the host must be approached: that of the bacteria with the innate immune proteins targeting the PGN and, more specifically here, with the lysozyme. In this regard, it must be stated that several bacterial species seem to have some active mechanisms to avoid the alleged bactericidal power of this humoral weapon that enzymatically cleaves the β-1,4 linkages between NAcMur and NAcGlc in the murein sacculus, finally leading to osmotic cell lysis (198). These bacterial mechanisms are generically called lysozyme inhibitors. We will try to highlight the most important lysozyme inhibitor-related findings for Gram-negative pathogens in the next lines and in Table 6, but for an exhaustive review on the topic, including findings for nonhuman pathogens and inhibitors not only of lysozyme but also of other muramidases that bacteria inject into competitors, an excellent review by Callewaert and colleagues (199) can be consulted.

First, it is worth mentioning that some works have suggested that the muramidase activity of lysozyme is not necessary for its bactericidal power, since it displays membrane permeabilization action, therefore actually working as a cationic peptide (198, 200–202). Additionally, it has to be stated that many works have proposed that lysozyme generally has low activity *per se* against Gram-negative bacteria, unless the outer membrane is permeabilized by some other innate immune compound (198, 203). However, different bacterial inhibitors of the three types of lysozyme that exist in the animal kingdom (chicken or conventional [c-type], goose [g-type], and invertebrate [i-type] lysozymes) have been described (204). The Gram-negative species in which these inhibitors have been studied more often is probably *E. coli*, in which Ivy and MliC (each one of which belongs to a different protein family) were first described to be inhibitors of c-type lysozyme (205, 206). An Ivy KO mutant showed hypersusceptibility to *in vitro* treatment with lysozyme and permeabilizers (such as lactoferrin) and also to saliva (207, 208), and similar features were found for an *E. coli* MliC mutant (205). Nonetheless, the importance of these two *E. coli* inhibitors has been assessed in additional assays (a serum resistance assay and a subcutaneous chicken infection model) with controversial results: disruption of *mliC* clearly decreased the *in vivo* virulence, whereas the absence of Ivy produced no effect. Interestingly, the double KO mutant showed behavior similar to that of the wild type, suggesting the existence of additional inhibitors and a regulatory interaction in the expression of these different elements (209). Meanwhile, the disruption of *mliC* was shown to reduce the intramacrophage survival of *S. enterica* serovar Typhi, similar to what happened with *S. enterica* serovar Enteritidis homologue (PliC) inactivation, which caused increased lysozyme susceptibility in the presence of lactoferrin (205). Therefore, the importance of these inhibitors is not always the same and heavily depends on the species that we are considering. In *P. aeruginosa*, for instance, the deletion of Ivy or MliC homologues has been shown to produce no effect with regard to lysozyme susceptibility, as happened for MliC of *Yersinia pestis*, which was not required for lysozyme resistance and the development of plague (203, 205, 210). On the contrary, the inactivation of Ivy in *Y. pestis* caused hypersusceptibility to lysozyme, neutrophil-mediated killing, and viru-

lence attenuation in a rat infection model (210). However, a closely related species, such as *Yersinia pseudotuberculosis*, did not require Ivy either to counteract the lysozyme activity or for virulence (210). On the contrary, in *Edwardsiella tarda*, an unusual human pathogen, both Ivy and MliC have been shown to be necessary for efficient tissue dissemination, resistance to serum, replication within macrophages, and overall virulence (211, 212). The presence of homologues of MliC/PliC in other Gram-negative pathogens has been reported, although to date, their impact on virulence has not been studied: *Aeromonas hydrophila* (213), *Salmonella enterica* serovar Typhimurium (214), *Brucella abortus* (215), etc. (199).

Finally, it has to be mentioned that some works suggest that the true physiological function of the Ivy proteins is to control the autolytic activity of lytic transglycosylases within the periplasm of Gram-negative bacteria and that the inhibition of exogenous lysozyme by Ivy is a fortuitous coincidence, which could add more controversy to the real role of lysozyme inhibitors in Gram-negative bacteria (216). In any case, in general terms, the above-mentioned inhibitors are quite specific and do not show significant cross inhibition against other lysozyme types, with the exception of Ivy, which weakly inhibits the g-type lysozyme (217, 218). Moreover, much scarcer information regarding the inhibitors of the other two types of lysozyme (i-type or g-type lysozymes) has been published. In the case of the invertebrate type, the PliI protein of *Aeromonas hydrophila* has been shown to contribute to resistance in the presence of an outer membrane permeabilizer (218). In the same work, the existence of similar i-type inhibitors in *Serratia marcescens* and *Proteus mirabilis* was proposed (218). Besides, PliG has been proposed to be a g-type inhibitor in *E. coli* and shows no activity against the other lysozyme types (219). Deletion of *pliG* increased the *in vitro* susceptibility to the g-type lysozyme (220) but showed no effect on serum resistance or virulence in a chick infection model (Table 6) (209).

Therefore, to conclude, as can be seen in these last few paragraphs, the impact and importance of the lysozyme inhibitors are complex issues, since they are quite variable, depending on the species and conditions. Nevertheless, what does seem clear is that they have at least some degree of participation in virulence. This would be also supported by the fact that the levels of expression of some inhibitors seem to be tightly regulated and under the control of two-component systems, presumably responding to the stress on PGN that the presence of lysozyme entails. This has been proposed for *E. coli* (the Rcs two-component system regulon includes the Ivy- and MliC-encoding genes [221]) and *Moraxella catarrhalis*. In this last species, the two-component system MesRS has been shown to finely tune several genes necessary for infection development; among them are the genes encoding the proteins LipA and LipB, which show structural homology to bacterial lysozyme inhibitors. These two proteins were shown to inhibit human lysozyme activity *in vitro* and in saliva, and a double KO mutant was impaired for resistance to lysozyme in the presence of permeabilizers (Table 6) (222).

THE REGULATION OF INTRINSIC β-LACTAMASES AND PEPTIDOGLYCAN METABOLISM: INTIMATELY LINKED PROCESSES INFLUENCING VIRULENCE

Overview of Interaction between Intrinsic β-Lactamase Regulation and Peptidoglycan Metabolism

As shown in the previous sections, the amount of information on structure, metabolism, host detection, and the inflammation implications for the PGN-virulence interplay is notable. However, many fewer works have linked PGN biology to virulence together with the production of β-lactamase regulation, mainly if we consider only the intrinsic β-lactamases. Indeed, it is tempting to speculate that the production of an intrinsic β-lactamase, at least at regular levels, should not entail any biological cost, but what about when the enzyme is overproduced? Very few works have tried to answer this question (203, 223). Either way, the targets that we have identified in the contexts mentioned above are displayed in Table 7.

Although the array of mechanisms of intrinsic β-lactamase regulation in Gram-negative bacteria is very wide (13), one of the most extended models and, conse-

quently, the model for which a higher number of virulence-related publications exists is for AmpC from *P. aeruginosa* or certain *Enterobacteriaceae*, among other species. Several elements take part in this mechanism (which is intimately linked to PGN recycling), with the most important probably being AmpG, NagZ, AmpD, and AmpR, although additional actors have been described (13, 19, 224). For the sake of clarity, Fig. 2 shows a scheme of this regulatory pathway with *P. aeruginosa* as a model, which is taken from the work of Juan et al. (13), and is a useful reference on this specific topic of intrinsic β-lactamase regulation. Briefly, the products of PGN remodeling released during growth or degradation, thanks to the effects of β-lactams, reach the cytosol through the permease AmpG (specific for NAcGlc-1,6-anhydromuropeptides). Once there, these PGN fragments are processed by the β-*N*-acetylglucosaminidase NagZ to generate 1,6-anhydromuropeptides, which under regular conditions are metabolized by the *N*-acetylanhydromuramyl-L-alanine amidase AmpD to release the stem peptides, which enter the recycling circuit to finally generate UDP-NAcMur-pentapeptides (therefore, AmpD is a very important enzyme for PGN recycling). A certain proportion of these last elements, besides being exported to the nascent PGN, bind the LysR-type transcriptional regulator AmpR, and this complex is a repressor of *ampC* expression (allowing only basal levels of AmpC production) (225). On the contrary, when the amount of PGN fragments is higher because of the action of an inducer β-lactam, such as cefoxitin or imipenem, AmpD may get saturated; the 1,6-anhydromuropeptides, not metabolized by AmpD, could displace the UDP-NAcMur-pentapeptides from AmpR; and the new complex is an inducer of *ampC* expression (18, 226). Similarly, the mutational inactivation of AmpD (usually selected during β-lactam treatments) would presumably lead to the accumulation of 1,6-anhydromuropeptides and lead to *ampC* stable hyperexpression, resulting in clinically relevant levels of β-lactam resistance (13, 227, 228). Besides these phenomena, the nonessential penicillin binding protein 4 (PBP4) has been shown to act as a kind of sentinel for PGN damage against inducer β-lactams. Under these conditions or when its encoding gene (*dacB*) gets disrupted by mutation (which is very usually selected during treatment; in fact, it was the first cause of β-lactam resistance among *P. aeruginosa* clinical strains [229]), the situation is sensed by CreC (which belongs to the two-component system CreBC [BlrAB]), which in turn activates a complex and not fully understood parallel response that finally leads to enhancement of the effectiveness of AmpC production and the derived resistance level (Fig. 2) (230). Interestingly, whatever the route of AmpC hyperproduction, it has been shown that it is always AmpR dependent, since this transcriptional regulator finally controls the levels of *ampC* expression (13). Among the actors cited to be involved in the process, AmpG and NagZ have been shown to be indispensable for AmpC hyperproduction, since they allow, respectively, the physical entrance into the cytosol and an essential biochemical transformation (scission of NAcGlc) to obtain future *ampC*-inducing signals (via AmpR binding) (229, 231). Interestingly in this regard, in a closely related species such as *Xanthomonas campestris*, NagZ has been shown to be indispensable not only to allow a significant expression of its intrinsic class A AmpR-regulated β-lactamase but also for its full pathogenicity (Table 7) (232). This work revealed a novel connection between β-lactamase regulation, PGN biology, and virulence never found in any other species. Intriguingly, in the same work, an AmpG mutant was found to be more virulent than the wild type. Whether this last fact is related to an increased release of PGN fragments into the extracellular medium, as described before for *N. gonorrhoeae*, remains to be elucidated (136). It is also unknown why the cleavage of NAcGlc could be essential for virulence in *X. campestris* (232), but it opens up interesting horizons in the interplay with β-lactamase virulence that may be exportable to other species of clinical interest.

Besides the effects of these two elements (AmpG and NagZ), the roles of AmpD (and its homologues) (233, 234) in β-lactamase production but also in virulence have been investigated in some species (Table 7). To start with, in *S. enterica* serovar Typhimurium, although it has lost its *ampC* and *ampR* genes during evolution, the inactivation of AmpD has been shown to cause a dramatic decrease in virulence (99). More specifically,

TABLE 7 Elements simultaneously involved in regulation of expression of intrinsic β-lactamases and PGN metabolism

Target	Role(s)	Species	Effect(s) of target disruption	Reference(s)
AmpR	A LysR-type global transcriptional regulator controlling the expression of *ampC* and other genes related to virulence, quorum sensing, biofilm formation, etc.	*P. aeruginosa*	A significant reduction of the ability to kill *Caenorhabditis elegans* with AmpR inactivation; with the G154R AmpR mutation, prevalent in the ST175 high-risk clone, reduced virulence in the same model of infection	267
CreBC/CreD	For CreBC, a two-component system and global metabolic regulator involved in the response against stress or biofilm growth, among others; for CreD an inner membrane effector protein of the CreBC system	*P. aeruginosa*	A decrease in fitness in *in vitro* competition experiments compared with that of the wild type, mainly in the presence of β-lactams; a decrease in the formation of biofilms, mainly in the presence of β-lactams; decrease of *exoS* expression, especially in the presence of ceftazidime	276
AmpD-AmpDh2-AmpDh3	*N*-Acetyl-anhydromuramyl-L-alanine amidases that are involved in PGN turnover and recycling as well as AmpC repression and that are cytoplasmic (AmpD) or periplasmic and involved in stem peptide cleavage (AmpDh2 and AmpDh3)	*P. aeruginosa*	Severely compromised growth rates, motility, and cytotoxicity in the triple mutant; repression in key virulence factors, such as protease LasA, phospholipase C, or T3SS components; decreased virulence of circa 100-fold in *Galleria mellonella* model	223
NagZ, AmpG, and AmpD-AmpDh2-AmpDh3	Key elements allowing PGN recycling (see the text for further details)	*P. aeruginosa*	Increased susceptibility to lysozyme and human PGRPs; if PGN blockade is combined with AmpC hyperproduction (for the AmpD-AmpDh2-AmpDh3 triple mutant), an even higher increase in susceptibility to lysozyme and PGRP2 specifically	203
CreBC	A two-component system and global metabolic regulator involved in the response against stress	*S. maltophilia*	A decrease in secreted protease activity	277
AmpD	A cytosolic 1,6-anhydro-*N*-acetylmuramyl–L-alanine amidase cleaving the stem peptide from 1,6-anhydro-*N*-acetylmuramic acid in a key step for PGN recycling	*Salmonella enterica* serovar Typhimurium	Upregulation during infection; in a knockout mutant, a decreased capacity to invade macrophages and more potent induction of the nitric oxide response of bacterial killing; decreased competitiveness in a BALB/c mouse model of infection	85, 99
AmpD	A cytosolic 1,6-anhydro-*N*-acetylmuramyl–L-alanine amidase involved in PGN recycling	*N. meningitidis*	An inability to cause systemic infection in an infant rat model; in wild-type strains, hyperexpression of AmpD during infection	84
AmpD	A cytosolic 1,6-anhydro-*N*-acetylmuramyl–L-alanine amidase cleaving the stem peptide from 1,6-anhydro-*N*-acetylmuramic acid in a key step for PGN recycling	*Ralstonia solanacearum*	Upregulation during infection; in a knockout mutant, reduced virulence in eggplant and tomato models	85, 98
NagZxc	A β-*N*-acetylglucosaminidase that cleaves the bond between 1,6-anhydro-*N*-acetylmuramic acid and *N*-acetylglucosamine	*X. campestris*	Reduced average lesion areas in cabbage infection model	232
AmpGxc	A specific permease for PGN fragments containing the disaccharide *N*-acetylmuramic acid–*N*-acetylglucosamine	*X. campestris*	Slightly increased average lesion areas in cabbage infection model	232
AmpC	A class C β-lactamase (noninducible due to the lack of the AmpR regulator in *E. coli*)	*E. coli*	In AmpH-AmpC double mutants, uneven contours and aberrant and asymmetric constrictions between dividing cells	255

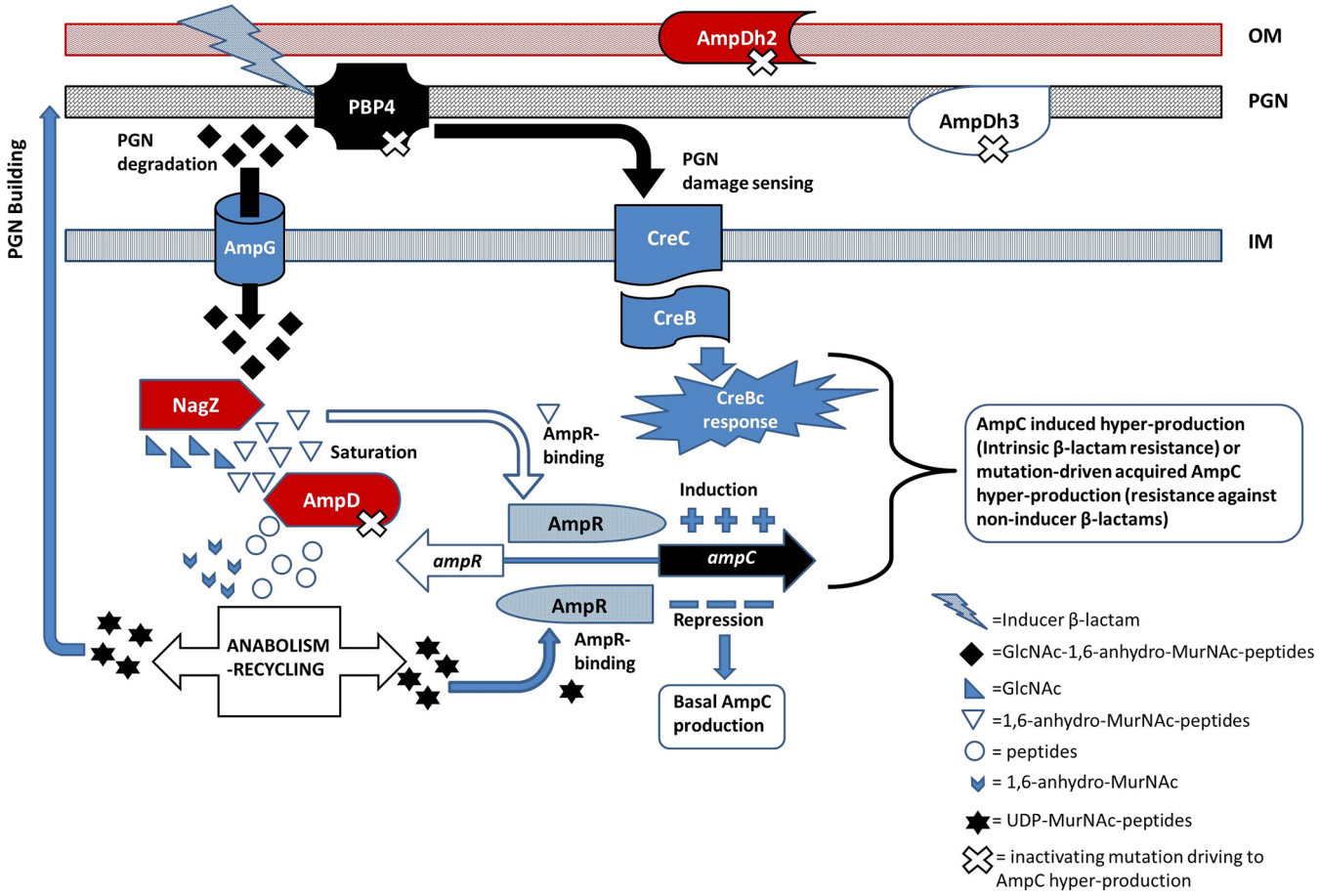

FIG 2 Overview of intrinsic β-lactamase induction and overexpression mechanisms (under the control of the LysR-type regulator and CreBC system) and their relatedness to PGN metabolism, taking *P. aeruginosa* AmpC as a model.

the reduced capacity for macrophage invasion and for intracellular growth is likely linked to the more potent activation of the bactericidal inducible nitric oxide synthase in the macrophage that was measured. An AmpD knockout mutant also showed impaired competitiveness in a BALB/c mouse model of infection. Interestingly, in the same work, the authors demonstrated that an AmpG mutant behaved like a wild-type strain in the cited parameters, and then they concluded that, more than the impairment of PGN recycling (obviously achieved through AmpG disruption but also through the disruption of AmpD), the basis for virulence attenuation was the cytoplasmic accumulation of PGN fragments derived from the absence of an active AmpD. In this regard, the authors suggested that, as well as a signaling role for β-lactamase induction through AmpR binding, some PGN fragments could also bind to other regulators, such as SinR or SpvR, finally modulating the expression of genes related to virulence (99). A similar hypothesis (although opposite) could be formulated for the above-cited case of *X. campestris*, in which NagZ would be essential to allow a reaction indispensable for the generation of PGN-derived inducing signals that would work as activators of virulence genes after binding to unknown regulators (232).

Interestingly, and as an almost unique feature in the microbial world (a few exceptions do exist, such as *Yersinia enterocolitica* [235]), *P. aeruginosa* shows two additional homologues of the cytosolic AmpD amidase, namely, AmpDh2 and AmpDh3 (19, 233, 234, 236, 237). Other species, such as members of the family *Enterobacteriaceae*, show only one additional homologue (AmiD). AmpDh2 has been shown to be anchored to the outer membrane with the active site oriented toward the periplasm and to be the orthologue of the above-mentioned AmiD, whereas AmpDh3 is a

periplasmic amidase (233, 234, 236, 237). Therefore, in contrast to AmpD, AmpDh2 and AmpDh3 have been proposed to start the reactions of PGN turnover already in the periplasm (233, 234). Moreover, the presence of two additional homologues of AmpD has been interpreted to be an advantage for *P. aeruginosa*, since this feature would allow the selection of AmpD-inactivated mutants showing increased levels of constitutive AmpC production and β-lactam resistance but still retaining full fitness and virulence levels, since the mutants would still maintain two active homologues (237). Moreover, it was shown that through the consecutive inactivation of the AmpD homologues, growing levels of AmpC production were achieved, reaching constitutive derepression of the chromosomal cephalosporinase in the triple AmpD-AmpDh2-AmpDh3 knockout mutant (236, 237). The high biological cost recently demonstrated for the triple AmpD mutant supports the idea that this mutant is unable to compete in the natural environment, although the existence of natural double mutants (AmpD-AmpDh2 or AmpD-AmpDh3, both with high levels of AmpC production, in contrast to AmpDh2-AmpDh3, which showed wild-type levels) needs to be further investigated (223). In this regard, it was shown that the inactivation of the three amidases, besides AmpC derepression and PGN recycling impairment, caused a dramatic effect on fitness and pathogenicity, severely compromising growth rates, motility, and cytotoxicity, with the effect on cytotoxicity likely being achieved thanks to the repression of key genes, such as those for protease LasA, phospholipase C, or T3SS components (223) (Table 7). Further, the two circumstances that were shown to be indispensable for obtaining this virulence-attenuated phenotype were AmpC derepression in a background of PGN recycling impairment, and hence, other pathways triggering similar levels of AmpC production and resistance, such as those involving *dacB* disruption, showed no biological cost, even under conditions of CreBC system deletion (223). Although the energetic burden of AmpC derepression or of the PGN recycling blockade or the total loss of amidase activity *per se* was ruled out as the molecular basis for this attenuated phenotype, the molecular basis for this attenuated phenotype has not yet been ascertained. Therefore, the hypothesis of recycling impairment plus derepressed AmpC-derived accumulation of PGN fragments potentially acting as virulence inhibitory signals (allegedly through binding to transcriptional regulators), similar to that proposed by Folkesson and colleagues (99), still needs to be studied.

Finally, an additional gene in *P. aeruginosa* with potential virulence implications that has recently been shown to take part in PGN recycling and whose disruption causes increased levels of *ampC* expression (circa 20-fold compared with that of the wild type) and derived increases in β-lactam MICs (1.5-fold for ceftazidime, for instance) is *mpl*, which encodes a UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase and which allows the cytosolic ligation of the new UDP-NAcMur units with recycled tripeptides (238, 239). Although its inactivation has been reported in strains from patients with chronic infections (cystic fibrosis) but also acute infections (ventilator-associated pneumonia), with strains with inactivated *mpl* showing a progressive loss of virulence during the development of disease and with *mpl* inactivation clearly being linked to selection during β-lactam treatment, the direct cause-effect relationship in terms of *mpl* disruption influencing virulence has not yet been established (240, 241). Thus, although the attenuation of certain virulence features during cystic fibrosis and other chronic infections is a classically accepted trait for many species of opportunistic Gram-negative bacteria (242) and a general inverse correlation between the levels of antibiotic resistance and virulence is often established (also in acute infections) (17), the identified targets simultaneously related to intrinsic β-lactamase production, PGN metabolism, and virulence are still scarce.

Interplay among β-Lactamase Production, Peptidoglycan Recycling, and Susceptibility to Innate Immune Proteins Targeting the Cell Wall

Further, the analysis of a set of *P. aeruginosa* mutants very similar to those used in the above-mentioned work by Pérez-Gallego and colleagues (223) has recently revealed that, besides fitness and virulence attenuation, PGN recycling impairment

(together or not with AmpC derepression) determines a great increase in bacterial susceptibility to innate immune weapons targeting the PGN, such as lysozyme and the four human PGN recognition proteins (PGRPs) (203). Lysozyme (also known as muramidase or *N*-acetylmuramide glycanhydrolase) is especially abundant in secretions and in neutrophil granules and catalyzes the hydrolysis of 1,4-beta linkages between NAcMur and NAcGlc. Therefore, as stated above, its bactericidal activity was initially linked to the PGN-degrading capacity, although some evidence of its nonenzymatic action has also been reported, with a detergent-like activity of lysozyme being suggested (200, 201). Besides, the PGRPs were initially described to be PGN binding, but in the case of PGRP1, -3, and -4, they do not show any PGNase activity. However, they have bactericidal power through their induction of a complex response mainly based on overactivation of the two-component system CpxA-CpxR (entailing oxidative, thiol, and metal stresses), finally leading to bacterial suicide (243, 244). Conversely, PGRP2 shows amidase activity (and, hence, a certain PGNase capacity), but its alleged bactericidal power or its implication in the regulation of inflammation in response to PGN detection is still controversial (170, 245–247). In fact, in some work with knockout mice, PGRP2 seems to be protective against the pathogen (*S*. Typhimurium infection [246]), whereas in other work, it looks like a proinflammatory actor, finally leading to a worse outcome for the host (*P. aeruginosa* keratitis, as described by Gowda et al. [245]), which indicates the complexity of these innate immune compound roles (170, 248) (Table 6). Besides, PGRP2 has also been linked to certain chronic pathologies allegedly unrelated to infections, hence adding even more interest to this protein's capacities and potential interplays (249, 250).

Either way, the work of Torrens and colleagues (203) showed that, once the permeability barrier is overcome, the activity of lysozyme and the four human PGRPs is dramatically enhanced when inhibiting key peptidoglycan recycling components (such as the 3 above-mentioned AmpD homologues, AmpG, or NagZ), indicating a decisive protective role for cell wall recycling against the innate immune aggressions cited earlier (Table 7). Even though the exact way in which PGN recycling could exert this protective role remains elusive, some hypotheses can be formulated: it has been described that PGRP1, -3, and -4, besides overactivating CpxA-CpxR, exert their activity by affecting the biology of PGN by dampening the initial steps of its anabolic pathways, and thus, in this situation, PGN recycling would become essential to avoid perturbation of the metabolism and of the cell wall itself that the activity of the above-cited innate proteins could entail as bactericidal mediators. Obviously, under these circumstances, PGN recycling would become a key protection (203). Furthermore, it was specifically shown for lysozyme and PGRP2 that an even higher bactericidal activity level was achieved by blocking PGN recycling and simultaneously overexpressing AmpC (which happens in the AmpD-AmpDh2-AmpDh3 triple knockout mutant). This phenotype was found to be associated with a circa 30% decrease in the amount of PGN per cell (203). The reasons why the PGN blockade together with AmpC hyperproduction negatively influences the resistance of *P. aeruginosa* to lysozyme and PGRP2 have not been determined to date, although they are presumably linked to the reduction in the PGN amount per cell mentioned above. It has been accepted that *P. aeruginosa* has a PGN relatively thinner (approximately 30%) than that of other Gram-negative bacteria (33, 34), and this could be the basis for the increase in susceptibility to lytic aggressions, such as those exerted by lysozyme and PGRP2, especially if it affects certain regions likely to be essential for counteracting osmotic pressure (33, 34, 203). The explanations for these results could involve the energetic burden of producing very high levels of AmpC, added to a direct effect of the β-lactamase on PGN physiology, likely due to residual peptidase activity reminiscent of its potential PBP ascendance, which has been widely reported in the past (Table 7) (48, 251–254). In fact, at least in *E. coli*, AmpC has been shown to somehow contribute to its regular morphology, since its inactivation, if it occurs together with that of LMM-PBP AmpH, rendered aberrant morphologies, which could somehow support the idea of a β-lactamase directly acting on the PGN structure and/or composition (255, 256). It still needs to be elucidated whether these

effects are exclusive to the AmpC-type β-lactamases (and, consequently, linked to a potential exclusive remaining activity) or could be achieved by any kind of β-lactamases.

The Effect of Global Regulators: Controlling Not Only Intrinsic β-Lactamase Production but Also Virulence

The previously cited LysR-type regulators (denominated AmpR, in most cases), whose activity allegedly occurs through the binding of different PGN fragments, have been shown to be indispensable for allowing the expression of significant levels of intrinsic β-lactamases, such as AmpC from *P. aeruginosa* and some *Enterobacteriaceae*, PenA and AmpC from BCC, and L1 and L2 from *S. maltophilia* (13, 257, 258). These circumstances have been known for decades for some species (259, 260), for which even a detailed characterization of the structure of the regulator but also the PGN fragments capable of binding to it have recently been published. In this sense, AmpR from *P. aeruginosa* and its PGN-derived activating ligands during induction have been studied in depth (18, 19, 225). Nevertheless, the knowledge regarding the capacity of LysR-type regulators to regulate the expression of wider features of the bacteria, in addition to β-lactamase production, is more recent. In *P. aeruginosa*, for instance, AmpR has been shown to regulate not only the expression of *ampC* but also the expression of a repertoire of more than 500 genes; it is a positive promoter of virulence factors relevant in acute infections, while it is a repressor of biofilm formation, a key driver of chronic infections. The above-mentioned array also includes genes involved in iron acquisition, heat shock, and oxidative stress responses, among others (261–264). Moreover, Sánchez-Diener and colleagues have recently reported that the inactivation of AmpR but also some specific polymorphisms strongly linked to certain high-risk clones of *P. aeruginosa* (clones with the Gly154Arg substitution, which is very prevalent in sequence type 175 [ST175] isolates, and isolates that constitutively promote *ampC* expression [265, 266]) entail significant impairments of virulence in a *Caenorhabditis elegans* model (Table 7) (267). Even though a very high number of studies regarding the control of virulence gene expression under the control of LysR family regulators in a wide range of species were published decades ago (268–272), it is noteworthy that, except for the works mentioned in this section, no link to the regulation of intrinsic β-lactamases or PGN metabolism has yet been found. Therefore, this interplay among PGN metabolism, β-lactamase production, and virulence, all of which are under the control of a common LysR-type regulator, is still barely studied and is worthy of more investigation in order to find future therapeutic targets.

With regard to global transcriptional regulators other than those of the LysR type, two-component systems could be good candidates to coordinately control the expression of genes related to virulence, β-lactamase production, and/or PGN metabolism. These systems consist of a first element (sensor), anchored to the cytosolic membrane, which is usually a histidine kinase that autophosphorylates when activated by an appropriate stimulus. The second element (cytosolic) is activated through phosphorylation, performed by the sensor, and acts as a transcription factor over distinct sets of genes to respond to certain environmental conditions and/or punctual stresses (273). Examples of these systems are BlrAB (where Blr stands for "β-lactam resistance"), which has been described to be the regulator of the multiple β-lactamases in *Aeromonas* spp. (274), or CreBC (where Cre stands for "carbon source responsive") of *E. coli*, which has been proven to be not only a β-lactamase regulator but also a global one involved in metabolic control (275). Similarly, the previously mentioned CreBC system from *P. aeruginosa* (276) has been shown to be an essential actor in the response against β-lactams, entailing a complex metabolic response based on hyperproduction of its effector inner membrane protein, CreD, and on a better output for a given AmpC production level, improving its effectiveness and derived resistance. For these reasons, it has been considered a global regulator especially important during stresses (such as exposure to β-lactams): disruption of the system significantly impaired the fitness of the mutants in competition experiments and biofilm formation. Interestingly, among the

genes with decreased expression (circa 2-fold) after CreBC inactivation, mainly during exposure to ceftazidime, there was *exoS*, encoding an exotoxin injected through the T3SS in the host cells. Nevertheless, the real impact of this hypoexpression has not yet been determined (276); in any case, the impact seems minor, since inactivated mutants with mutations in CreBC have recently been shown to display wild-type levels of virulence in the *G. mellonella* model (223). Similarly, the study of the same system in *S. maltophilia* has very recently provided data also linked to virulence, since a significant decrease in secreted protease activity was observed in CreBC double KO mutants but also in a CreC single mutant. Besides, an increase in swimming motility was also documented and shown to be CreD upregulation dependent (Table 7) (277). As happened with LysR-type regulators, many works relate the function of two-component systems to the control of pathogenesis in bacteria (278), but apart from the works cited in this section, no additional relatedness to PGN metabolism and/or β-lactamase regulation has been reported. This is the contrary to what happens with Gram-positive bacteria, where the volume of information on the topic is very important (279).

To conclude, some other different kinds of regulators have been shown to control intrinsic β-lactamase production in certain Gram-negative bacteria, presumably within complex regulatory networks also affecting features of the bacteria that could potentially include virulence (13). In this sense, it has been published that *Elizabethkingia meningoseptica* harbors an intrinsic class B β-lactamase (BlaB) which is not controlled by any known regulator but which is capable of increasing its production in response to stress conditions likely resembling those that bacteria find when causing infections, which suggests a coordinate regulation of β-lactamase production and virulence (280). Similarly, in *Ralstonia pickettii*, its intrinsic β-lactamases (OXA-22 and OXA-60) have been shown to be under the control of the barely studied ORF-RP3 element, to which global regulatory capabilities have been attributed, since it affects the lag phase of bacterial growth as well as the survival capacity against some stresses, such as pH changes, heat exposure, and osmolarity (281). Finally, in *Caulobacter crescentus*, the peculiar environment of the CAU-1 β-lactamase gene suggests that its regulation is under the control of a gene linked to the ArsR family of transcriptional regulators, which have been shown to be responsive against the stressing concentrations caused by heavy metals (282). Additionally, and more interestingly, these ArsR regulators have also been reported to control the expression of virulence genes in species such as *Vibrio cholerae* and *Mycobacterium* spp. (283, 284). Nevertheless, it still needs to be studied whether these clues suppose that there are more virulence genes within the cited regulatory networks and whether these models could be extended to more species for the future discovery of antivirulence targets.

EFFECTS OF HORIZONTALLY ACQUIRED β-LACTAMASES ON FITNESS AND VIRULENCE

The issue of the potential fitness and virulence costs derived from the expression of horizontally acquired β-lactamases has been widely studied mainly in *Enterobacteriaceae* but also in other Gram-negative bacteria, but with the information available, we are not able to draw any general conclusions. Moreover, the potential influence of these acquired enzymes (the genes for which are usually carried in integrons located on plasmids) on virulence has to be analyzed from different points of view: besides the alleged energetic burden that the massive production of the enzyme (or even the replication of the large natural plasmids carrying the genes) could entail or even the potential effects of the β-lactamase on the PGN, other factors have to be taken into account, such as the potential codification of virulence genes in the mentioned plasmids (which could, consequently, even increase the virulence of the strain) or the influence of some genes carried by the plasmid on the expression of chromosomal ones (48, 251–253, 285, 286). In this sense, it has been reported that *ampR* (as mentioned above, a transcriptional regulator of intrinsic β-lactamases), which is usually cotransferred on the same plasmid with some AmpC-type β-lactamases, can also regulate some virulence-related features in the host.

More specifically, *ampR* from a plasmid containing the DHA-1 enzyme in a clinical strain of *K. pneumoniae* was shown to be involved in capsule synthesis and the derived serum resistance, biofilm formation, type 3 fimbrial gene expression, adhesion to cells in culture, as well as colonization of the gastrointestinal tract in mice (287). These results add strength to the previously mentioned idea on the wide pleiotropic power of *ampR* (and other LysR regulators) to regulate not only intrinsic or horizontally acquired β-lactamases expression but also many other bacterial processes, including virulence. Some other works proposed a correlation between the expression of acquired β-lactamases and increased levels of virulence. In this regard, Sahly and coworkers studied more than 200 clinical isolates of *K. pneumoniae* (producers of different extended-spectrum β-lactamases [ESBL] versus nonproducers) and found a positive association between increased adhesion, invasion capacities, and ESBL production. The molecular basis for this observation was that the acquisition of some ESBL-carrying plasmids appeared to upregulate the expression of some chromosomal genes (those encoding fimbrial adhesins, for instance), finally leading to increased invasiveness (285). In the same sense, Schaufler and colleagues studied seven clinical isolates from pandemic *E. coli* lineages (such as B2-ST131) carrying plasmids harboring CTX-M-type ESBLs and demonstrated (using a *C. elegans* infection model) that the expression of these plasmids did not dampen fitness but increased virulence (286). The presence of different virulence-related genes in the cited plasmids (*finO* or *traT*, for instance) but also the presumptive influence of an ESBL-positive plasmid on chromosomally encoded virulence-associated features would support the conclusions of this work (286). In this sense, the work of Ramirez and colleagues also collected information supporting the idea of a clear cotransfer of resistance determinants and virulence genes on the same plasmids specifically in *K. pneumoniae* (288). Another recent work with *K. pneumoniae* allows us to draw similar conclusions: a significant association between ESBL carriage and increased biofilm production and serum resistance was documented (289).

In an important number of publications, conversely, no significant increase/decrease in fitness or virulence was observed for different species carrying horizontally acquired β-lactamases. For instance, the reference strain of *P. aeruginosa* PAO1 was transformed with a vector containing a cloned bla_{IMP} metallo-β-lactamase gene, and its virulence in terms of invasion of MDCK cells in culture and in a mouse bacteremia model did not significantly change (290). Similarly, in *E. coli*, the production of the β-lactamase CTX-M-1 did not seem to affect virulence: Dubois et al. (291) reported the isolation from a patient with neonatal meningitis of an *E. coli* strain harboring three different plasmids, one of which produced the aforementioned β-lactamase. The curation of these plasmids did not alter the incidence of meningitis in a neonatal mouse model, hence suggesting that the production of the cited β-lactamase does not influence the virulence of *E. coli*. Furthermore, even though the presence of virulence-related genes (*iss*, *aer*, and *iroN*) in the cited plasmids was documented, their curation did not impair the pathogenesis capacity (291).

Furthermore, given the widespread presence of horizontal β-lactamases in successful or epidemic clones from different Gram-negative bacteria (mainly *Enterobacteriaceae*), it is tempting to speculate that their carriage does not represent an important handicap for fitness and virulence or, at least, that under antibiotic pressure their carriage is positively selected as it adds more advantages than drawbacks for the bacteria. Some examples of this would be an ST131 strain (of the O25:H4 serotype) of *E. coli* carrying the ESBL CTX-M-15, which has emerged internationally as a successful multidrug-resistant strain (292). The latter β-lactamase and other CTX-M-type variants are also very prevalent in other virulent clones of *E. coli*, such as Shiga toxin-producing serotypes O104:H4 and O26:H11, and even in strains from farm animals (293, 294). Some other works have tried to understand the virulence capabilities of successful clones, such as the above-mentioned ST131 strain carrying the CTX-M or NDM β-lactamase (295), revealing a very complex interplay and balance between the expression of β-lactamases and derived resistance and levels of virulence. Therefore, what seems plausible is that certain

β-lactamases and virulence genes can successfully coexist, mainly in specific clones of internationally spread strains and possibly as a result of gradual coevolution processes intimately linked to antibiotic pressure.

Finally, several works have demonstrated that the expression of acquired β-lactamases could entail a handicap for the bacterium, specifically, for fitness and/or virulence, as published by Lavigne and coworkers (296). In this paper, however, the authors reported that not all the plasmids harboring the acquired β-lactamases and not all the strains responded equally to these alleged biological costs. In this regard, assessing the virulence of the clinical strains and their plasmid-cured derivatives in a *C. elegans* model, it was shown that epidemic strains, such as the ST258 strain, harbor KPC-carrying plasmids associated with a biological cost lower than that for plasmids from nonepidemic strains (296). Another example is the recent work of Göttig and colleagues (297), in which the expression of the carbapenemase NDM-1 in *E. coli* or *K. pneumoniae* did not, apparently, significantly change the cytotoxicity in cell culture or virulence in the *G. mellonella* larva model. Nevertheless, and intriguingly, a significant fitness cost was observed in pairwise competition assays, where the wild-type strains of the cited species significantly outcompeted their respective transformants (obtained through transformation of the natural plasmid or a construct with the NDM-1 gene cloned on a vector), with the conclusion of this work being that efficient horizontal gene transfer probably has a higher impact on the dissemination of NDM-1 than potential fitness or virulence changes (297). Similarly, Marciano et al. reported a significant fitness cost associated with cloned SME-1 β-lactamase expression in *E. coli*, according to the results of growth and competition assays (298). Some more evidences in this paper were a decrease in culture viability (for the strain harboring a vector with the cloned SME-1 versus that for the wild type) and an increase in the rate of plasmid loss compared to the rate of loss of isogenic plasmids carrying a different β-lactamase gene (bla_{TEM-1}). Moreover, the exchange of the SME-1 signal sequence for the TEM-1 one alleviated the fitness cost in the strain transformed with this new construct, and therefore, the authors suggested that the SME-1 signal sequence was the most responsible for the fitness cost, potentially due to the accumulation of the SME-1 signal sequences (when SME-1 is exported to the periplasm) resulting in inner membrane pore formation that dampened viability. The authors concluded that the fitness costs associated with some β-lactamases (whatever is the basis of this cost) may be the origin of their limited dissemination in comparison with the level of dissemination of others (298). In this sense, additional explanations for the virulence and fitness costs associated with certain β-lactamases were formulated some years ago: Fernández et al., using *E. coli* both for *in vitro* assays and in a murine model of systemic infection, linked the reported costs to quantitative changes in the composition of the PGN (a decrease in the level of cross-linking of the muropeptides and an increase in the average length of the glycan chains), presumably due to the residual DD-endopeptidase activity of the acquired enzymes (OXA-10, OXA-24, and SFO-1) (299). In fact, the potential residual activity of certain β-lactamases, due to or not due to their presumed PBP ascendance, has been proposed in the past and in this review (48, 251–255) and could still have some barely studied effects on the PGN (presumably, a weakening of its structure) that could entail a handicap for the bacterium under certain conditions (203, 223). These findings support the importance of the interaction between β-lactamases and PGN metabolism and the potentially associated biological costs and, as mentioned before, may even provide information on the epidemiology of β-lactamases and help explain the low incidence of some of them in specific pathogens, such as *Salmonella* spp. In this regard, several studies have highlighted the effect of β-lactamases on the fitness and virulence of *S. enterica* serovar Typhimurium. For instance, Cordeiro et al. observed that the synthesis of VIM-2 entailed a very important reduction in the growth rate, motility, and invasiveness in cell culture in this species, together with alterations in the micro- and macroscopic morphology of the cells, which the authors related to the extremely low number of reports of *Salmonella* isolates carrying acquired metallo-β-lactamases (300). Meanwhile, Morosini and coworkers

associated the unregulated (in the absence of AmpR) expression of *ampC* in *Salmonella* with changes in colony morphology and a high reduction in the growth rate and invasiveness in cell culture, concluding that the expression of the AmpC in this species probably represented an almost unbearable cost (253). In fact, very few reports of *Salmonella* strains harboring AmpC-type β-lactamases have been published (301, 302). Nevertheless, some studies proposed that to make the AmpC-type enzyme production in *Salmonella* bearable, more than a low expression level of the enzyme (kept under the control of an active AmpR regulator) is needed, and the essential point could be the presence of additional encoded functions in the plasmid carrying the enzyme. This would compensate for the biological cost of AmpC-type enzyme overexpression (specifically, the CMY-7 β-lactamase in the previous work [303]).

In conclusion, and as reviewed in the work of Beceiro et al. (17), the issue of the antibiotic resistance/virulence interplay is still a controversial and very complex topic that is still a long way from being completely understood. With this review and specifically this section, we provide more data not only to support this idea of complexity but also to make it clear that the interplay between PGN–β-lactamases and virulence does exist and, thus, is a potential target to be exploited in the future.

CONCLUDING REMARKS: DELINEATING NEW THERAPEUTIC STRATEGIES BASED ON THE INTERPLAY BETWEEN PEPTIDOGLYCAN BIOLOGY, β-LACTAMASES, AND VIRULENCE

As stated throughout this review, much evidence of PGN being indispensable for virulence, in connection or not with the regulation of β-lactamases, has been gathered. Therefore, it is hoped that the existence of different bacterial targets leading to an attenuation of fitness/virulence could be deduced in this context, and a hope for alternatives to the current scenario of an almost useless antibiotic arsenal may exist. In fact, some current projects try to take advantage of targets in the β-lactamase–PGN biology interplay intended to block or revert the bacterial mechanisms leading to resistance to β-lactams but also to exert some bactericidal power. In this sense, several inhibitors of elements in PGN metabolism are currently being studied. These inhibitors affect, for instance, NagZ, AmpG, the septal protein FtsZ, PBP1a-PBP1b, PBP2, and even the membrane-bound lytic transglycosylases, with the inhibitors showing diverse levels of success as treatments or adjuvants (16, 229, 231, 304–311). Nevertheless, with the connection to virulence that we reviewed here, a new, additional horizon could be opened to, if not kill the bacteria or block/revert the β-lactam resistance, at least attenuate its pathogenesis in the host to ameliorate the clinical outcome (hence, the field of antivirulence therapies) (14). Nevertheless, in spite of all the evidence in this review, we are still a long way from using the identified weak points as antivirulence targets. The answers to many questions are still unknown and need to be resolved prior to finding therapeutic solutions; for instance, we need to know whether some PGN-derived fragments can act as signals to attenuate virulence (through binding to a transcriptional regulator, similar to what happens with AmpR-AmpC or two-component system models or not) or even whether the same β-lactamases can have a residual activity that could negatively affect PGN under certain conditions. Interestingly, the future development of these therapies could be approached from different perspectives, which improves the possibilities of success: the development of therapies based on the reduction of inflammation in response to PGN detection by our immune system (197), drugs intended to weaken the cell wall in order to make our innate immune weapons more effective in attacking the PGN (such as lysozyme and PGRPs), or drugs that block the motility/cytotoxicity of the pathogen to avoid the dissemination of the infection are only some of the possibilities that the issues that we have reviewed here could open. Hence, although the information dissected here clearly identifies PGN to be a structure essential for the virulence of Gram-negative bacteria, we have to take into account the fact that not all the actors playing a role on its biosynthetic, degradation, or recycling pathways have the same importance and the same chance to be used as therapeutic targets, and this is even more marked if we compare different species. According to this and since we have reached the so-called era of antibiotic resistance, perhaps we should change our conception of antimi-

crobial treatments and adapt them more specifically to the features of specific pathogens and their virulence factors (312). Thus, to conclude, although much work is still needed, the clues shown here clearly display weak points in the biology of Gram-negative bacteria (the PGN–β-lactamase–virulence interplay) to be exploited as targets for the design of novel therapeutic options in the future.

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