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From Input to Output: The Lap/c-di-GMP Biofilm Regulatory Circuit

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Abstract

Biofilms are the dominant bacterial lifestyle. The regulation of the formation and dispersal of bacterial biofilms has been the subject of study in many organisms. Over the last two decades, the mechanisms of *Pseudomonas fluorescens* biofilm formation and regulation have emerged as among the best understood of any bacterial biofilm system. Biofilm formation by *P. fluorescens* occurs through the localization of an adhesin, LapA, to the outer membrane via a variant of the classical type I secretion system. The decision between biofilm formation and dispersal is mediated by LapD, a c-di-GMP receptor, and LapG, a periplasmic protease, which together control whether LapA is retained or released from the cell surface. LapA localization is also controlled by a complex network of c-di-GMP-metabolizing enzymes. This review describes the current understanding of LapA-mediated biofilm formation by *P. fluorescens* and discusses several emerging models for the regulation and function of this adhesin.

Keywords

biofilm; LapA; c-di-GMP; signal transduction; type I secretion; *Pseudomonas*

INTRODUCTION

Bacteria exist predominantly within biofilms (46). During biofilm formation, free-living, planktonic cells encounter a surface and undergo numerous physiological changes to attach to that surface (120). Once attached, cells in a developing biofilm produce proteins, polysaccharides, and extracellular DNA (eDNA) that contribute to an adhesive matrix securing the cells to the surface and each other (18, 47, 85, 139, 155). Bacterial cells are able to leave the biofilm through a process of dispersal and become free-floating, planktonic cells

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(54, 55, 84, 100, 129). These cells are then free to move to other sites, where they can seed new biofilms.

Whether a cell will continue to exist in a planktonic state or attach to a surface and commit to a biofilm lifestyle is often determined by its level of cyclic di-guanosine monophosphate (c-di-GMP), a ubiquitous second messenger in bacteria (124). This cyclic dinucleotide is synthesized by the condensation of two GTP molecules into one molecule of c-di-GMP by enzymes called diguanylate cyclases (DGCs), which contain so-called GGDEF domains (113), and it is degraded by enzymes called phosphodiesterases (PDEs), containing either conserved EAL or HD-GYP amino acid motifs (29, 125). The complex role of c-di-GMP in regulating biofilm formation involves specific impacts on many different functions, including motility, surface sensing, surface adhesion, and gene regulation (7, 13, 63, 90, 95, 98, 105, 149). c-di-GMP signals impact these functions through the activity of c-di-GMP-binding proteins called effectors. Many such effector proteins have been described in recent years; they impact diverse functions such as extracellular polymeric substance (EPS) synthesis (3, 44, 83, 96, 145), motility (7, 28, 60, 112, 119, 127, 149, 154), protein secretion (123), metabolism (152), transcription (3, 9, 63, 78, 82, 98), subcellular protein localization (39), adhesin localization (105), and cell development (1, 39, 110).

Many organisms encode large numbers of proteins that can metabolize or bind c-di-GMP (85, 124). While these proteins have in some cases been shown to impact multiple behaviors (61, 106), in many cases they appear to impact only one function (2, 33, 34, 61, 79, 87, 105, 106, 152). Untangling the mechanisms by which complicated biofilm-related behaviors are specifically regulated by a diffusible cyclic nucleotide signaling molecule is difficult. However, the bacterium *Pseudomonas fluorescens* has emerged as an important model organism for the study of biofilms due to the relative simplicity of its c-di-GMP-regulated biofilm output, that is, control of localization of the LapA adhesin, which is required for the first committed step in biofilm formation. In this review, we discuss recent advances in our understanding of how localization of LapA is regulated by c-di-GMP, as well as current models of how *P. fluorescens* organizes its c-di-GMP signaling network around this output. The regulatory system controlling biofilm formation by *P. fluorescens*, from environmental input(s) to localization of the LapA adhesin, is one of the best-understood of such circuits and has yielded general insights into c-di-GMP signaling pathways.

THE Lap SYSTEM AS A BIOFILM REGULATORY UNIT

P. fluorescens, which is commonly isolated from soil and freshwater, plays important roles in agriculture (62) and industrial processes (135) and is linked to human disease in severely immunocom-promised individuals (117, 118, 130). This bacterium can form a biofilm on a variety of biotic (37, 38, 131) and abiotic (20, 108) surfaces. Hinsa and colleagues (64) showed that *P. fluorescens* forms a biofilm through regulation of the cell-surface localization of the adhesive protein LapA (large adhesive protein A; also from the Spanish *lapa*, for limpet) (69). The LapA adhesin is secreted to the cell surface by the type I secretion system (T1SS), composed of the LapB, LapC, and LapE proteins (64), where it remains tethered to the bacterial outer membrane and acts to adhere the cell to biotic or abiotic surfaces through various adhesive domains (20, 40, 41, 69) (Figure 1). The retention of

LapA on the outer membrane is controlled by the proteins LapD and LapG (55, 65, 104). LapG is a periplasmic protease that can proteolytically process LapA, removing the globular N-terminal portion that anchors LapA to the cell and liberating the cell from the surface (19, 20, 104, 132, 134). The activity of LapG is, in turn, regulated by the protein LapD. LapD is an inner membrane–spanning protein with a periplasmic Cache domain and cytoplasmic HAMP, GGDEF, and EAL domains. The LapD Cache domain can bind to LapG and thereby sequester this protease from its LapA target (25, 26, 103–105). The cytoplasmic GGDEF and EAL domains detect intracellular c-di-GMP signals, which are then transduced across the inner membrane via the HAMP domain to determine the affinity of the periplasmic Cache domain for LapG (26, 30, 103–105). When cytoplasmic c-di-GMP levels are high, LapD is activated and binds to and sequesters LapG, preventing LapA processing (Figure 1). However, when intracellular c-di-GMP levels are low, LapD does not bind to LapG, leaving LapG free to process LapA and release this adhesin from the outer membrane (Figure 1). Together these proteins constitute what we refer to as the Lap system.

Lap systems have been described in several bacteria (103, 134), including *P. fluorescens* (26, 103–105), *Pseudomonas aeruginosa* (31, 126), *Pseudomonas putida* (54, 55, 92, 94), *Legionella pneumophila* (25, 26), *Bordetella bronchiseptica* (5), *Shewanella oneidensis* (153), *Marinomonas primoryensis* (56, 58), *Vibrio cholerae* (14, 77, 132), and *Pectobacterium atrosepticum* (116). However, mechanistic insight into the Lap system is most complete for *P. fluorescens*; thus, this organism is the focus here. Studies in other organisms are discussed in the context of the *P. fluorescens* Lap system.

LapA

LapA is a 520-kDa protein required for biofilm formation (64), is exposed on the cell surface (69), and is a documented adhesin based on atomic force microscopy (AFM) studies: ~100 nN of force is required to remove LapA from a glass substratum (20, 40, 41, 69). LapA is typically encoded among a cluster of genes that contains its cognate T1SS and regulatory proteins (Figure 2a). However, in some organisms, Lap-system secretory and regulatory genes may be encoded at distant sites in the genome (50, 64, 77).

LapA homologs frequently differ dramatically from one another at the primary sequence level (134). The most variable portion is the central repeats region (Figure 2b), which often contains dozens of tandem repeats thought to be involved in surface binding (20, 40) and to extend functional C-terminal domains away from the cell (51, 58, 59, 142, 143). LapA homologs are also characterized by the presence of key domains on either end of the polypeptide chain that are much more conserved among homologs. We discuss these functional domains below.

FUNCTIONAL SIGNIFICANCE OF LapA EXTRACELLULAR DOMAINS: C TERMINUS

The LapA C terminus contains a T1SS secretion signal that targets the adhesin to its cognate secretion machinery and a cluster of repeat sequences of the repeats-in-toxin (RTX) family (147). These RTX repeats are thought to discourage protein folding in the low-calcium

environment of the cytoplasm (27, 71), and they may be involved in substrate tethering by the LapB protein and protection from proteolysis as observed for another T1SS (81). Therefore, in the cytoplasm LapA is thought to remain in a disordered, unfolded state, which allows it to be secreted as a linear polypeptide chain through the LapEBC T1SS (Figure 2c), which is thought to be too narrow for a folded polypeptide to pass through (114). As T1SS proteins are secreted C terminus first, the RTX repeats reach the relatively high-calcium extracellular space first and initiate folding as the rest of the protein is secreted (21, 86).

FUNCTIONAL SIGNIFICANCE OF LapA EXTRACELLULAR DOMAINS: CORE REPEATS REGION

The core repeats region is highly variable among LapA homologs (Figure 2b). It has been proposed that LapA homologs have a modular structure in which the central region can be exchanged by horizontal gene transfer (35). There are large differences in these repeats even between highly related organisms; for example, the repeats found in the RtxA protein of *L. pneumophila* differ dramatically between strains of the same species (35). Indeed, it has been demonstrated that LapA can be effectively secreted and retained on the surface of the cell with a number of structurally diverse cargo proteins in place of this core repeats region, such as the fluorescent protein tdTomato and metal-binding domains (133). The modularity of the core repeats regions may suggest specific function of the repeats in each strain, while the conservation of the N and C termini may indicate a shared molecular mechanism of secretion and retention. However, the functional differences between the various classes of repeats found in these proteins are not yet clear.

Structural studies of the repeats regions of ice-binding proteins of *M. primoryensis* and *Shewanella frigidimarina* suggest that these proteins adopt a long chain of β sheets in tube-like conformations rigidified by calcium binding (58, 59, 142, 143). It has been proposed that these repeats function as extender domains to hold the ligand-binding and adhesive C-terminal domains far from the cell (Figure 2b), where they can interact with their binding substrates.

That large tandem-repeats regions extend adhesive domains far from the cell would be consistent with the large size of some of these proteins (1.5 MDa in the case of the *M. primoryensis* ice-binding protein), but this does not explain the variations in sequence and predicted structure seen among LapA orthologs. For example, the RtxA protein orthologs encoded by the *L. pneumophila* strains Paris and Lens contain core repeats regions of roughly the same size but with entirely different amino acid sequences (23), perhaps indicating functional differences between LapA orthologs that may not be sufficiently explained by a common extender function. Alternatively, since length is conserved but not sequence, perhaps the repeats serve an extender function that does not require sequence conservation.

FUNCTIONAL SIGNIFICANCE OF LapA EXTRACELLULAR DOMAINS: ADHESIVE PROPERTIES

In addition to the roles described above, the C-terminal domains of LapA have been shown to play a role in surface adherence. Experiments using AFM show that distinct regions of LapA are involved in adhesion to different surface types (40): For hydrophilic surfaces, rupture distance was higher than for hydrophobic surfaces, indicating that different portions of LapA attach to hydrophilic and hydrophobic surfaces. Also, the force profile of LapA on a hydrophilic surface had a sawtooth pattern, characteristic of a protein unfolding incrementally. These data indicate that on a hydrophilic surface, LapA attaches with its C terminus while the core repeats remain unattached and form a regularly folded structure (40). Indeed, Boyd et al. (20) found that the C-terminal von Willebrand A domain in LapA is required for attachment to hydrophilic substrates. Conversely, for a hydrophobic surface, the rupture length was much lower and the force profile did not exhibit a sawtooth pattern. These data indicate that attachment to a hydrophobic surface is mediated by LapA's core repeats—the repeats, being bound to the surface, are not free to unfold when pulled upon by the cantilever.

Interestingly, the AFM patterns observed were dependent on the density of LapA on the surface (40). When *P. fluorescens* cells were allowed to form a biofilm on a hydrophilic surface before the cells were dispersed, enabling LapA to accumulate on the substratum, the degree to which individual LapA molecules stretched was greatly reduced and the force of resistance was increased. These data indicate that, in addition to mediating binding to the surface, LapA is capable of strong intermolecular adhesion. However, not all LapA homologs are similarly able to contribute both adhesive and cohesive roles in the biofilm matrix. For *P. putida*, LapA contributes only to cell-to-surface attachment, while a second large adhesin, LapF, which is not thought to be a LapA homolog, is required for cell-cell interactions (42, 94).

The ability to bind multiple ligands has been described for a LapA ortholog in another organism. Guo et al. (58) showed that the 1.5-MDa ice-binding protein of *M. primoryensis* has at least three distinct ligand-binding domains in the extracellular portion of the protein that separately bind ice, peptides, and sugars. They proposed that the bacterium tethers itself to the ice of antarctic lakes using the ice-binding domain and then recruits photosynthetic microorganisms to this relatively high-light environment using the peptide- and sugar-binding domains.

FUNCTIONAL SIGNIFICANCE OF LapA PERIPLASMIC DOMAIN: REGULATION OF LapA SURFACE LOCALIZATION VIA THE N-TERMINAL ANCHORING DOMAIN

LapA is secreted through a T1SS composed of three proteins encoded by genes adjacent to the *lapA* gene on the chromosome: an inner-membrane ATP-binding cassette transporter, LapB; a membrane fusion protein, LapC; and a TolC-like outer membrane pore, LapE (Figures 1 and 2a). Like all other T1SS substrates described to date, LapA is likely secreted

in a C-to-N terminus manner once LapA's C-terminal secretion signal is recognized by LapB (67, 68). Historically it has been thought that in all cases type I secretion proceeds in a single-step process where, upon substrate recognition, the T1SS forms a continuous secretion pore, allowing the substrate to be directly secreted from the cytoplasm into the extracellular environment with no periplasmic intermediate (66, 68, 74, 136). However, in the case of LapA homologs, a portion of the LapA N terminus is thought to fold, thereby stalling secretion (58, 59, 132, 134). The role of the N-terminal portion of LapA homologs as a retention domain appears to be well conserved (Figure 2b,c), as the N-terminal portion of LapA homologs can be swapped and functional retention is observed (132). The newly described two-step secretion process may represent a phylogenetically distinct subclass of T1SSs—the function and distribution of LapA-like adhesins and their associated T1SS are detailed in a recent review by Smith et al. (134). The LapD and LapG proteins have been identified as regulators of LapA localization (55, 65, 104)—LapD is an inner membrane–spanning protein with cytoplasmic and periplasmic domains (65, 105), while LapG is found in the periplasm (104). Considering the previously described one-step type I secretion model and the regulation of LapA function by a periplasmic protease, the mechanisms by which LapA remains associated with the cell surface to mediate cell-to-surface attachment have, until recently, remained enigmatic.

Studies probing the features of LapA required for its regulation by LapG found that LapG can proteolytically process LapA in vitro and remove the N-terminal ~100 amino acids (104) (Figures 1 and 2c). This processing event depends on the double-alanine motif of LapA that is characteristic of this group of adhesins. When this motif was mutated from 108AA109 to 108RR109 in LapA, processing was no longer observed in vitro, although in vivo processing still occurred at a low rate (104). It was only when all of the double-alanine sequences in the N-terminal portion of LapA were deleted that LapG processing was completely prevented, indicating that in vivo LapG is capable of cleaving LapA at multiple AlaAla sites (20). The ability of the periplasmic protease LapG to access and regulate LapA by directly binding and cleaving its N terminus indicates that at least that portion of LapA is exposed to the periplasm.

Recent data (57, 58, 132) support the view that LapA is secreted in a two-step process during which the N-terminal portion of the protein is able to fold in the periplasm, forming a stable, globular N-terminal domain that prevents complete secretion of LapA through LapE (Figures 1 and 2c). Biochemical and genetic data supporting this two-step secretion model were provided recently by Smith et al. (132), who expressed a short secretion substrate, HA-C235, composed of the LapA C-terminal T1SS secretion signal fused to a 3X hemagglutinin tag but lacking the N-terminal domain required for cell association. The investigators assessed whether two substrates can be secreted through the same T1SS by observing competition between secretion of LapA and the HA-C235 secretion substrate through the LapBCE T1SS. When full-length LapA remained anchored to the surface of the cell in a Δ*lapG* mutant [i.e., lacking the periplasmic protease required to cleave the N terminus and release LapA from the cell surface (Figure 1)], the HA-C235 protein could not be detected in the supernatant. However, when HA-C235 secretion was assessed in a Δ*lapD* mutant (i.e., in which LapA is cleaved by a constitutively active LapG protease and LapA is therefore released from the cell), they found that HA-C235 was detected abundantly in the

supernatant. These studies showed that LapA obstructs the Lap T1SS outer membrane pore during its secretion, preventing the secretion of other substrates. In further support of this model, overexpression of the LapE outer membrane pore in a Δ*lapG* mutant was sufficient to recover HA-C235 secretion, indicating that the component of the secretion machinery that LapA is occupying, and is limiting for secretion, is indeed LapE (Figure 1). Thus, when the LapE protein is occupied by LapA, no further protein can be secreted, consistent with the model where LapA is anchored to the cell surface via the outer membrane component of the T1SS. These observations are further supported by previous data indicating that when cellular levels of LapA are compared between wild-type and Δ*lapD* mutant cells, the level of intracellular LapA in a Δ*lapD* mutant is roughly half that in a wild-type cell (105). We interpret these data to mean that a fraction of the cellular pool of LapA is trapped inside the cell because cell-surface-localized LapA is preventing secretion of cytoplasmic LapA.

The two-step secretion model proposed here requires a well-folded, stable N-terminal domain that prevents complete secretion of LapA by obstructing the LapE outer membrane pore. Evidence for a globular N-terminal domain was presented recently by Guo, Davies, and colleagues using small-angle X-ray scattering (SAXS) data analysis of the N-terminal portion of the LapA ortholog *Mp*IBP of *M. primoryensis* (57, 58). The SAXS-derived envelope suggests that the N terminus forms a structure comprising a thin cylindrical section small enough to fit into the pore formed by the LapE β barrel, and it also indicates that there is a wider globular region at the very end of the N terminus that is too large to pass through the pore and may therefore act as a plug (58). In addition, a solution nuclear magnetic resonance structure solved in the same study indicates that the dimensions of the globular domain are roughly 30 Å by 29 Å, too large to fit through the roughly 20-Å LapE pore (Figure 2c). Biochemical analysis of the N terminus of LapA is consistent with these findings, in that this retention domain comprises both a well-ordered, heat-stable, protease-resistant subregion of this domain and an unstructured subregion (132).

LapG AND LapD REGULATE LapA LOCALIZATION

Localization of LapA to the cell surface in *P. fluorescens* is required for biofilm formation. Individual cells appear to express multiple copies of LapA on their cell surface (69), and LapA levels on the cell surface are proportional to the amount of biofilm formation observed (19, 20, 101, 104, 105). *P. fluorescens* can thus fine-tune its tendency for surface attachment by controlling the amount of LapA presented on its surface, which in turn is achieved through the regulated activity of LapG and LapD.

LapG CAN PROTEOLYTICALLY PROCESS LapA AND RELEASE IT FROM THE CELL SURFACE

LapG belongs to the bacterial transglutaminase-like cysteine protease family of proteins (a.k.a. DUF920/COG3672) (53). LapG controls LapA localization by cleaving LapA at a double-alanine motif that is conserved in many LapA homologs and other LapG targets (19, 20, 104, 126, 132). This cleavage event separates the adhesin's anchor region from the extracellular adhesive portions (Figures 1 and 2c).

The activity of LapG depends on two conserved features: a calcium-binding region and an active site containing the cysteine-histidine-aspartate catalytic triad (Figure 3a). Mutation of residues in either of these conserved sites, or the use of calcium chelators, is sufficient to abolish LapG activity (19, 20, 25, 26). Consistent with key structural features of LapG being broadly conserved, studies have shown that LapG orthologs are able to process LapA homologs from other species (19, 25, 77, 132). However, the efficiency of this between-species processing is sometimes as much as 375-fold lower than that of processing by LapG homologs of their native targets (25), indicating that there are elements of the LapG-LapA interaction interface that confer specificity, and there are some clues to what these elements may be. The region neighboring the predicted LapA-binding pocket of LapG is reasonably well conserved and hydrophobic, suggesting a possible interaction interface (25). Also, LapA has two conserved hydrophobic surfaces that may interact with the hydrophobic surface of LapG: (*a*) a predicted α-helical region adjacent to the major TAAG processing site that seems to be required for LapG processing and (*b*) a region of the globular N-terminal domain. Both domains seem to have conserved hydrophobic properties that could be involved in recognition by, or interaction with, the LapG protease (20, 57) (Figures 2c and 3a). One possibility is that the hydrophobic interaction interface between LapA and LapG acts as a recognition site and if necessary can also allow LapG to bind LapA and pull the TAAG motif out of the LapE pore in order for LapG to access and process LapA (Figures 1 and 2c).

Interestingly, in addition to chelators like EGTA, weak organic acids such as citrate can also function to chelate calcium (70). Boyd et al. (19) have suggested a broader significance of this observed activity for citrate in the context of LapG. Citrate is able to increase biofilm formation by *P. fluorescens*, even in a Δ*lapD* mutant, where LapG should be constitutively active (65), and this biofilm enhancement can be suppressed by supplementation of $CaCl_2$ (19). Availability of citrate as an environmental regulator of LapG activity via calcium chelation may have biological significance, as this organic acid has been found in appreciable concentrations in the exudates of the roots of plants commonly colonized by *P. fluorescens* (36, 70, 73). Thus, citrate may deplete available calcium to enhance *P. fluorescens* root colonization. Additionally, some common biofilm-matrix components, including adhesins, alginate, and extracellular DNA, can chelate calcium (45, 93, 102). Therefore, while the periplasm can concentrate calcium above extracellular levels to support LapG activity (72), environmental calcium chelators may be capable of suppressing the activity of LapG and thus promote biofilm formation.

LapD CONTROLS LapG ACTIVITY THROUGH TRANSDOMAIN SIGNALING INDUCED BY c-di-GMP BINDING

LapD is an inner membrane–spanning, c-di-GMP-responsive effector protein. Its periplasmic portion is composed of a Cache domain that physically interacts with LapG to sequester this protease and thus inhibit it from processing LapA (Figure 1): A tryptophan exposed on the tip of the Cache domain of LapD inserts into a pocket on LapG close to the latter's calcium-binding domain and active site (25, 26, 103–105, 141). This tryptophan is conserved in LapD homologs (Figure 3b,c; also see Figure 3d), and binding between

LapD and LapG in vitro can be competitively inhibited by addition of a short, tryptophan-containing peptide that mimics the LapD interface (26).

The cytoplasmic portion of LapD is composed of a HAMP domain and signaling helix (S-helix)—common in transmembrane signaling proteins (6, 43, 137)—and two degenerate c-di-GMP-metabolizing domains, a GGDEF domain and an EAL domain (Figures 1 and 3b). These domains would be expected to have DGC and PDE activity, respectively, but comparison with active enzymes reveals that key residues important for catalytic function are degenerate, and purified LapD was not found to possess catalytic activity in vitro (105). However, LapD's EAL domain retains the ability to bind c-di-GMP, which is essential for LapD to transduce cytoplasmic c-di-GMP signals out to the periplasm—thus LapD was posited to mediate inside-out signaling by Newell et al. (105).

Crystallography and cross-linking studies indicate that LapD exists as a constitutive dimer through interactions of its cytoplasmic HAMP domains and periplasmic Cache domains (25, 26, 103). In the contracted, autoinhibited state, the EAL domain is positioned to share a large contact area with the S-helix (Figure 3b,c). In this state, the GGDEF domain occludes the EAL domain's c-di-GMP-binding pocket, making this conformation incompatible with c-di-GMP binding. In the other, more relaxed and open state, the EAL domain dislodges from the S-helix, enabling it to engage with c-di-GMP if cellular levels are sufficiently high. The EAL domain interface that accommodated the S-helix in the autoinhibited state is now available for homotypic, c-di-GMP-dependent dimerization (30). Thus, inside-out signaling would occur through binding of c-di-GMP to the LapD EAL domain, which stabilizes LapD in the relaxed, signaling-competent conformation. The conformational change between the autoinhibited and relaxed states is transduced across the membrane through conformational changes in the HAMP domain, which in turn causes the periplasmic Cache domain to adopt a conformation that is more favorable to LapG interaction (103).

LapD is unable to bind to c-di-GMP in the autoinhibited conformation observed in crystals (103); this raises the question of how LapD is able to sense second messenger levels. SAXS data indicate that in a low-c-di-GMP environment, one half of the LapD dimer may switch between the two states (Figure 3c), therefore allowing the EAL domain to sample the c-di-GMP binding-competent conformation (30). c-di-GMP binding to the EAL domain shifts the equilibrium away from the autoinhibited state and stabilizes the open, active conformation. Consistent with the model of equilibrium conformations, mutations in the S-helix that inhibit interaction between the S-helix and EAL domain double the affinity of LapD for c-di-GMP and result in a hyperbiofilm formation phenotype (103). These observations suggest that LapD is more active when pushed toward the open conformation. The conformation adopted by LapD when binding to c-di-GMP results in a sixfold increase in affinity for LapG (30), and in vitro studies of the dynamics of this interaction have shown that LapD affinity for LapG increases abruptly, not linearly, with c-di-GMP concentration (26). These findings suggest that LapD is responsive to cytoplasmic c-di-GMP in an all-or-nothing, switch-like manner once a threshold signal strength is reached.

More recent data suggest that LapD can signal in both directions simultaneously through a mechanism dubbed "coincidence detection" (26, 30). The first clues to bidirectional

signaling came from experiments conducted by Chatterjee, Sondermann, and colleagues that characterized interaction between LapD's periplasmic domain and LapG (26). LapD and LapG were found to have transient, low-affinity interactions even in the absence of c-di-GMP. Chatterjee et al. proposed that perhaps these transient interactions cause a conformational change that is transduced through the LapD TM and HAMP domains and causes the cytoplasmic portion of LapD to transiently switch from the autoinhibited conformation to the open, c-di-GMP binding-competent conformation to allow activation. In a follow-up study, Cooley et al. (30) demonstrated an important role for this LapG binding and clarified many important features of LapD's bidirectional signaling. By adding cross-linkable cysteine residues at sites predicted to be proximal in certain conformations, they were able to capture the conformational changes in the TM domain of LapD. This conformational change was observed when c-di-GMP was present, consistent with previous data describing LapD's inside-out signaling (103, 104). In addition, the same conformational change was observed when LapD was incubated with LapG, even in the absence of c-di-GMP; crucially, it was not observed in a LapD mutant lacking the tryptophan required for interaction with LapG, showing that this LapD conformational change is due to specific interactions between LapD and LapG at the same site where c-di-GMP-activated LapD binds LapG to prevent this protease from targeting LapA. Together, these data suggest that weak interactions of LapG with the periplasmic domain of LapD promote LapD binding of c-di-GMP in the cytoplasm—when such c-di-GMP is present (i.e., in biofilm-promoting conditions), then LapD can rapidly shift to its LapG-binding conformation.

Interestingly, while activation of LapD dramatically increases its affinity for LapG, it seems that only one LapG is bound by each activated LapD dimer (Figure 3b,c), suggesting asymmetry or anticooperativity within an activated LapD dimer (26). This stoichiometry seems to be essential for the proper functioning of LapD and LapG as a regulatory unit, as the expression of either of these proteins from a multicopy plasmid dramatically impacts biofilm formation, leading to either a hyperbiofilm or a biofilm-negative phenotype, respectively. In addition, mutations in the HAMP domain of LapD that would be expected to interfere with dimerization abolish LapD binding to c-di-GMP but yield a hyperbiofilm phenotype, perhaps by forcing both LapD dimer halves into active-like conformations while also trapping the sensing module in an autoinhibited conformation (26, 104). Alternatively, the mutations may create a monomeric LapD fraction, thus disrupting the LapD:LapG stoichiometry. Whether the actual stoichiometry in *P. fluorescens* cells is at this 2:1 ratio, or how this stoichiometry is maintained, is not yet clear. However, expression data from RNA-seq studies in several organisms suggest that functional LapD and LapG stoichiometry may be regulated via differential expression of the *lapD* and *lapG* genes. In *P. fluorescens* (89), *P. aeruginosa* (15, 22, 24, 121, 128), and *V. cholerae* (97), median expression of *lapD* is roughly twice that of *lapG*, consistent with the stoichiometry observed in structural and biochemical studies described above (26). Interestingly, in *P. putida* (17, 48, 49, 76, 99), *Shewanella oneidensis* (138), and *L. pneumophila* (146), median expression of *lapD* and *lapG* is roughly the same, perhaps indicating a lesser role for transcriptional regulation in these microbes.

FORMATION OF A LapD BASKET

To further understand the conformational changes that LapD undergoes in its various signaling modes, Cooley et al. (30) employed SAXS and cross-linking to study the conformation of full-length LapD. They saw LapD adopt three distinct configurations (Figure 3c), in all of which LapD forms a dimer, with interactions evident between the HAMP and Cache domains. In the absence of c-di-GMP, apo-LapD forms a heterogeneous dimer, where one half of the dimer adopts an autoinhibited conformation while the other seems to switch between the autoinhibited and open conformations. In the presence of c-di-GMP, one half of the dimer is stabilized in the open conformation while the other remains in a conformation that is indistinguishable from the autoinhibited state at the resolution of the analysis. Finally, in the presence of both c-di-GMP and LapG, LapD forms a tetrameric dimer-of-dimers. In this conformation, each dimer consists of one autoinhibited-like half and one active half; the dimers are joined into a dimer-of-dimers by an interaction between the EAL domains of the two active halves (Figure 3c,d). Experiments using SEC-MALS (size exclusion chromatography multi-angle light scattering) confirmed this observation, showing that LapD in the presence of c-di-GMP predominantly exists as a dimer, with a small fraction of the population forming a dimer-of-dimers. However, when LapD is incubated with both c-di-GMP and LapG, the dimer-of-dimers form, dubbed the “LapD basket,” is the dominant conformation observed.

CONTROL OF THE Lap SYSTEM THROUGH A c-di-GMP NETWORK

As described above, LapD plays an important role in the regulation of biofilm formation. However, it is becoming clear that a crucially important and fascinating layer of biofilm regulation exists upstream of LapD, in the network of c-di-GMP-metabolizing proteins that produce the signals to which LapD responds. Two distinct mechanisms by which LapD activity is controlled are now well described: One is the modulation of total cellular c-di-GMP concentrations, and the other is the production of small quantities of c-di-GMP in a local environment to specifically signal to LapD.

CONTROL OF THE Lap SYSTEM THROUGH GLOBAL c-di-GMP SIGNALING

In *P. fluorescens*, when phosphate levels are low the Pho regulon is activated. Included in this regulon is a dual-domain GGDEF/EAL-containing protein, RapA, that acts as a PDE. Monds et al. (100) showed that RapA activity reduces the cellular c-di-GMP concentration and reduces the rate of irreversible attachment during the initial stages of biofilm formation. In established biofilms, RapA activity leads to the loss of LapA from the cell surface and biofilm dispersion through inhibition of LapD sequestration of LapG (105). These observations indicate that LapD is therefore responsive to global changes in c-di-GMP concentration.

Phosphate exerts a strong influence on biofilm formation in *P. fluorescens* (100) and may be an important signal to *P. fluorescens*, as in its soil habitat, free phosphate will be very low (109). Phosphate tends to be encountered here when the cell is close to phosphate-containing soil particles, as this nutrient is liberated from inorganic minerals by microbially produced

organic acids (111). Therefore, phosphate may serve as a nutritional surface signal for *P. fluorescens* (100, 109). In addition, *P. fluorescens* has been observed to accumulate phosphate from its growth medium upon surface attachment (75). *P. fluorescens* cells may therefore sense phosphate when close to or engaging a soil particle, leading to a repression of RapA activity and allowing the initiation of biofilm formation. Upon attachment to the soil substratum, cells may then accumulate phosphate in their cytoplasm to buffer against transient decreases in available phosphate and to slow the rate of phosphate starvation when phosphate on the surface is depleted. Once phosphate is depleted, cytoplasmic phosphate levels would decrease, leading to increased *rapA* expression and dispersion of cells in the biofilm.

CONTROL OF THE Lap SYSTEM THROUGH LOCAL c-di-GMP SIGNALING AND PHYSICAL INTERACTION

In some c-di-GMP signaling systems, specific signaling outcomes are achieved by colocalization of DGCs and PDEs with their target c-di-GMP effector proteins. For example, colocalization of an oxygen-sensing DGC and PDE from *Escherichia coli*, DosC and DosP, and their target, PNPase, allows specific c-di-GMP-mediated regulation of RNA degradation (140). Additionally, in *P. aeruginosa*, the DGC NicD physically interacts with the PDE DipA and another protein, BdlA, allowing the three proteins to function in a biofilm dispersion-triggering signaling cascade (12). The DGC NicD senses a dispersion-stimulating signal and is activated to produce c-di-GMP; elevated c-di-GMP contributes to activation of BdlA, which activates DipA, which in turn degrades c-di-GMP, leading to reduced overall cellular levels of c-di-GMP and to biofilm dispersal. While production of c-di-GMP is canonically a biofilm-stimulating process, localization of c-di-GMP production near BdlA and DipA allows this dinucleotide signal to have the opposite effect. Physical interaction is therefore a mechanism by which c-di-GMP signaling can be targeted to achieve specific outcomes, even if those outcomes are opposed to the canonical activity of an enzyme.

Recent studies have also shown that LapD activity can be regulated by local signaling through physical interaction (33, 34, 52). GcbC is an inner membrane–localized DGC that contributes to biofilm formation through LapD (33, 106). Using a bacterial two-hybrid system, Dahlstrom et al. (33) identified the α5 helix of the GGDEF domain as an interface involved in the interaction with LapD. They further identified the complementary surface on LapD's EAL domain as the α2 helix. Interestingly, the α2 helix on LapD's EAL domain is buried when LapD is in the autoinhibited state, so the autoinhibited conformation may not be compatible with interaction with GcbC, raising the question of whether interaction with GcbC contributes to stabilization of the open, active LapD conformation (Figure 3d).

That a DGC is able to interact physically with LapD, and in doing so stimulate LapD inhibition of LapG, raises the question of how signaling through physical interaction is regulated to achieve biologically useful control of LapD activity. An explanation of how the interaction between a DGC and LapD is regulated was offered recently by Giacalone et al. (52), who showed that GcbC interaction with LapD is specifically enhanced by the addition of citrate, which is sensed by GcbC through its periplasmic Cache domain. They also

presented evidence showing that stimulation of GcbC-LapD interaction was coupled with an increase in GcbC's synthesis of c-di-GMP. Together, these two enhanced behaviors resulted in a GcbC-dependent increase in biofilm formation in the presence of citrate. Interestingly, while it has previously been observed that citrate is able to stimulate biofilm formation in a Δ*lapD* mutant due to calcium chelation by citrate (19, 65), this stimulation was not observed in a Δ*gcbC* mutant (52). This finding may indicate that while citrate is able to indirectly inhibit LapG activity through calcium chelation in a Δ*lapD* mutant, under the conditions used by Giacalone et al. LapG is already inhibited enough by LapD that there is no additive effect (52).

One very surprising result from this study and others is that when tested in the heterologous bacterial two-hybrid system, some DGCs are only capable of producing c-di-GMP when their cognate binding partner is coexpressed (8, 52). Surprisingly, even GcbC mutants with disrupted I-sites, which contribute large quantities of c-di-GMP when expressed in *P. fluorescens*, are not observed to produce any c-di-GMP when expressed in *E. coli* in the absence of LapD (34). It is possible that contingency of DGC activity upon interaction with an effector protein is a regulatory mechanism, requiring for signaling both a specific environmental cue and engagement of an appropriate c-di-GMP receptor. Also, the requirement of some DGCs for interaction with a partner in order to signal may act as a checkpoint. That LapD switches back and forth between an autoinhibited and an open, active conformation in the absence of c-di-GMP (30) may indicate that a high concentration of cellular c-di-GMP primes LapD for interaction with a partner such as GcbC by stabilizing the open, interaction-competent conformation.

The mechanisms by which LapD may modulate DGC activity are unclear. However, there are two hypotheses that we believe offer reasonable explanations of these data. The first is simply that LapD may hold the DGC in an active conformation. DGC catalytic activity requires dimerization of two GGDEF domains in a conformation that brings the catalytic residues into alignment (151); each LapD monomer that makes up the basket (Figure 3c,d) may bind to one of the DGC monomers and coordinate them in a conformation that promotes catalysis. The second mechanism by which LapD may modulate DGC activity is direct interaction with catalytic residues of the GGDEF domain. Previous work characterizing the interaction interface between LapD and GcbC has shown that LapD interacts with the α5 helix of the GcbC GGDEF domain, which contains an aspartic acid that is invariant at this position in active DGCs and is thought to coordinate the c-di-GMP product of DGC catalysis (150). That LapD is physically interacting with a residue involved in binding and releasing the product of DGC catalysis raises the possibility that LapD can somehow influence the release of the c-di-GMP product by an interacting DGC and therefore allosterically modulate the activity of that DGC.

Giacalone et al. (52) have shown that in addition to the interaction between GcbC and LapD in response to citrate, the Cache domain–containing DGC Pfl01_2295 can interact with LapD and signal in response to succinate. The observation that at least two DGCs can interact physically with LapD to signal in response to environmental stimuli raises interesting questions about possible regulatory mechanisms governing the large number of partners known to interact with LapD (32). One question is whether proteins that can

interact with LapD compete for access to this receptor. Evidence that LapD interaction may be required for, or enhance catalytic activity of, DGCs (33, 52) and the observation that many proteins that interact with LapD are predicted to function as PDEs (32, 106) indicate that competition to interact with LapD could serve as a key step in c-di-GMP signaling. This model is supported by Giacalone et al. (52), who have shown that when the *gcbC* gene is introduced on a multicopy plasmid in *P. fluorescens*, biofilm formation is enhanced. However, when a catalytically inactive mutant allele of GcbC in which the catalytic GGDEF motif is mutated (GcbC-GGAAF) is introduced on a multicopy plasmid, biofilm formation is reduced compared to that of the wild-type strain. These findings are consistent with a model in which a catalytically inactive GcbC is able to occupy LapD and reduce the ability of other DGCs to signal to LapD; that is, the GcbC-GGAAF mutant has a dominant negative phenotype. Alternatively, that the interaction of a catalytically inactive DGC with LapD leads to reduced biofilm formation may indicate that interaction between LapD and a DGC is insufficient for full signaling. Perhaps for LapD to enter a fully signaling-competent conformation, both interaction with a signaling partner and the presence of c-di-GMP are required.

The examples of global and local signaling described above are two mechanisms by which c-di-GMP enzymes can exert control over biofilm formation. Dahlstrom, Collins and colleagues (32) attempted to explore how the activity of the c-di-GMP network of *P. fluorescens* is organized through transcriptional regulation and physical interaction to achieve these scales of signaling. They found that most c-di-GMP enzymes are capable of physical interaction and that LapD is capable of interacting with at least 16 of the 50 DGC, EAL, dual-domain, and PilZ domain proteins tested. However, while Dahlstrom, Collins, et al. found that *rapA* expression is increased 16-fold when cells are incubated in low-phosphate medium, they observed only small changes (typically ~2-fold) in expression of other c-di-GMP-related genes. The apparently limited role of transcriptional regulation in organizing the c-di-GMP network may indicate a role for posttranscriptional regulation of enzyme activity and leaves open the question of how physical interactions between network members are organized.

TRANSCRIPTIONAL CONTROL OF THE Lap SYSTEM

Mechanisms of regulation of the Lap system in addition to specific regulation of LapD activity have been described in *P. fluorescens* and other organisms. Principal among these is transcriptional regulation. In *P. fluorescens*, a low-phosphate environment was shown to impact LapA localization not only through inducing expression of RapA leading to reduced c-di-GMP. In addition, Δ*pst* mutant cells, which behave as if phosphate-starved, exhibit reduced LapA secretion due to ~80% lower expression of the *lapE* T1SS outer membrane pore (presumably through competitive binding of the *lapEBC* promoter region by PhoB) (100).

c-di-GMP modulation has broad impacts on gene expression in various *Pseudomonas* species (144). One protein responsible for c-di-GMP-responsive transcriptional changes is FleQ (9, 11, 63, 98). FleQ was originally described as a factor influencing expression of EPS biosynthesis genes (11, 63) and the adhesin CdrA (18), which is a noncanonical LapG target

in *P. aeruginosa* (31). Identification of a consensus binding motif for the FleQ transcription factor allowed in silico analysis of bacterial promoters that predicted that many *lap* genes in the genus *Pseudomonas* and related organisms may be transcriptionally regulated by FleQ (10).

The organism in which transcriptional regulation of *lap* genes has been best described, by Espinosa-Urgel and colleagues, is *P. putida* (16, 92, 98, 122, 148). In *P. putida*, biofilm formation is determined by the activity of multiple factors, i.e., the type 1 secreted adhesins LapA and LapF (the latter not thought to be a LapG substrate) and the polysaccharides Pea, Peb, cellulose, and alginate (91, 94, 107). Transcription of these biofilm components is regulated by numerous factors, including FleQ (98), the stationary phase sigma factor RpoS (88, 94), and the DNA-binding protein Fis (4, 80). A curious compensatory transcriptional response is also observed whenever either of the protein adhesin genes is mutated: Mutants lacking either adhesin or both have increased expression of the EPS Pea (91). The two Lap adhesins, LapA and LapF, are differentially regulated by two transcription factors: *lapA* gene expression depends on FleQ (148), while *lapF* gene expression depends on RpoS (94). This differential expression means that LapA, which is required for cell-surface interactions, is produced when cells accumulate c-di-GMP in conditions favorable for biofilm formation, while LapF (required for cell-to-cell binding) is expressed in response to physiological changes that cells experience as the biofilm develops and perhaps as cells in this biofilm become nutrient limited. This regulatory pattern leads to a temporal separation of expression that supports the differing roles of these two adhesins in biofilm formation.

REGULATION OF THE Lap SYSTEM AT THE LEVEL OF SECRETION

While a comprehensive mechanism has not yet been described, studies of the Lap system suggest that LapA localization is regulated not only via release from the surface by LapG but also by modulation of secretion of LapA to the cell surface. The most compelling evidence for a role for secretion as a regulatory mechanism comes from a study by Pérez-Mendoza et al. (115) in *Pectobacterium atrosepticum*. These investigators found that secretion of the *P. atrosepticum* LapA ortholog, MRP, is regulated by the product of the adjacent gene, *ECA3265*, a putative ortholog of the *P. fluorescens* Pf0-1 *lapA*-adjacent gene, *Pfl01_0132* (57% amino acid identity; Figure 2a). Pérez-Mendoza et al. observed that when they disrupted the *ECA3265* gene, the amount of MRP detected in the supernatant increased dramatically while the level in the cytoplasm decreased. They also found that two c-di-GMP-metabolizing proteins encoded near the *mrp* gene on the chromosome were able to alter the secretion of MRP through their modulation of c-di-GMP levels in the cell. While no obvious orthologs of the two c-di-GMP enzymes described by Pérez-Mendoza et al. exist in *P. fluorescens*, homologs of the *ECA3265* gene commonly neighbor the *lapA* homologs in many organisms; therefore, it is possible that this gene product is important in regulating secretion.

While *P. fluorescens* does not encode the two c-di-GMP-metabolizing enzymes described by Pérez-Mendoza et al. (115), differences in LapA secretion have been observed in some mutants of *P. fluorescens*. For example, while wild-type cells grown in low-phosphate medium will secrete and process all of their LapA, depleting the cytoplasmic pool, Δ*lapD*

mutants will retain LapA in the cytoplasm in spite of LapG being constitutively active. Conversely, in high-phosphate conditions, wild-type cells retain LapA in the cytoplasm while the Δ*lapD* mutants secrete a higher proportion of LapA (100, 104). In addition, as noted above, low phosphate level impacts expression of the *lapBCE* T1SS genes in *P. fluorescens*, leading to decreased secretion. However, differences in c-di-GMP levels, rather than PhoB binding to the *lapBCE* promoter, seem to be responsible; this difference in secretion was not observed in a *rapA* mutant, which had wild-type levels of LapA on the cell surface (104). Together, these data suggest that secretion of LapA in *P. fluorescens* is impacted by c-di-GMP and may depend on the activity of LapD; however, the mechanism by which this occurs, and of possible involvement of Pfl01_0132 protein, is not clear.

CONCLUSIONS

The Lap system provides a versatile mechanism by which bacterial cells can attach to surfaces with different properties to form biofilms. The success of this strategy is demonstrated by the breadth of LapA's distribution throughout the *Proteobacteria* (132). While a small number of the core repeats regions have been characterized in some LapA homologs, the vast majority are still of unknown function. In addition, the mechanisms by which the Lap secretion complex is able to dissociate and the LapA N-terminal retention domain is able to fold in the periplasm during secretion are important areas of study for future research.

LapA also provides a rapid mechanism of dispersal if conditions no longer favor a biofilm lifestyle. For biofilms containing complex mixtures of secreted proteins, polysaccharides, and DNA, dispersal of a biofilm entails disruption of this matrix, which in turn requires the coordinated action of many matrix degradation processes. LapA can be quickly removed from the cell surface by the activation of a single periplasmic protein, allowing for rapid dispersal of bacterial cells during early periods of biofilm formation in which LapA is likely the major matrix component.

After almost two decades of work, we now have a detailed understanding of how LapD is able to transduce signals across the inner membrane to influence LapA localization to the cell surface. In addition, recent developments have enhanced our understanding of how c-di-GMP signals are communicated to LapD through global signaling (100, 104, 105) and physical interaction (33, 34, 52). How these processes function to regulate LapD activity in the broader context of a c-di-GMP signaling network containing the ~50 c-di-GMP-related proteins encoded by *P. fluorescens* is not yet clear. However, the relative simplicity of the Lap system makes it a promising system for increasing our understanding of these processes.

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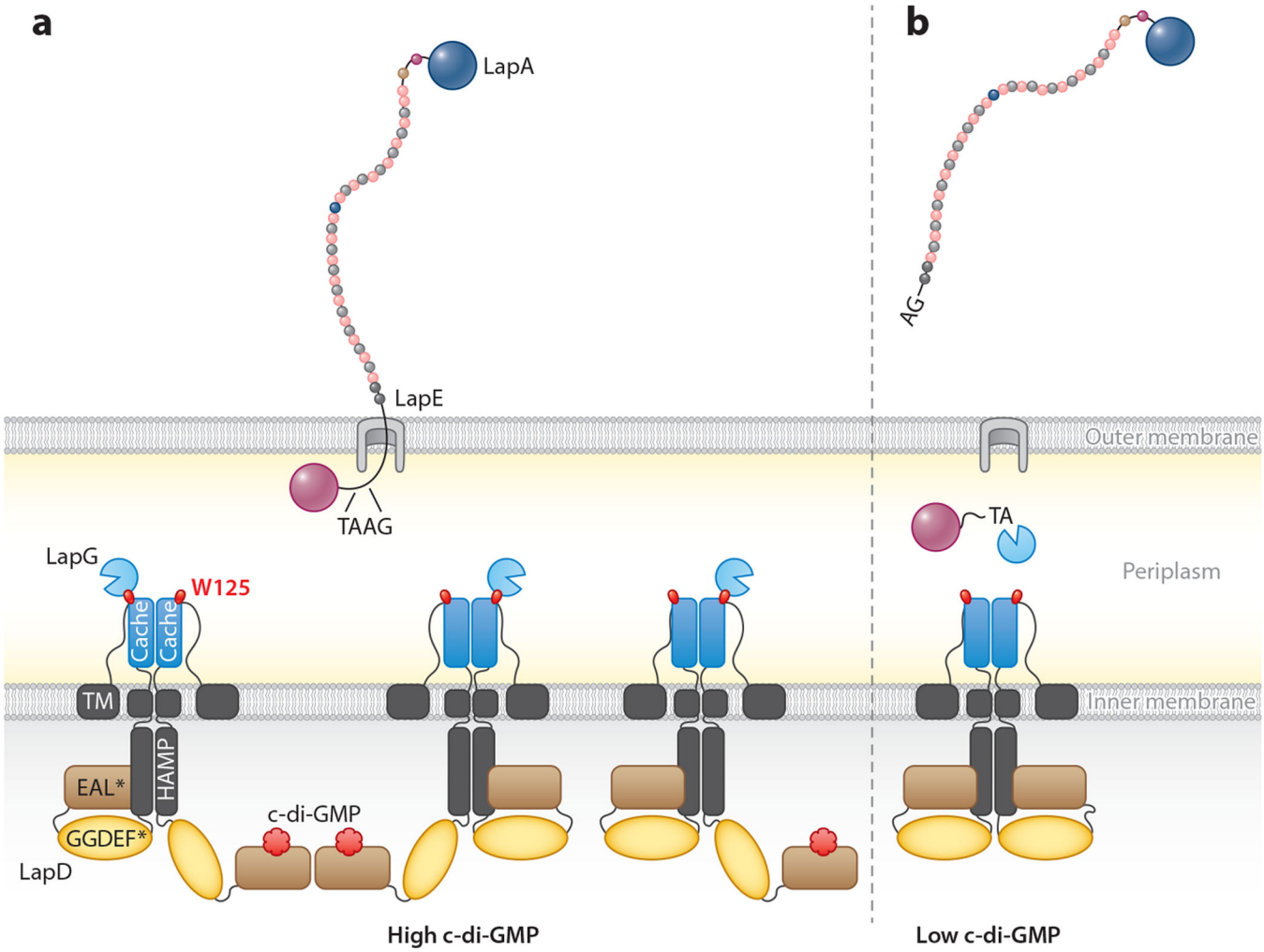

Figure 1.

The Lap system. (*a*) When in a biofilm-competent state, LapA is tethered in the outer membrane pore, LapE, and exposed on the cell surface. During secretion, the N-terminal portion of LapA folds into a globular plug domain in the periplasm (*large pink sphere*) that is too large to pass through the LapE pore; thus, LapA is anchored to the cell surface. (*b*) When LapA's plug domain is cleaved by the periplasmic protease LapG, LapA is released from the cell (i.e., is fully secreted), enabling the cell to return to a planktonic lifestyle. LapD is a c-di-GMP receptor that binds this dinucleotide via a degenerate phosphodiesterase domain in the cytoplasm. LapD, in turn, can bind LapG via LapD's periplasmic domain, thereby restricting the access of LapG to the N-terminal processing domain of LapA. The small red oval on LapD indicates the W125 residue critical for LapD-LapG interaction, and c-di-GMP is represented by a red flower. The asterisk indicates an enzymically inactive domain.

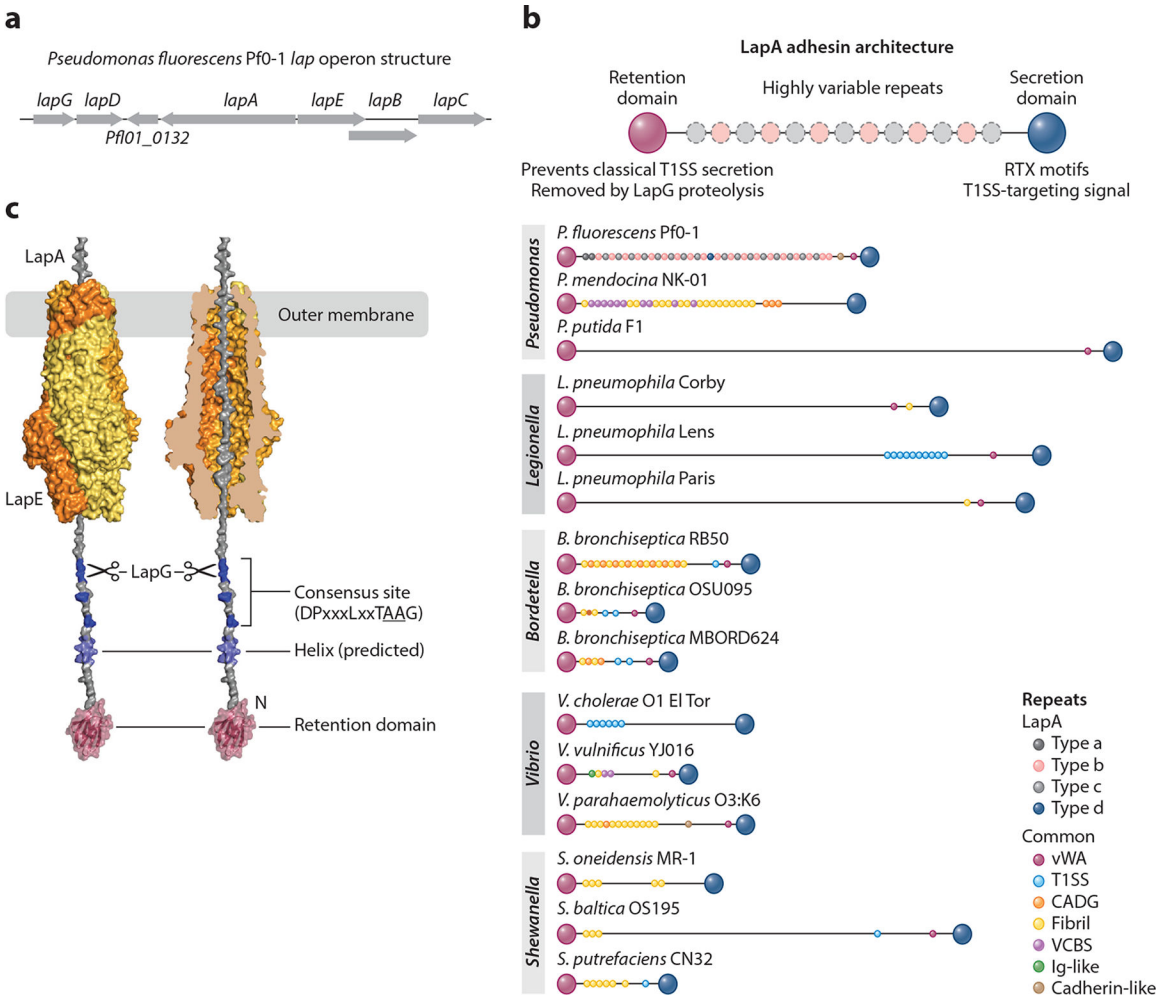

Figure 2.

The LapA biofilm adhesin. (*a*) In *Pseudomonas fluorescens*, LapA is encoded by a gene adjacent to the genes coding for the LapEBC T1SS and the regulatory proteins LapD and LapG. The *Pfl01_0132* gene, which is a putative homolog of a gene whose product is implicated in the regulation of secretion of a LapA homolog in *Pectobacterium atrosepticum*, is also found in this region. In some organisms, components of the Lap system are encoded at different locations in the genome from their LapA-like protein (not shown). (*b*) LapA homologs have relatively conserved N and C termini but variable core repeats regions. The variation in size between these proteins is primarily due to differences in the number and type of these repeats. Large differences are seen in the content of the core repeats regions between species and, in some cases, between strains of the same species. (*c*) LapA is secreted C terminus first through the LapBCE T1SS. During secretion of the C-terminal portion through LapE, the N-terminal portion of LapA folds in the periplasm or secretion channel. This folded plug (the retention domain) prevents complete secretion of LapA and anchoring of the adhesin to the cell surface. Once LapA has occupied the LapE pore, the rest of the secretion machinery (i.e., LapB and LapC) is presumably able to dissociate from this secretion complex, thus exposing the N-terminal region of LapA to

the periplasm for regulated processing by LapG. Abbreviations: Ig, immunoglobulin; RTX, repeats-in-toxin; T1SS, type I secretion system; vWA, von Willebrand A domain.

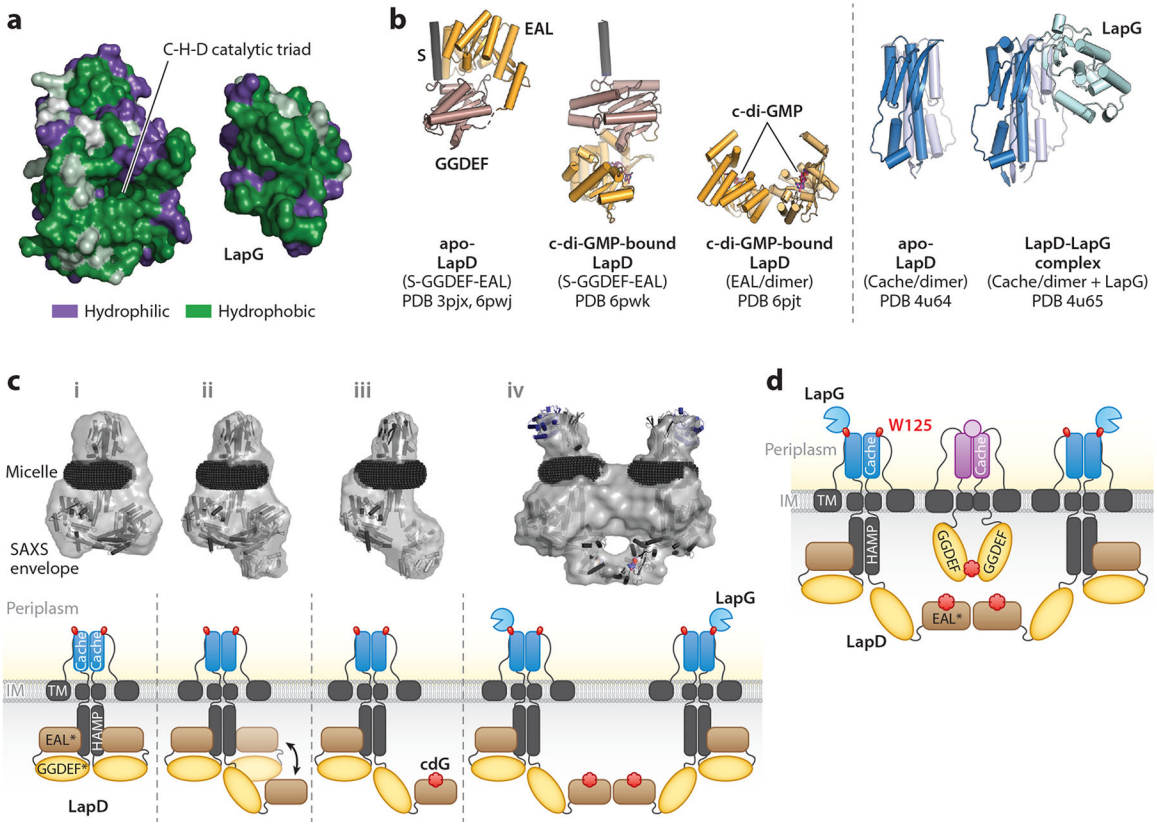

Figure 3.

Structural insights into the Lap system. (*a*) A large patch of hydrophobic residues is exposed on the surface of LapG near the C-H-D catalytic triad. The hydrophobic patch on LapG may interact with a hydrophobic predicted α helix of LapA near the TAAG LapG-processing site. (*b*) Structures of the cytoplasmic domain of LapD (*left*) and the periplasmic domain of this receptor (*right*). When LapD is not bound to c-di-GMP, the EAL domain is positioned in contact with the S-helix in an autoinhibited conformation (apo-LapD, *left*) and the periplasmic Cache domain is not competent to bind LapG (apo-LapD, *right*). The binding of c-di-GMP to the EAL domain stabilizes an extended, open conformation in which the LapD EAL domain can dimerize (c-di-GMP-bound LapD, *left*). The conformational change is transduced to the periplasmic Cache domain, making LapD competent to bind LapG (LapD-LapG complex, *right*). (*c*) In the absence of c-di-GMP, LapD exists as a dimer that remains in an autoinhibited state in which either half of the dimer switches at some unknown frequency to the open, active conformation, allowing the detection of cytoplasmic c-di-GMP concentration (subpanel *i* depicts a fully closed LapD dimer, and subpanel *ii* shows half of the dimer in transition to the open conformation). The favorability of adopting the open conformation is increased by either the binding of c-di-GMP to the EAL domain (*iii*) or the transient binding of LapG to the periplasmic Cache domain (not shown). When both c-di-GMP and LapG are bound to LapD, the open conformation is further stabilized, allowing the formation of a tetrameric form of LapD as a dimer-of-dimers or “basket.” In this basket form LapG will be stably sequestered by LapD, indicating that this is the active form ultimately responsible for the retention of LapA on the cell surface (*iv*). (*d*) The basket

conformation of c-di-GMP-bound LapD provides a space into which a protein such as the diguanylate cyclase GcbC could fit in order to interact with the LapD EAL domain and produce a c-di-GMP signal. A protein interacting with LapD in the basket would occlude the space and prevent other proteins from interacting with LapD. This occlusion may represent a mechanism of coordinating LapD-focused c-di-GMP signaling due to competition for access to LapD baskets, which would limit the number of proteins that can effectively signal to LapD, contributing to signaling specificity. The small red oval on LapD indicates the W125 residue critical for LapD-LapG interaction, and c-di-GMP is represented by a red flower. The asterisk indicates an enzymically inactive domain. Abbreviations: IM, inner membrane; PDB, Protein Data Bank; SAXS, small-angle X-ray scattering; TM, transmembrane domain.