Chemical Biology Reveals Involvement of N-acetylglucosaminidase LytG In Cell Elongation and Division.

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Abstract:

The growth and division of the Gram-positive cell requires the coordinated action of enzymes involved in the synthesis and degradation of the heteropolymer peptidoglycan. Herein, we present the use of the diamide masarimycin, an inhibitor of the *exo*-N-acetylglucosaminidase (GlcNAcase) LytG, as a chemical biology probe to elucidate biological role of this cell wall degrading enzyme. Using a combination of chemical biology and genetic approaches we provide the first evidence that LytG activity influences the elongation and division complexes in *Bacillus subtilis.* Chemical inhibition of LytG results in dysregulated cell elongation and localization of division plane and the induction of the cell wall stress response. In the presence of masarimycin, cells are elongated 3-fold compared to wild-type cells and formation of daughter cells along the sidewall was observed. The use of genetic and synergy/antagonism screens established connections to late-stage peptidoglycan synthesis, particularly related to cross-linking function. These results stand in stark contrast to those observed for the Δ*lytG* knockout, which does not exhibit these phenotypes. The results presented here emphasize the difficulty in assigning mode-of-action using solely genetic screens. This is the first report of assigning physiological role of LytG beyond being the major GlcNAcase during vegetative growth.

Introduction

Bacillus subtilis is a fast-growing, aerobic, rod-shaped bacterium and is one of the best studied Gram-positive organisms. [1] The cell shape of most bacteria (with the exception of the

Mycoplasma) is maintained by the cell wall, which protects the cell from the outward facing turgor pressure. [2] The cell wall of *B. subtilis* contains two major cell wall components, peptidoglycan (PG) and the anionic polymers wall teichoic acid (WTA) and lipoteichoic acid (LTA).[3] The heteropolymer PG is composed of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues linked by a β-(1→4) glycosidic linkage (Figure 1). Adjacent polysaccharide strands are cross-linked via stem peptides attached to the C-3 lactyl moiety of MurNAc to form a three-dimensional mesh. [2] The current model of PG synthesis in Gram-positive organisms invokes the ‚essential' degrading enzymes for the incorporation of nascent PG into the stress bearing layer.[4-6] Controlled degradation of PG is required for cell maintenance and division. This tightly regulated process is suggested to involve the interplay between synthetic and degradative enzymes.[4, 7, 8] Bacterial autolysins are a highly diverse group of enzymes capable of cleaving bonds in polymeric PG, and participate in cell wall growth and its regulation.[9] The degradative enzymes fall into 4 broad classes based on their activity: lytic transglycosylases, *N*-acetylglucosaminidases (GlcNAcases) and muramidases, *L*-alanine amidases, and endopeptidases. Deciphering physiological role of autolysins has been a formidable task as functional redundancy complicates attribution of biological activity.[10] Recent biophysical [4, 11] and computational studies [12] of bacterial autolysins have begun to unravel their roles in the release of stress in the cell wall to allow for incorporation of new material. A renaissance in PG metabolism research has started to provide new chemical biology tools to study synthesis [13-16] and elucidate the role endopeptidases play in methicillin resistance.[17] While the cell wall, and PG in particular, have provided a wealth of clinically relevant antimicrobial targets [7], our understanding of the complex interplay between degradative and synthetic steps is still developing.

We have previously demonstrated the utility of glycosyl triazoles[18] and diamides (Figure 1 insert) [19, 20] as a scaffold for developing inhibitors to bacterial cell wall hydrolases in *B. subtilis* and *Streptococcus pneumoniae*.[20] To our knowledge these are the first inhibitors of autolysins that also inhibit cell growth. These probes can be used to interrogate Gram-positive cell wall metabolism in an orthogonal manner to traditional genetic approaches. Here we report on the application of the bacteriostatic inhibitor of LytG, masarimycin (Mas)[19, 20] to interrogate cell wall metabolism in *B. subtilis*. LytG is the major active GlcNAcase during vegetative growth[21] yet its exact role has remained elusive. We demonstrate the the ability of Mas to inform on biological function when used in tandem with genetic and molecular approaches. Our results provide fresh insight into the role LytG plays in cell growth and division.

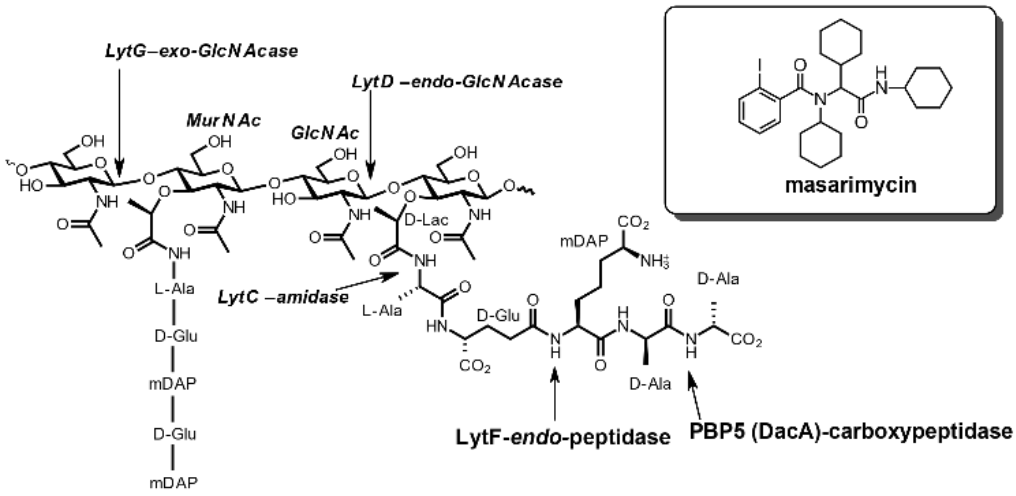

Figure 1. Structure of peptidoglycan showing the cleavage sites of the major autolysins in *Bacillus subtilis.* Inset, structure of Mas, an inhibitor of the *exo*-acting GlcNAcase LytG.

Results and Discussion

We have previously shown that sub-MIC Mas-treated *B. subtilis* demonstate a sausage-link-like phenotype.[19] To better understand the physiological changes that give rise to this morphology, cells were investigated by negative stain electron microscopy (Figure 2 A,B). Masarimycin induces a morphology in which *B. subtilis* cells are enlarged with an inability to complete separation of daughter cells. The average cell length of 15 µm, is 3 times greater than typical wildtype *B. subtilis* cells. Cells presented a pronounced cleavage furrow (Figure 2B) indicating an inability to complete constriction of the septum. Additionally, electron microscopy identified cells with abberant division planes, with daughter cells formed from the sidewall (Figure S1). This suggests disregulation of the assembly of the FtsZ ring. The localization of the FtsZ ring at the midcell is in part controlled by MinC in *B. subtilis* which prevents FtsZ ring formation at the poles and in the timing of cell division.[22] Gram-positive bacteria are believed to employ two different modes of cell wall synthesis that are characterized by differences in the PBPs employed.[23] It has been demonstrated that the shuttling of the major bifunctional PBP1[24] between these complexes is carried out by ErzA and GpsB.[23] Previous biochemical evidence has demonstrated that Mas inhibits the exo-GlcNAcase LytG[19], the major active GlcNAcase during vegetative growth and does not exhibit promiscuous behavior.[21] This data provides evidence for the participation of LytG or the products of its enzyme catalyzed reaction in influencing these two PG synthesis complexes.

Given the disruption in cell shape and impaired separation of daughter cells, we further evaluated the changes to PG metabolism via metabolic labeling with the fluorescent D-amino acid analog HADA.[25, 26] Early exponential phase *B. subtilis* (OD_{600nm} = 0.2) were incubated with 1mM HADA for 1h in the presence of 3 µM Mas (0.75x MIC) or vehicle control (DMSO). After fixation to halt de novo cell-wall metabolism, cells were imaged by confocal microscopy. Control cells demonstrated incorporation of HADA into the cell wall with diffuse distribution along the sidewalls, and with actively dividing cells showing a concentration of HADA at

division sites (Figure 2C). In the presence of 0.75x MIC Mas, HADA incorporation was significantly reduced ($p<0.05$) (Figure 2D). Of particular note is the reduction of HADA incorporation at the septum site in Mas-treated cells. Previous work has shown that septa in *B. subtilis* are enriched in unprocessed pentapeptides, indicating a reduction in cross-linked PG at the division site.[27] HADA is incorporated exclusively into muropentapeptides at the fifth position via an extracellular mechanism using predominantly D,D-transpeptidases. [25, 26] Additionally, HADA does not appear to be incorporated when the D-Ala in the fourth position is involved in a cross-link. [25] Based on this, Mas treatment appears to increase the degree of cross-linking of PG, including at the septum. While HADA is incorporated into stem-peptides via D,D-transpetidases, it can be removed via the action of carboxypeptidases like DacA (Pbp5).[26] To further investigate whether Mas treatment was inducing a change in HADA hydrolysis by DacA, we repeated the experiment in a Δ*dacA* (*Δpbp5)* background. In the absence of DacA, increased fluorescence was observed in both control and treated samples, as DacA is not present to hydrolyze incorporated HADA (Figure 2E,F)[26]. However, Mas-treated cells demonstrated significantly lower fluorescence incorporation in the Δ*dacA* ($p<0.05$) (Figure 2G). Taken collectively, Mas treatment appears to results in increased DacA activity, likely an attempt to control the degree of cross-linking at the septum. Increasing the degree of cross-linking of PG results in increased stiffess of the cell wall.[28] Control of cell wall stiffness is managed through PG hydrolases including GlcNAcases, carboxy- and endo-peptidases.[11, 29] In the absence of LytG activity it appears the cell compensates by increasing endo-peptidase activity.

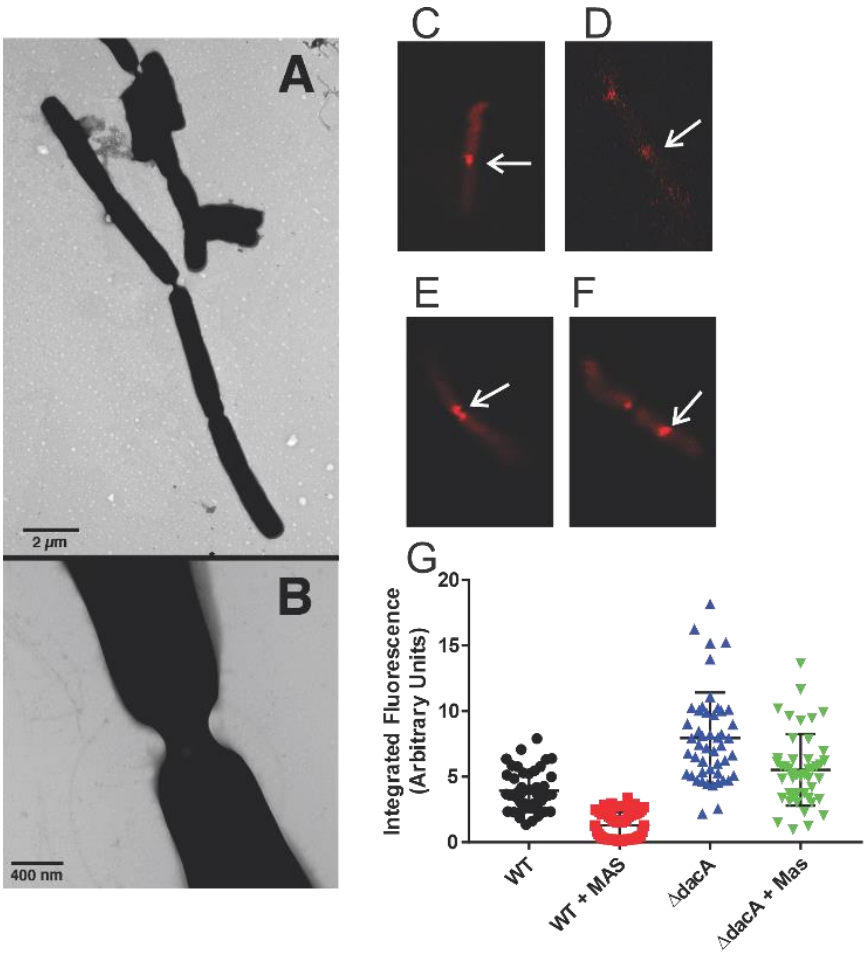

Figure 2. Negative stain transmission electron microscopy of control **(A,B)** and 0.75x MIC Mas-treated *B. subtilis* exhibiting incomplete cell division**.** Early exponential phase *B. subtilis* (OD_{600nm} = 0.2) were treated for 1 hour with 0.75x MIC Mas in liquid culture. (A) Under low magnification, (**B**) under high-magnification. (**C-F**) Confocal fluorescence microscopy of *B. subtilis* cell wall stem-peptides metabolically labeled with HADA. Early exponentially growing *B. subtilis* cells ($OD_{600\ nm}$ = 0.2) labeled with HADA in the absence (**C**) or presence (**D**) of 0.75x MIC Mas.HADA labeling of *ΔdacA* strain in absence (**E**) or presence of Mas (**F**). Arrow indicates septum. (**G**) Integrated fluoresence measurement of cells shows statistically significant decrease in labeling in the presence of Mas (t-test $p<0.05$)

With the observed reduction of HADA incorporation in Mas-treated cells and the impact that a Δ*dacA* strain had on overall HADA incorporation, the muropeptide profiles were analyzed (Figure 3A). Given the number of L,D- and D,D-transpeptidases encoded by *B. subtilis*,[30, 31] this approach will allow for the assessment of global transpeptidase activity in Mas-treated cells. Additionally, this will further assess whether reduced HADA incorporation is due to increased cross-linking. *B. subtilis* was grown to early exponential phase (50 mL LB, OD_{600nm} = 0.2) and treated with 0.75x MIC Mas or vehicle control. After 2 h incubation, cells were harvested and PG was isolated using established procedures. [32] Soluble muropeptides were obtained by digestion with mutanolysin followed by reduction of reducing ends with sodium borohydride. Separation of muropeptides by RP-HPLC was achieved using a linear gradient of 10 mM $(NH_4)_2HPO_4$ pH 5.6 containing 20% methanol.[33] Muropeptide profiles of Mas-treated cells

showed an increase in cross-linking (longer retention times) compared to control cells. [33] This increased cross-linking would prevent transpeptidase reactions where HADA is used as a substrate. The increased cross-linking also demonstrates that Mas is not inhibiting transpeptidase activity. Taken in conjunction with the elevated HADA incorporation in the Δ*dacA* background suggests that DacA activity is increased upon exposure to Mas in an attempt to control the degree of cross-linking.

This remodeling of PG in response to Mas resulting in a highly cross-linked and stiff cell wall in conjunction with the unique morphology raised the question of whether the cell wall stress response was induced. The bacterial alarmone (p)ppGpp acts as an important second-messenger linking both intra- and extracellular environmental cues with global changes in transcription.[34] In *B. subtilis* (p)ppGpp levels are predominantly controled by the bifunctional synthase/hydrolase RelA and can serve as a reporter for cell wall stress.[35, 36] Levels of (p)ppGpp has been shown to influence Sigma D (SigD) controlled genes and high levels of (p)ppGpp are associated with the stringent response.[34] Several SigD controlled autolysins (LytD, LytC, LytF)[37] have been shown to influence sensitivity to Mas.[19] Primers for *relA* produced a single product of 97bp and demonstrated a linear response to template concentration (Figure S2). Incubation in the presence of 3x MIC (15 µM) masarimycin for 20 min showed a 7-fold increase in *relA* transcript levels (Figure 3B). As a positive control for cell wall stress response the β-lactam cefoxitin (3x MIC) was used. Increased levels of (p)ppGpp, as measured by *relA* transcript levels, result in a SigD „off" state, which has implications for cell chaining in *B. subtilis*[34] and is likely influencing the Mas-induced sausage-link-like phenotype. PG hydrolases have been shown to be required for controling the stiffness of the Gram-positive cell wall.[11] Increased levels of (p)ppGpp and the corresponding decrease in GTP levels alleviates repression of the global transcriptional regulator CodY.[38] In addition to activating branched chain amino acid synthesis[38] CodY modulates clhAB2 operon which is involved in cell shape and regulating autolytic acitivity in *B. cereus*.[39] Inhibition of LytG by Mas, initiates a cellular response of disregulated cell elongation and division and an increased level of PG cross-linking that results in elevated stiffness in the cell wall. This increased stiffness is likely the inducer of the cell wall stress response which in turn influences operons and genes involved in cell shape and elongation.

In light of the changes to the cell wall and induction of cell wall stress response,we wanted to investigate potential Mas altered fitness using a chemical genetics interaction approach. Previously we screened several *B. subtilis* SigD controlled autolysins for changes in fitness to Mas, all showed increased sensitivity[19], suggesting that the GlcNAcase (LytD), amidase (LytA), and endopeptidase (LytF) are conditionally required for adaptation to Mas exposure. Given the clear disruption in cell division and peptidoglycan metabolism in the presence of Mas, an array of *B. subtilis* strains lacking autolysins GlcNAcase, carboxypeptidase, transpeptidases, and cell wall division (MinC/D) were screened for altered fitness to Mas (Figure 4A). Given the previous biochemical evidence indicating Mas inhibits the exo-GlcNAcase LytG, the observation that the Δ*lytG* mutant strain demonstrates reduced sensitivity (increased fitness) to Mas with no MIC observed up-to a concentration of 40 µM (10x wild-type MIC) is in line with those results. This is further supported by the fact the Δ*lytG* strain does not produce the characteristic phenotype in the presence of up to 24 µM Mas (6x MIC) (Figure S2A,B). The lack of altered morphology in Δ*lytG* strain further supports LytG being the major target of Mas. Interestingly, screening of several transpeptidase and cell division mutants resulted in increased fitness to Mas. The class A (bifunctional) PbpA (PBP2a) and the class B PbpH are implicated in elongation and rod shape

determination, which given the Mas-induced phenotype is suggestive of their involvement.[40] Analysis of morphology changes upon Mas treatment of Δ*pbpA* presented an increase in irregular cell shape, with the pressence of a bulge in the side wall (Figure S3C,D). The class A PbpD (Pbp4) has been shown along with PbpA and H to directly interact with MreB (cytoskeletal element) in the elongation complex. [24] The lack of the elongated morphology upon Mas treatment in these strains suggests a functional connection with the activity of LytG. The phenotype presented in Mas-treated *B. subtilis* (Figure 2) and the apparent inability to complete separation of daughter cells led us to screen mutants strains lacking cell division associated proteins MinC, MinD, and FtsH. All three strains demonstrated increased fitness to Mas.In addition, upon treatment with Mas, the Δ*minC* strain demonstrates a reduction in clumping and the pressence of bulges along the side wall and poles is reminicent of the bulgecin phenotype in *E. coli*, an inhibitor of the soluble lytic transglycosylase 70 (Slt70).[41-43] This broad based improved fitness to Mas with elongation and division associated proteins argues that Mas may disrupt membrane polarization or impact global protein, or nucleic acid levels in the cell. Given this potential promiscuity of Mas, we screened *B. subtilis* for disruption in membrane polarization,total protein and nucleic acid synthesis by flow cytometry (Figure S4). In both cases, no disruption to membrane polarization or protein synthesis was observed up to 2x MIC. This supports an argument that Mas inhibition of LytG results in disregulation of elongation and division complexes in *B. subtilis.* Based on this information we hypothesize that the absence of LytG activity (through Mas inhibition) results in: 1) altered cell wall stiffness that is compensated for through increased cross-linking, and 2) a reduction in muropeptide production influences the elongation and division complexes.

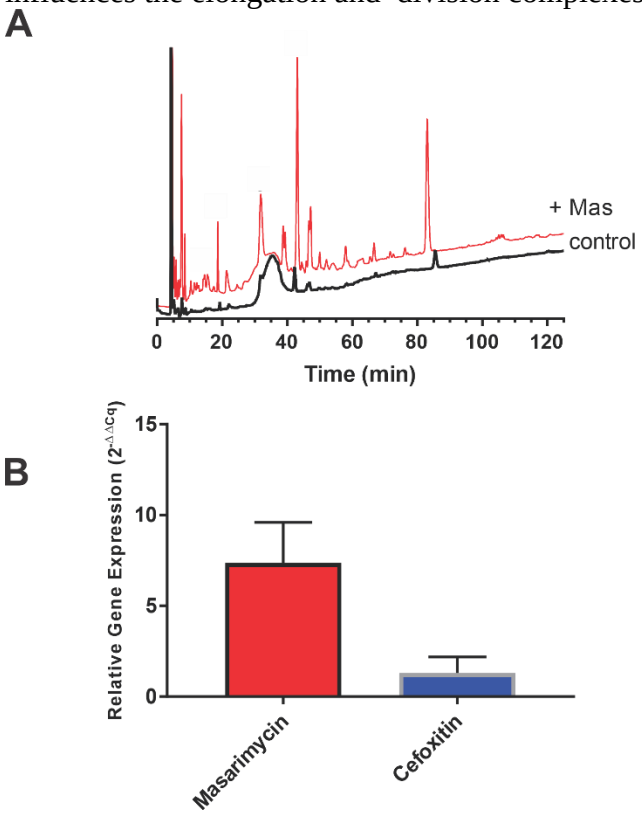

Figure 3. .(**A**) RP-HPLC muropeptide profile of *B. subtilis* in the absence or presence of Mas. (**B**) Relative quantitation of *relA* (ppGpp) synthase as a measure of induction of stringent

response upon treatment with Mas or the cell wall antibiotic cefoxitin. Experiments were run in biological and technical duplicate. Average $2^{-\Delta\Delta Cq}$ with standard deviation are presented.

With the essential complex coordination between PG-synthesis and degradation required for the inside-to-outside model of PG synthesis, we investigated the morphological differences of these mutant strains. Analysis of several mutant strains in the presence of Mas revealed that the Mas-induced wildtype phenotype was not present and in several of the mutant backgrounds bulges observed (Figure S3). One explanation for the increased fitness to Mas in these genetic backgrounds is a change in exo-GlcNAcase (LytG) activity in these strains. To explore this further, wildtype *B. subtilis* and *ΔpbpA* and *ΔminC* strains were subject to a whole cell exo-GlcNAcase activity assay.[18] *B. subtilis* cells were grown to early exponential phase, washed in PBS and incubated with 4 mM pNP-GlcNAc for 16 h, followed by measurement of released pNP (Figure 4B) In both mutant strains exo-GlcNAcase activity was below the level of detection, observed suggesting this improved Mas fitness is in part due to a reduced presence/activity of the biochemically confirmed Mas target LytG. This result highlights the difficulty in molecular target identification using a strictly genetic approach for cell wall targeting compounds as observed phenotypes could be the result of pleotropic effects.

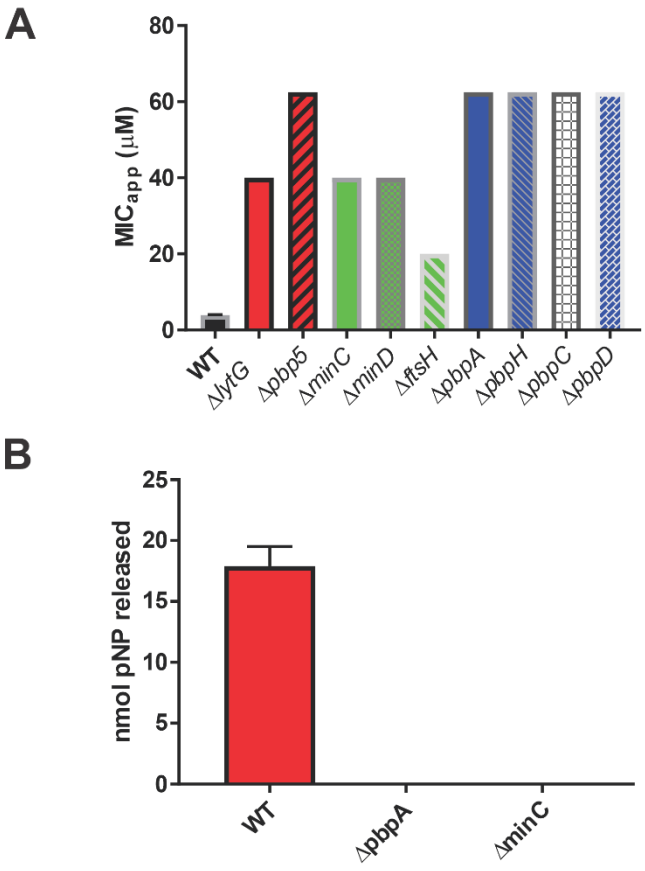

Figure 4 (**A)** Chemical genetic screen of autolysin (*lytG,pbp5*) cell division (*minC,minD,ftsH*), and transpeptidases (*pbpA/H/C/D*) mutant strains for changes in Mas sensitivity. (B) Whole cell assay measuring exo-GlcNAcase activity in two of the mutant strains from the genetic screen. Results shown are the average of 2 biological and technical replicates.

Given the complex interactions at play upon Mas inhibition of LytG, Mas was screened in checkerboard assays to identify potential synergistic or antagonistic relationships with antibiotics with well-defined modes-of-action (Table 1). [44-46] Synergistic interactions can reveal functional connections among cellular components.[44] Conversely, antagonistic interactions can reveal underlying network connectivity.[47] Synergy was observed with ampicillin (FIC_{Index} 0.134), a PBP2a/b (encoded by *pbpA/H*) and PBP4 selective β-lactam [48], and vancomycin (FIC_{Index} 0.235) which binds to the terminal D-Ala-D-Ala in Lipid-II and interfering with the PG maturation process.[49] Vancomycin binding obstructs PBP cross-linking activity, resulting in a compromised cell envelope integrity. In light of the information that the cellular response to Mas treatment is an increase in cross-linking, this functional connection with PG maturation further strengthens a cell wall mode-of-acton. Antagonism was observed with bacitracin (FIC_{Index} 5.88) an inhibitor of undecaprenyl-pyrophosphate recycling, cefuroxime (FIC_{Index} 4.91) a PBP1 selective β-lactam[48], and kanamycin (FIC_{Index} 4.89) a protein synthesis inhibitor (30s subunit). PBP1 is the major transglycosylase/transpeptidase in the cell and is associated with the cell elongation and division complexes in *B. subtilis.* [50] Bacitracin and cefuroxime lead to reduced Lipid-II levels for polymerization and maturation of PG respectively. The antagonism with kanamycin was an intriguing result. While Mas does not interfere with total protein levels in the cell (Figure S4), kanamycin has previously been shown to confer short-term protection to a variety of functionally unrelated antibiotics, including β-lactams.[51] Additionally, the induction of cell wall stress reponse (RelA) and (p)ppGpp production results in the direct inhibition of translation through inactivation of GTPase initiation factor-2.[38]

Table 1. Synergy and antagonism screen with masarimycin.

Antibiotic	**MIC_{app} Antibiotic (μM)**	**FIC Antibiotic**	**MIC_{app} Mas[a] (μM)**	**FIC Mas**	**FIC Index[b]**
Cell-wall targeting					
Ampicillin	3.12	0.0975	0.460	0.121	0.134
Cefoxitin	4.70	1.04	5.85	1.54	2.59
Cefuroxime	0.49	0.035	18.5	4.87	4.91
Bacitracin	577.5	3.30	10.0	2.63	5.88
Vancomycin	0.293	0.073	0.62	0.164	0.235
Fosfomycin	18.75	2.60	1.40	0.367	2.97
Protein synthesis					
Kanamycin	0.03	2.3	2.31	0.60	4.89
Tetracycline	0.0035	1.25	0.28	0.073	1.8215

Nucleic acid synthesis					
Rifampicin	0.08	0.96	3.9	1.03	1.99

[a] MIC masarimycin 4.0 μM; [b]FIC index: <0.5 – synergistic; >0.5-1 – additive; >1-4 – indifference; >4 – antagonistic.

Conclusion

PG metabolism is a tightly coordinated, spatially separated system of biosynthetic and degradative processes. There are several reports of inhibitors of bacterial autolysins (lytic transglycosylases, GlcNAcases) reported in the literature[52-54], however Mas is to our knowledge the first to show inhibition of bacterial growth. The application of the small molecule inhibitor of the *exo*-GlcNAcase LytG, masarimycin as a chemical probe to investigate the role of LytG has provided the first evidence for LytG involvement in the elongation and division complexes. Inhibition of LytG results in an increase in rigidity of the cell wall that induces cell wall stress response (RelA) and production of the alarmone (p)ppGpp (Figure 5). Using both chemical biology and genetic approach we have identified interactions between LytG activity and key players in cell wall elongation and division. While these results do not completely elucidate the role of LytG in these processes, it does clearly identify several lines of inquiry for future studies. Our combined approach highlights the difficulty in elucidating the mode-of-action of cell wall targeting molecules utilizing solely a genetic approach. We demonstrate that deletion of cell wall metabolism and division genes (*pbpA, minC*) impacts the levels of *exo*-GlcNAcase activity, likely resulting in the increased fitness observed in genetic screens. The use of small molecule inhibitors of bacterial autolysins provides a new approach to understanding these enigmatic enzymes biological roles.

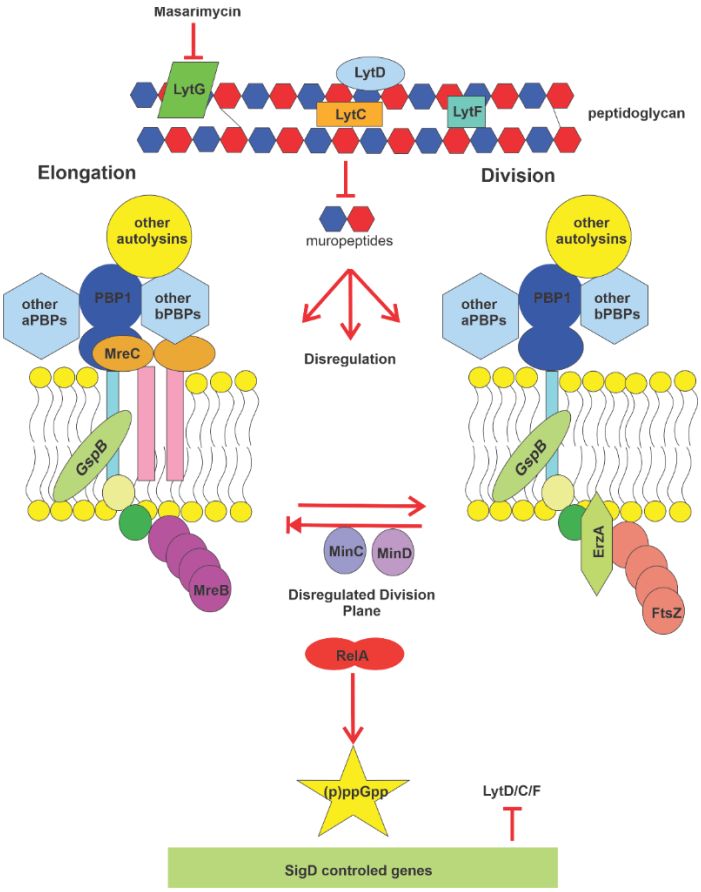

Figure 5 Summary of cellular effects of masarimycin inhibiton of the the *exo*-acting GlcNAcase LytG. aPBP: Class A penicillin binding proteins; bPBP: Class B penicillin binding proteins.

Acknowledgements

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Keywords:

antibiotics, carbohydrates, enzymes, metabolism, peptidoglycan, Gram-positive

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