dimeric AimR for both phi3T and SPbeta in the absence of peptide binding. In the presence of their peptides, the AimR proteins of different phages exhibit different conformational changes. Phi3T AimR (phAimR) turned from dimeric to monomeric in the presence of its peptide, SAIRGA. By contrast, GMPRGA, the peptide of SPbeta, stabilized the dimeric state of SPbeta AimR (spAimR), consistent with another structural study of spAimR published earlier suggesting that AimR regulation by the SPbeta peptide does not involve AimR monomerization [7].

In addition, the SPbeta peptide appeared to promote lysogeny to a lesser extent than the phi3T peptide [6]. This suggests that the aforementioned differences in AimR alteration allowed modulation of lysogeny promotion (or lysis inhibition), where AimR dimer disruption might inhibit lysis more strongly for phi3T. By disrupting phAimR dimerization, the phi3T peptide strongly inhibits the ability of phAimR to activate *aimX*, whereas, by stabilizing the spAimR dimer, the SPbeta peptide carried out a similar function. In the mutation/complementation analysis, the residues and regions important for AimR dimerization were identified, showing that, in the absence of dimerization, lysis was impaired with or without the peptide, and in both phi3T and SPbeta. By mutating the peptide-binding region of AimR, the paper showed that blocking peptide binding decreases the likelihood of lysogeny for both phages. From these results it is clear that the phi3T peptide induces a monomeric form of phAimR to promote lysogeny. For SPbeta, the results suggest that, although full abrogation of the spAimR dimer can promote lysogeny, the SPbeta peptide actually locks spAimR in its dimeric form to promote lysogeny. That the SPbeta peptide regulates decision-making by stabilizing the spAimR dimer, even though this could also be accomplished by dissolving the dimer, demonstrates that there is more complexity to this specific system than simple differences in oligomerization states.

It is important to note that SPbeta lysogeny may be enhanced more by the SPbeta peptide than phi3T lysogeny is enhanced by its respective peptide, as reported by Erez *et al.* [5]. These data differ from the study by Dou *et al.* [6], where SPbeta lysogeny was relatively impaired compared to phi3T, particularly without the external addition of the synthesized SPbeta peptide. Recall that different strains were used for infection with SPbeta – BEST7003 and CU1050 for the Erez *et al.* and Dou *et al.* studies, respectively. CU1050 carries a potentially relevant ochre-suppressor [8,9], and was derived distinctly from BEST7003 [10]. Regardless, it is clear that divergent biological behaviors can arise from subtle structural changes. Moving forward, it would be helpful to consider the strain backgrounds for similar studies, and this would aid in the interpretation of experimental results.

Overall, these types of studies which directly target the elucidation of detailed mechanisms are extremely valuable. Phage decision-making systems such as those of phi3T and SPbeta are not yet well characterized in terms of full genetic pathways and host interactions, generally limiting comprehension of these systems, and thus their potential applications. Therefore, it is exciting to see that Dou *et al.* have devoted effort toward revealing the mechanistic details of how decision-making is regulated by comparing the structural differences between these systems, where small changes in protein structure can shape the fates of entire cells and communities.

Acknowledgments

This work was supported by the National Institutes of Health (grant R01GM107597 to L.Z.).

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https://doi.org/10.1016/j.tim.2018.11.005

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Spotlight

Building Walls: Work That Never Ends

Helene Botella[1] and Julien Vaubourgeix[1,2,*]

Fluorescent amino acid analogs have proven to be useful tools for studying the dynamics of peptidoglycan metabolism. García-Heredia and colleagues showed that their route of incorporation differs depending on the adjunct fluorophore and applied this property to investigate mycobacterial peptidoglycan synthesis and remodeling with heightened granularity.

The bacterial cell wall constitutes a physical and functional barrier between a bacterium's interior and its environment. During its infectious cycle, *Mycobacterium tuberculosis* (*Mtb*), the bacterium that causes tuberculosis (TB), faces hostile and dynamic environmental conditions. For example, during transmission it may go from caseum in the lungs of one host, an environment that permits rapid replication; to aerosolized sputum, in which replication may be impaired by sunlight and/or desiccation; to the alveolar macrophages of its next host, which deploy an arsenal of defensive strategies that *Mtb* must overcome. These environmental perturbations not only demand that *Mtb* adjust its replication rate, but also require it to cope with physical challenges; as such, the success of *Mtb* as a species necessitates constant engagement in repair and synthesis of new cell wall components. This need explains the vulnerability of *Mtb* to cell wall inhibitors used for the treatment of TB, including the first-line drugs isoniazid and ethambutol.

The cell wall of *Mtb* is composed of a plasma membrane around which lies peptidoglycan (PG), itself enveloped by a layer of arabinogalactan esterified by mycolic acids to which a mat of various glycolipids is imbricated; all of this is cocooned in a capsule composed primarily of polysaccharides and proteins. PG, which gives a bacterium its shape, is a polymer of repeating disaccharide units amidated by a peptide chain composed of five amino acids, including two terminal D-alanine residues (D-ala). Crosslinks between peptide chains – consisting of 3–4 crosslinks and noncanonical 3–3 crosslinks, which bridge the third amino acid of a peptide chain to the fourth and third amino acid, respectively, of another – confer rigidity onto PG [1]. Although PG has proven to be an effective target for the treatment of other bacterial infections, no current regimens for TB treatment include a drug that cripples PG pathways. This stems from the fact that *Mtb* produces β-lactamases that degrade β-lactam antibiotics, a class of PG inhibitors, and from a lack of understanding of the nature and spatiotemporal regulation of the molecular mechanisms by which *Mtb* synthesizes, edits, and degrades its PG. The use of fluorescent D-alanine analogs (FDAAs), which allow *in situ* labeling of PG, has served to start bridging this gap [2–4]. FDAAs contain an adjunct that allows them to emit light at a particular wavelength; in lay terms, different adjuncts bestow different colors. In an overdue exercise for the community, García-Heredia and colleagues, in the laboratory of Sloan Siegrist at UMass Amherst, showed that these substituents were not inert but rather affected the route of incorporation of the FDAAs to which they were conjugated, a finding that the authors ingeniously exploited to magnify the granularity of molecular events related to PG metabolism that FDAAs can mark [5]. Particularly, the authors evaluated how deletion of genes or chemical inhibition of targets involved in PG biosynthesis affect labeling by FDAAs, and showed that they incorporate into PG via either extracellular or intracellular routes: while some are mostly substrates for L,D-transpeptidases (LDTs), which are enzymes that exchange D-amino acids in tetrapeptides and also catalyze 3–3 crosslinks, others are mainly incorporated into peptidoglycan precursors in the bacterial cytoplasm (Figure 1). Additionally, the authors surveyed which enzymatic activities ensured incorporation of labeled D-ala-D-ala and showed that the dipeptide complemented deletions of the D-alanine racemase and D-alanine ligase, both of which are indispensable for D-ala-D-ala synthesis, in *Mycobacterium smegmatis* (*Msm*). This suggests that the enzyme MurF catalyzes the addition of intact D-ala-D-ala onto UDP-MurNAc-tripeptide cytoplasmic precursors; of note, decreased incorporation of D-ala-D-ala in an LDT mutant revealed that a proportion of the dipeptide is likely cleaved to monopeptides to supply the LDT substrate pool. Collectively, these findings align with the ability of FDAAs to light up chiefly the poles of mycobacteria – the primary sites of PG elongation – as well as their side walls [4–8]. The latter, long overlooked as inert material, was the focus of further work by García-Heredia and colleagues as well as a concomitant study published by Baranowski and colleagues from the laboratory of Hesper Rego at Yale [8]. In both studies, the authors observed that FDAAs were incorporated into a gradient of decreasing fluorescence intensity from pole to mid-cell. The former study reported that FDAAs that were incorporated into PG via both intracellular and extracellular routes labeled lateral PG, indicating that both tailoring of existing PG and synthesis of new PG occurred along the bacterial longitudinal axis. The latter study reported that LDTs convert newly synthesized 4–3 crosslinked PG to a 3–3 crosslinked mesh at this site, bestowing upon *Msm* its shape and a mechanism by which it may control septum placement (Figure 1). Additionally, García-Heredia and colleagues documented that synthesis of PG intensified at the side wall when the bacterial cells were subjected to cell wall-damaging insults [5]. This functionalization of lateral PG is reminiscent of a previous study that used atomic force microscopy to demonstrate that physical cell-surface deformations indicative of future septum sites migrated from the polar region to mid-cell [9]. Altogether, these studies raise new questions: to what degree, and under which regulatory mechanisms, is PG edited to ensure proper septum placement? How are LDTs excluded from the poles or retained at the side wall, and, more generally, how is functional segregation of proteins maintained? Do stress-associated perturbations, including stresses imposed by host immunity or antibiotics, affect the

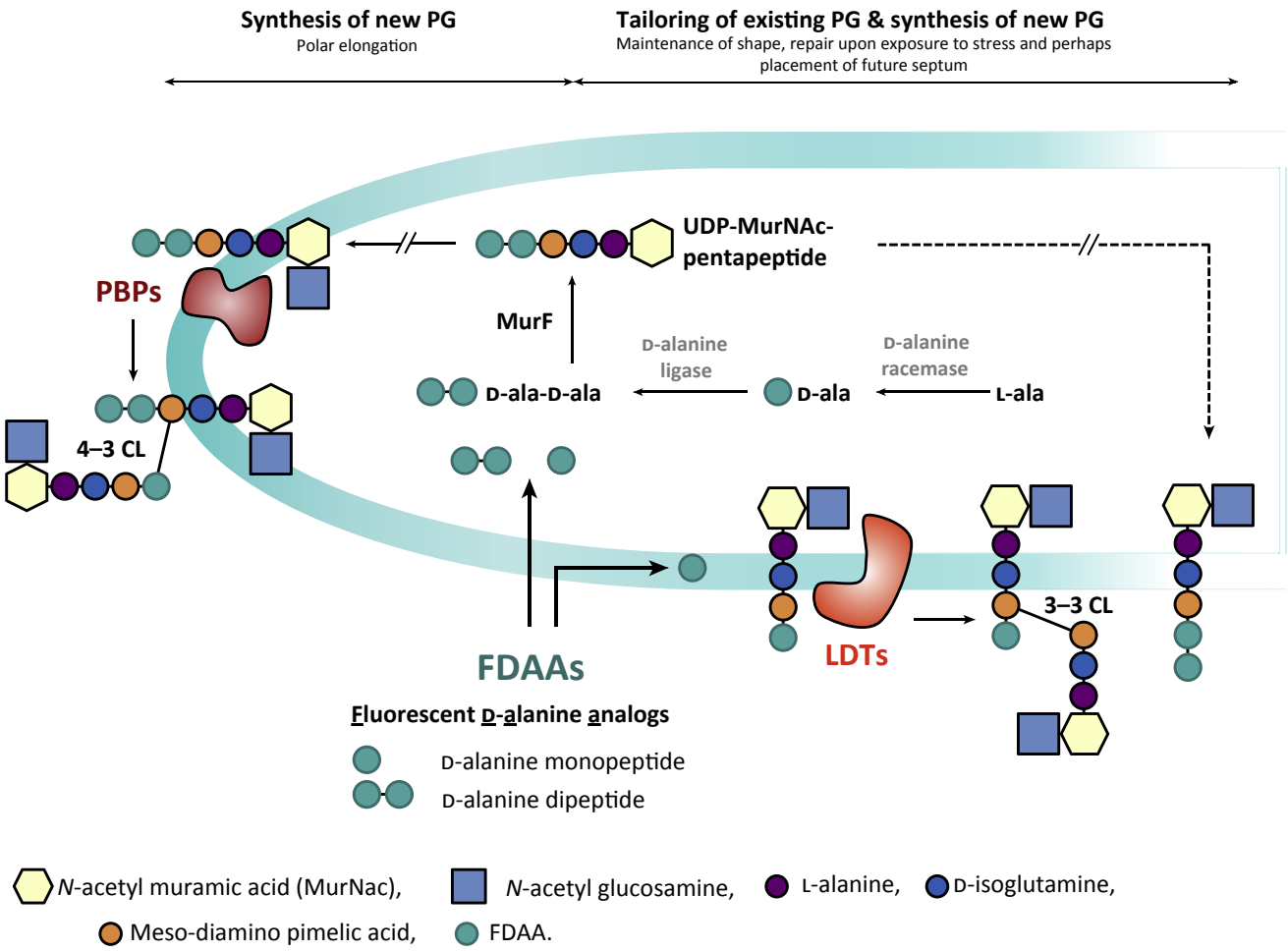

Figure 1. Incorporation Routes of Fluorescent D-Alanine Analogs (FDAAs) in Mycobacterial Peptidoglycan (PG). Depending on their adjunct, FDAAs can be incorporated into PG precursors by the MurF ligase, or into existing PG by L,D-transpeptidases. Addition of new PG occurs chiefly at the mycobacterial pole and to a lower extent at the side wall; the latter intensifies upon exposure to an external stress. The lateral PG is also subject to extensive tailoring that involves formation of 3–3 crosslinks. Abbreviations: D-ala, D-alanine; L-ala, L-alanine; CL, cross link; LDTs, L,D-transpeptidases; PBPs, penicillin-binding proteins.

functional distribution of enzymes involved in PG synthesis and editing, and does that affect septum placement or initiation of septation?

Most studies in similar lines of investigation have so far focused on *Msm*, for which some, but not all, biology pertains to *Mtb*. In this vein, while Baranowski and colleagues showed that inhibitors of LDTs and penicillin-binding proteins, which catalyze 4–3 crosslinks, are synergistic in both organisms, the Siegrist group's study confirmed that FDAA labeling of *Mtb* is more heterogeneous than in *Msm* [4,5]. The biological implications of this latter observation should be evaluated, and studies extended to examine the dynamics of PG metabolism in *Mtb* both *in vitro* and in macrophages, as some FDAAs can enter host cells [3].

Collectively, these studies provide significant scholarly advances and outstanding practical and intellectual tools to continue exploring PG metabolism in mycobacteria. For example, the use of FDAAs, in conjunction with the use of supravital markers for classes of macromolecules, and assisted by additional technologies such as atomic force microscopy and genomic variants, may support the development of automated computational algorithms to describe the morphology of a mycobacterial cell, including parameters such as area, length, width, circularity, curvature, sinuosity, and angularity. Such detailed descriptions could help to evaluate the

morphological impact of a compound on a bacterial cell, allowing for the clustering of compounds based on these morphometric parameters and even the assessment of the mode of action of unknown compounds based on where they cluster [10]. Similarly, this may constitute a new avenue to screen for compounds that block PG synthesis in *Mtb* and help us to better comprehend the mode of action of these inhibitors, which could prove to be powerful weapons in the continued fight against TB.

Acknowledgments

We thank Drs C. Nathan and S. Ehrt, and S. Schrader (Weill Cornell Medicine) for careful editing of the manuscript. The Department of Microbiology and Immunology is supported by the William Randolph Hearst Foundation. J.V. was supported by the Abby and Howard Milstein Program in Chemical Biology and Translational Medicine and the Potts Memorial Foundation. We apologize to those authors whose references were omitted due to space constraints.

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https://doi.org/10.1016/j.tim.2018.11.006

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