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Supramolecular structure in the membrane of *Staphylococcus aureus*

Jorge García-Lara a,1, Felix Weihs a, Xing Ma a,2, Lucas Walker a, Roy R. Chaudhuri a, Jagath Kasturiarachchi a,3, Howard Crossley a, Ramin Golestanian b,4, and Simon J. Foster a,4

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All life demands the temporal and spatial control of essential biological functions. In bacteria, the recent discovery of coordinating elements provides a framework to begin to explain cell growth and division. Here we present the discovery of a supramolecular structure in the membrane of the coccoid bacterium *Staphylococcus aureus*, which leads to the formation of a large-scale pattern across the entire cell body; this has been unveiled by studying the distribution of essential proteins involved in lipid metabolism (PisY and CdsA). The organization is found to require MreD, which determines morphology in rod-shaped cells. The distribution of protein complexes can be explained as a spontaneous pattern formation arising from the competition between the energy cost of bending that they impose on the membrane, their entropy of mixing, and the geometric constraints in the system. Our results provide evidence for the existence of a self-organized and nonpercolating molecular scaffold involving MreD as an organizer for optimal cell function and growth based on the intrinsic self-assembling properties of biological molecules.

Membrane curvature can act as a cue for localization of components (16). Raft aggregation of transmembrane proteins and the presence of compartment boundaries are insufficient explanations for such patterning. The physiological principles and molecular processes governing pattern formation are largely unknown.

*Staphylococcus aureus* is a coccoid bacterium that can grow and divide in three consecutive orthogonal planes with fidelity (19); however, it lacks key morphogenetic components, such as MinCDE and MreB (20). Hence, what are the spatial organizers in *S. aureus* (21)?

Here we present the discovery of a supramolecular structure in the membrane of *S. aureus* that has been unveiled by studying the distribution of essential proteins involved in lipid metabolism (PisY and CdsA) and the cell division component MreD. Such novel distribution of proteins complexes can be explained mainly as a by-product of the energy cost of bending that potential complexes exert on the membrane, and the geometric constraints imposed by the latter. A model based on such basic

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**Significance**

The fundamental processes of life are organized and based on common basic principles. Molecular organizers, often interacting with the membrane, capitalize on cellular polarity to precisely orientate essential processes. The study of organisms lacking apparent polarity or known cellular organizers (e.g., the bacterium *Staphylococcus aureus*) may enable the elucidation of the primordial organizational drive in biology. How does a cell choose from infinite locations in its membrane? We have discovered a structure in the *S. aureus* membrane that organizes processes indispensable for life and can arise spontaneously from the geometric constraints of protein complexes on membranes. Building on this finding, the most basic cellular positioning system to optimize biological processes, known molecular coordinators could introduce further levels of complexity.

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PlsY is an acyltransferase required for the in-
SI Ap-
C
promoter demonstrated septal lo-
muts revealed phenotype. Loss of MreD can be com-
SI Appendix
G
and S8
SI Appendix
SI Appendix
SI Appendix
SI Appendix
and
and
and
and
EzrA~GFP
repression led to a decrease of PlsY (Fig. 3
D
PlsY depleted
with Fig. 3
D
PlsY depleted
and
Movie S3
muta-
and
and
and
and
gene (
JGL166
(SI Appendix
mreC
with Fig.
DIC
and
S. aureus
SI Appendix
S. aureus
both fixed and unfixed samples, demonstrating that the observed
distribution, which also revealed a pattern (Fig. 2
D
Figs. S3
membrane throughout the cell cycle (Fig. 2
–
A–D
and
SI Appendix
Fig. S1F).
To ex-
amine the link between PlsY and cell cycle progression, we studied the distribution of divisome proteins EzrA and PBP2. EzrA forms a septal ring at the midplane of the cell and was found at unexpected division planes when cells are depleted for PlsY (Fig. 1
E–G
and
Movie S1) (25, 26). PBP2, a septal protein responsible for cell wall biosynthesis at the site of division (27), was also delocalized (SI Appendix
Fig. S2), suggesting PlsY involvement in septum placement and/or division progression.

A chromosomally encoded and functional PlsY–GFP fusion under the control of the plsY promoter demonstrated septal local-
alization, but also distributed in a pattern of foci around the membrane throughout the cell cycle (Fig. 2
A–C
, SI Appendix
Figs. S3–S5, and Movie S2). Immunoblot and FACS analysis demonstrated that PlsY is membrane associated (SI Appendix
Fig. S6), and plsY repression led to a decrease of PlsY (SI Appendix
Fig. S1 C–E).

The fluorescent translational fusion reporter data were sup-
ported by immunofluorescence microscopy observations of PlsY distribution, which also revealed a pattern (Fig. 2
D
and
SI Appendix
Fig. S7A), and optical sectioning of fluorescent or immunolabeled PlsY that showed a 3D distribution of foci (SI Appendix
Fig. S3 and
Movie S3). The pattern was present in both fixed and unfixed samples, demonstrating that the observed pattern is not an artifact of the sample preparation procedure (SI Appendix
Fig. S84). CdsA, a CDP-diacylglycerol synthase

downstream of PlsY in the glycerophospholipid biosynthesis
pathway, localized in a similar pattern and colocalized with PlsY (Fig. 2
E
, SI Appendix
Figs. S7C and S8B, and Movies S4–S6), whereas other membrane proteins did not (e.g., YmdA and SecY; SI Appendix
Fig. S7B).

Consistent with these observations, PlsY and CdsA also showed a weak interaction in a bac-
terial two-hybrid analysis (BACTH; SI Appendix
Fig. S9).
To confirm interaction between both components at the molecular level in their natural environment, we implemented a FRET-based system in
S. aureus
using fluorescent protein fusions. A donor bleaching strategy was applied and revealed a specific interaction between PlsY and CdsA (SI Appendix
Fig. S10).

To our knowledge, these are the first pieces of evidence of the suspected colocalization and interaction between the enzymes in this pathway, and support the notion of metabolic channelling. Lipid clusters are also organized with PlsY in the membrane of unfixed cells (SI Appendix
Fig. S11).
Such a distribution could correspond to the differential accumulation of specific fatty acid species or lipid conformations in the membrane (28).

MreD Is Needed for the Formation of a Supramolecular Structure in the Membrane. PlsY and/or CdsA seemed unlikely candidates as
organizers of the patterned distribution, as did FtsZ, despite its link to the localization of phospholipid synthases (29). The MreBCD complex in rod-shaped bacteria localizes along the membrane, acting as a spatial organizer of various processes (7, 9, 30–33). In
Escherichia coli,
MreB (an actin-like molecule) is required to regulate phospholipid and membrane biosynthesis (31). However, MreB is missing in
S. aureus
and other coccal cells, whereas MrcC and MrcD are present (9).

S. aureus
derived from MrcC grew identically to the parent, whereas lack of MrcD or MrcD led to a growth defect with larger cells of abnormal morphology (Fig. 3
A–C
and
SI Appendix
Figs. S12 and S13), reminiscent of the PlsY-depleted phenotype. The lack of an apparent phenotype for the single
mrcC
mutation was interesting given its importance in other organisms (34). Genome sequencing of two individual
mrcC
mutants revealed one single nucleotide polymorphism in each strain leading to an amino acid substitution (SI Appendix
Whole Genome Sequencing).

However, those substitutions seem unlikely candidates to justify the lack of an
mrcC
phenotype. Loss of MreD can be complemented by ectopic expression of the
mreD
gene (SI Appendix
Fig. S13 F and G). MreD is required for the observed pattern of PlsY (cf. Fig. 2
B
and
D
with Fig. 3
D
and
SI Appendix
Fig. S14A) and the characteristic placement of EzrA (cf. Fig. 1
G
with Fig. 3
E
, SI Appendix
Fig. S14B, and
Movie S7); this is consistent with the immunodetection of a MrcD–GFP fusion that revealed a
localization pattern equivalent to PlsY and CdsA (Figs. 2B and D and 3F and SI Appendix, Figs. S3 and S15). BACTH analysis also showed protein–protein interactions between MreD and CdsA (SI Appendix, Fig. S9). Similarly, FRET provided supporting evidence of the interaction between MreD and PlsY (SI Appendix, Fig. S10).

**Spontaneous Formation of a Patterned Distribution of Protein Complexes in the Membrane.** Without cytoskeletal components, how might MreD, PlsY, and CdsA adopt a pattern in the membrane? Their distribution could arise from the interplay between the potential complex they contribute to and the geometric constraints imposed on the membrane, doing what cellular organizers do, but spontaneously, and leading the way. If the integral membrane protein complex inflicts a sufficiently large local curvature on the membrane, a random distribution would be frustrated due to the high bending energy cost that results from accommodating the complexes across the membrane. This mechanism of protein organization can account for the development of slowly growing patterns that results from the sensing of 2D membrane geometry by the wall and the turgor pressure. Here, $\kappa$ is the bending rigidity, $H/2$ is the mean curvature, $\chi$ is the compressibility and $\zeta$ is the correlation length of the density fluctuations, and $k$ is an effective spring constant (per unit area) representing the confinement potential. Using this free energy, we can define the governing dynamical equations for $u(\theta, \varphi)$ and $\psi(\theta, \varphi)$, which involve the corresponding mobility (transport) coefficients $L_u$ and $L_\psi$ (SI Appendix, Fig. S16 and Fig. 4/4).

Fig. 4B shows the relevant growth rate $\lambda_\phi$ in units of $kL_u/R^2$ as a function of the mode number $\ell$ at selected values of $K$, $M$, and $P$ for $W=1$. It shows that $\lambda_\phi$ acquires a positive value (Fig. 4C) for the $\ell=8$ mode, which means that this mode becomes unstable. The instability in the intermediate $\ell=8$ mode develops as the parameter $W$ is changed from 0.8 to 1 (Fig. 4D), leading to the development of slowly growing patterns that are linear combinations of the $Y_{8m}(\theta, \varphi)$ for different values of $m$, which could lead to eight foci in a cross-section of the sphere (Fig. 4E). This example demonstrates that patterns such as those observed (Figs. 2B–E, 3F and G, and 4C and D, SI Appendix, Figs. S7A and C, S8A and B, and S11B, and Movies S3–S6) can be generated as a result of such intermediate wavenumber instability.

Assuming that the protein complexes are in the dilute regime—namely, $\rho_{p0}q_0 \ll 1$, we can estimate the compressibility as $\chi\approx1/(k_BT\rho_0)$ (38), and write the coupling parameter as...
\[ \lambda_\pm = \frac{1}{2} \left[ -K - \ell (\ell + 1) \left( M - 2 + \ell (\ell + 1)(MP + 1) \right) \right] \]
\[ \pm \frac{1}{2} \sqrt{ \left[ K - \ell (\ell + 1) \left( M - 2 + \ell (\ell + 1)(MP - 1) \right) \right] + 4 MW \ell (\ell + 1)(\ell + 2) (\ell^2 + \ell + 2) } \]

**Fig. 4.** (A) Calculated formula of the growth of the system as a function of its modes (I) and the adimensional variables \( K, P, M, \) and \( W \) (SI Appendix, Fig. S16). (B) The growth rate \( \lambda \) as a function of the mode number \( \ell \), corresponding to \( K = 84, M = 1, P = 0.00015 \), and \( W = 1 \); a magnified section of the latter (C) shows that the \( l = 8 \) mode becomes unstable. (D) Instability is exclusively sensitive to \( W \) but not to the other variables (SI Appendix, Fig. S17). (E) Density plots of the real parts of various spherical harmonics \( Y_{\text{esp}}(\theta, \phi) \) for different values of \( M \). (F) See extended explanation in SI Appendix, Fig. S17. (I) The bacterial membrane (e.g., \( S. \) aureus membrane) is a lipid-based surface, under cytoplasm-induced turgor pressure and tethered to the cell wall, which contains protein complexes whose distribution is a 3D phenomenon that can be defined by a mathematical function (SI Appendix, Fig. S16). (II) The latter depends on multiple independent variables that can be grouped into dimensionless variables \( P, K, M, \) and \( W \) for convenience. (III) This enables one to solve the differential equations corresponding to the various components \( \mathcal{F}_p, \mathcal{F}_o, \mathcal{F}_i, \mathcal{F}_s \) of the overall free energy of the system \( \mathcal{F} \). The \( P, K, M, \) and \( W \) variables do not directly correspond to biological variables, but they relate to them in terms of controlling the relative competition between the energy contributions; \( K \) relates to \( \mathcal{F}_p, \mathcal{F}_o \), \( P, M \) relate to \( \mathcal{F}_p, \mathcal{F}_i \), and \( W \) relates to \( \mathcal{F}_i, \mathcal{F}_s \). The solutions to the equation are two functions \( \lambda_+ \) and \( \lambda_- \), representing the growth \( \lambda > 0 \); pattern formation of protein complexes) or decay \( \lambda < 0 \); random distribution of protein complexes in the membrane. A combination of selected values of \( K, P, M, \) and \( W \) (Fig. 4 B–D) as a function of the characteristic modes of the system (I) results in positive \( \lambda_+ \) (SI Appendix, Figs. S16 and S17). Linear analysis (Fig. 4 B–D) reveals \( W \) as the key variable determining the distribution of protein complexes. Hence, the presence of a protein complex in the membrane induces a membrane deformation that results in localized membrane curvature \( \mathcal{H}_p \) and will entail a bending cost. If the curvature is large enough, the \( \mathcal{H}_p \) will result in a system that will enable the growth of patterns. If \( \mathcal{H}_p < \mathcal{H}_p \), the resulting system will lead to the decay of patterns. (IV) The separation of variables into spherical coordinates leads to spherical harmonics of the form \( Y_{\text{esp}}(\theta, \phi) \) (Fig. 4B) that can be represented as a density plot.

\[ W \approx \left( k_B T / \rho a_0 \right)^2 H_p^2 \]

A critical threshold \( W \) is defined as the value for which the pattern forms when the spontaneous curvature of the protein complex is larger than a critical value—namely,

\[ H_p > H_p \approx \left( k_B T / \rho a_0 \right)^2 \]

Considering the characteristic values of \( k_B T / \rho a_0 \sim 100 \) and \( \rho a_0 \sim 0.01 \), we find a critical radius of curvature of the order of the lateral size of the protein complex. SI Appendix, Fig. S17 illustrates the robustness of the model with respect to the choice of the parameters. We choose reasonable values for the parameters, but show that changing them does not alter the main features of our linear stability analysis, which only depend sensitively on \( W \), which is essentially controlled by the curvature imposed on the membrane by the protein complex. A simple graphic explanation of the model is presented in Fig. 4F and SI Appendix, Fig. S17.

**Conclusion**

The model of cell organization based on molecular scale self-organization, emerging spontaneously and without guidance, offers a new fundamental organizing framework for proteins in cocal organisms, such as \( S. \) aureus; this establishes an early cue that dictates the position of a target protein in the overall ensemble (22). The competition between the various forces at play leading to the ordered distribution of protein complexes in the membrane constitutes in essence an intrinsic amplifier that can act as a sensitive switch enabling the fine-tuning of important cellular processes. Less-favorable \( \lambda \) values leading to different protein patterns or pattern decay could still be preferentially selected as an expansion of the model; this could occur through the anisotropy introduced by forces generated by molecular structures (e.g., cell curvature) (39) or by the recruitment of specific proteins to subcellular sites, such as the assembly of penicillin binding proteins by MreB or FtsZ (40). Such molecular networks may form a basic system to coordinate life.
In eukaryotes, membrane partitioning is a well-described process with features at different length scales (41). In prokaryotes, it would also be envisaged that multiple mechanisms are at play, resulting in the coordination of processes from the molecular to the cellular level. The ability to colocalize members of the same macromolecular biosynthesis pathway provides a dynamic mechanism to permit efficient metabolism. Phospholipid biosynthesis involves complex intermediates and potential substrate channelling between enzymes, which would require their colocalization. The development of FRET in *S. aureus* has provided the experimental framework to test juxtaposition of proteins in their native membrane setting, which has shown the interaction between *PlsY* and *CdsA*. It will be of great interest to determine the localization pattern of other phospholipid biosynthesis enzymes in the pathway and to extend this to further proteins involved in other metabolic processes and beyond. It may be that such protein assemblies generate localized physiobiological environments propagating effects to other membrane components in the same or different processes. The observation of apparent differential partitioning of *PlsY* and a lipid dye in the membrane supports such a hypothesis (SI Appendix, Fig. S11).

It has previously been shown that MreB, in rod-shaped cells, acts as a structural scaffold for the cell membrane and other cellular components, and that it provides a mechanism for multiple biosynthetic components (11). However, the roles of MreC and MreD have remained more obscure. Here we find an important role for MreD in growth in an organism lacking MreB. Deletions of *mreD* results in pleiotropic effects, and we suggest that in rods, MreB may spatially constrain MreD, but it is MreD that is itself a key player in the actual coalescence of molecules. In *S. aureus* we also see evidence of cellular morphology effects on MreD-associated processes during the cell cycle. Thus, other spatiotemporal cues will be brought into play to allow coordination during growth and division. We have hypothesized that cell wall peptidoglycan architectural features can provide a template for division site recognition (42). It is likely a combination of intrinsic properties of proteins, such as MreD, that may provide localized perturbation to the membrane, coupled with larger scale morphological features derived from other cellular components and morphological dynamics generated from apparent cytoskeletal elements (such as FtsZ) that determine the overall mapping of membrane components. Such a dynamic accumulation of cues optimizes cellular reactions within a given environment.

In a drive to understand the basis of life, a key goal is the ability to assemble a protocol capable of self-assembly (43). Unraveling the mechanisms underpinning such assembly is crucial. Our observations provide a novel and basic organizational drive of proteins in membranes, and catalyze further research to determine their behavior in vitro and in vivo to understand patterning in a lipid environment and in the interplay within and between cellular processes. The mathematical model presented here gives a theoretical framework to be tested and to begin to predict the behavior of components underpinning life.

**Materials and Methods**

This section briefly recapitulates the data analysis methods. A detailed description of methods can be found in SI Appendix. IPTG-inducible expression constructs of *PlsY* in single copy in the staphylococcal chromosome, as well as *mreC* and *mreD* deletion strains, were made through allelic replacement by double crossover recombination. The process involved the use of episomal vectors with temperature-sensitive replications, initially constructed in *E. coli* (TOP10), followed by passing of the resulting constructs through a restriction-deficient strain (*S. aureus* RN4220) and subsequent transduction-mediated integration into the final test strain (*S. aureus* SH1000). The *mreD* complementation plasmid was based on *S. aureus* replication-stable episomes. A similar procedure was followed to generate GFP-transcriptional fusions but mediated through single crossover recombination that resulted in an intact copy of the gene of interest under the control of the regulatable promoter Papc, and another copy of the gene fused to GFP under the control of the native promoter of the gene of interest. The rest of the techniques, including protein and antibody production, immunolabeling, epifluorescence and electron microscopy preparation, flow cytometry analysis, bacterial two-hybrid analysis, FRET, and cell fractionation, were performed according to standard techniques optimized to our project. Imaging was undertaken with a DeltaVision RT Deconvolution microscope (Applied Precision) linked to an Olympus IX70 microscope system (Olympus U-RFT-T and IX-HLS100 lamps, and Olympus UPlanApo 100×/1.35 oil irs lens), with SoftWorx 3.5.1 software. Cell enumeration and perimeter, major axis, and minor axis measurements were performed using ImageJ 1.46o.

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