Published in final edited form as:

Curr Opin Microbiol. 2017 April ; 36: 76–84. doi:10.1016/j.mib.2017.02.001.

Exploring functional membrane microdomains in bacteria: an overview

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Abstract

Recent studies show that internal organization of bacterial cells is more complex than previously appreciated. A clear example of this is the assembly of the nanoscale membrane platforms termed functional membrane microdomains. The lipid composition of these regions differs from that of the surrounding membrane; these domains confine a set of proteins involved in specific cellular processes such as protease secretion and signal transduction. It is currently thought that functional membrane microdomains act as oligomerization platforms and promote efficient oligomerization of interacting protein partners in bacterial membranes. In this review, we highlight the most noteworthy achievements, challenges and controversies of this emerging research field over the past five years.

Introduction

The integrity of a cell depends on the organization of its limiting membrane, although the mechanisms involved in membrane organization are far from being understood. The pioneering fluid mosaic model suggested that membrane constituents diffuse freely and are thus homogeneously distributed[1]. Recent advances in the field nonetheless demonstrated that biological membranes have a variety of lipid species that tend to segregate laterally into discrete regions, as well as proteins that concentrate preferentially in specific membrane domains[2].

Although the principle of lateral organization of biological membranes might be anticipated to apply in equal measure to eukaryotic and prokaryotic membranes, the existence of submicrometric lipid domains is described mostly in eukaryotes, and was long thought unlikely to occur in prokaryotes[3]. For instance, a landmark in eukaryotic membrane organization is the presence of lipid rafts. Eukaryotic cells organize a number of proteins related to signal transduction and membrane trafficking into discrete cholesterol- and sphingolipid-enriched microdomains, or rafts, whose integrity is central to correct function

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of these proteins[4]. Protein association with rafts is related to protein affinity for specific lipids[2] and the scaffold activity of the flotillin protein[5]. As flotillin becomes concentrated in lipid rafts, it recruits other proteins and facilitates interaction among them[6]. Lack of flotillin thus compromises many lipid raft-associated cell processes [7–10] and is associated with severe conditions such as Parkinson's or Alzheimer's diseases[11].

Lipid rafts were originally associated exclusively with eukaryotic cells, because their assembly requires aggregation of membrane cholesterol, absent in many prokaryotic membranes. In addition, compartmentalization of cell processes (e.g., organelle formation) to optimize metabolic reactions is a phenomenon found largely in eukaryotic cells, but generally absent in microbes (with exceptions such as bacterial carboxysomes or magnetosomes[12,13]). It is thus assumed that bacteria do not need subcellular compartmentalization to organize their cellular processes correctly[14,15]. Bacteria nonetheless compartmentalize cell processes in membrane regions termed functional membrane microdomains (FMM)[16], which concentrate proteins associated with signal transduction (sensor kinases)[16–18], membrane trafficking (protein secretion systems)[19–21] and metabolism regulation (AAA+ protease complexes)[19,22,23] in close proximity, thus increasing the likelihood of interaction and the efficiency of related cell processes.

FMM were originally discovered in studies of regulation of biofilm formation by the *Bacillus subtilis* membrane-associated sensor kinase KinC. KinC lost function in the *yisP* mutant, which is defective for production of farnesol and probably other membrane-associated polyisoprenoid lipids; this mutant also loses its characteristic KinC focal localization[16]. These membrane regions can be purified because they resist detergent solubilization, resulting in larger fragments that can be separated in a sucrose gradient. The detergent-resistant membrane fraction (DRM) contains KinC as well as signaling proteins and the flotillin-homolog proteins FloT and FloA. Fluorescent microscopy showed that FloT, FloA and KinC colocalize in discrete membrane regions[16,21], and this colocalization is disturbed in *yisP* cells.

Controversies in the field of FMM

YisP is necessary for the production of certain membrane-related polyisoprenoid constituent lipids of FMM[16]. Characterization of YisP suggested that, similar to other squalene synthases, this enzyme catalyzes the condensation of two farnesyl pyrophosphate molecules, probably into squalene, with release of inorganic phosphate[24]. Further studies did not detect squalene, which suggested that YisP catalyzes only dephosphorylation of farnesyl pyrophosphate; this would yield farnesol as the reaction product rather than squalene[25].

KinC activity is reduced in the absence of FloA and FloT[16]. A recent report nonetheless shows that activation of KinC can occur in *B. subtilis* cells lacking FloA and FloT[26], although there are marked experimental differences that prevent direct comparison of the two studies[27]. Independent laboratories used pull-down analyses, bacterial two-hybrid analyses and BN-PAGE coupled to immunoblotting to show that FloA and FloT interact physically and with other FMM-associated proteins[17,19]. In another recent publication, fluorescence microscopy detected reduced colocalization between these proteins[28].

Evidence from various studies does not come to a conclusive agreement on the functional relationships between flotillins, lipid modification enzymes, and KinC.

Structural organization of FMM

Because they share similar molecular constituents, FMM organization could resemble that of eukaryotic lipid rafts. Even so, the biological role of FMM and lipid rafts could nonetheless be different, and it is possible that they are two different types of membrane microdomains with different biological functions[29,30] (Fig. 1).

Lipids

Genetic and biochemical studies in *B. subtilis* pointed to farnesol and/or farnesol-derived polyisoprenoid lipids as constituent lipids of FMM[25]. Although other lipids are likely to participate in FMM assembly, we lack a precise notion of their molecular structure, which probably varies from one species to another. For example, a number of bacterial species produce cyclic polyisoprenoid lipids termed hopanoids, which are structurally similar to eukaryotic cholesterol (sporulenes in *B. subtilis*[31,32]); they organize in microdomains in certain bacterial species, such as the *Methylobacterium extorquens* outer membrane microdomains[33–36]. In Gram-negative and -positive bacteria, hopanoids are important for membrane integrity and adaptation to stress conditions, signal transduction and cell division [31,37–40]. Hopanoids play a role in guiding cell division in *Rhodopseudomonas palustris*, and their mislocalization leads to growth defects in this bacterium[41]. Non-cyclic polyisoprenoid membrane lipids like carotenoids have analogous chemical properties to hopanoids[42] and also regulate bacterial membrane rigidity[42–45]. Many bacterial species produce non-cyclic polyisoprenoid lipids[29,46]. Some *Bacillus* produce carotenoids[47], and a carotenoid-deficient *Staphylococcus aureus* mutant shows mislocalization of flotillin and FMM-related proteins[16], which suggest a structural role for carotenoids in FMM assembly. Hopanoids and carotenoids have a more hydrophobic molecular structure than amphipathic phospholipids. The hydrophobic region of phospholipids contains two long, flexible fatty acid chains whereas hopanoids have a rigid, planar hydrophobic region of several aromatic rings that could help stabilize FMM in bacteria[29,46].

Flotillins

Flotillin proteins were initially identified as scaffold proteins in eukaryotic lipid rafts, in which they appear to recruit other proteins that must be in lipid rafts to interact. Eukaryotic lipid rafts have two flotillin homologs to each other, FLO-1 and FLO-2, which associate with each other in hetero-oligomers[8,9,48,49]. The FLO-1 and FLO-2 N-terminal regions anchor to membrane rafts via myristoyl and palmitoyl lipid moieties, respectively[50]. FLO-1 and FLO-2 have a prohibitin (PHB) domain (also termed the SPFH domain), part of a larger protein family known as the SPFH family (stomatin, prohibitin, flotillin and HflK/C) [51,52], which is necessary for flotillin function, although its precise role is unknown[51,52]. The C-terminal, a coil-coiled region with several glutamine–alanine repeats[53,54], participates in flotillin oligomerization[50]. Bacterial flotillins are structurally similar to those of eukaryotes, except that bacterial flotillins associate with FMM via an N-terminal hairpin loop[55]. Flotillin-encoding genes are found in most

bacterial genomes[29]; the flotilin gene is usually the second gene of an operon in which the first gene encodes a NfeD-homolog protein. NfeD interacts physically with flotillin in bacteria and archaea[56]. The third gene of the operon shows no homologies and is absent in some species.

One important role of bacterial flotillins is regulation of membrane fluidity[19]. Lack of flotillin leads to coalescence of distinct domains of high membrane order and reduces the heterogeneity of membrane lipid types. [19]. Another flotillin role is to facilitate efficient protein complex assembly, which is probably why flotillin perturbation affects the function of FMM-associated signal transduction pathways[5,54,57]. Flotillin scaffold activity stabilizes protein complexes by tethering interacting partners and increasing the likelihood of interaction[58]. Protein-protein interaction experiments show that flotillin promotes efficient homodimerization of FMM-associated sensor kinases, such as KinC and KinD, which regulate biofilm formation in *B. subtilis*[18], as well as ResE and PhoR kinases that respectively regulate antibiotic production and cell wall turnover in *B. subtilis*[17,18]. A third role of the scaffold flotillins is to prevent non-specific protein aggregation and guide protein assembly into productive complexes[59], e.g., FloT promotes KinC interaction specificity and KinC confinement in FMM prevents its non-specific aggregation with other sensor kinases that are not part of the FMM protein cargo, such as KinB[17,18].

Functional protein cargo

DRM composition has been analyzed in *B. subtilis*[16], *S. aureus*[16] and *Borrelia burgdorferi*[60]. Fluorescence microscopy and pull-down experiments show interaction of DRM-associated proteins with flotillin and confirm these proteins as *bona fide* FMM-associated proteins. A common feature of these FMM-associated proteins is their multimeric nature (Table 1), as is the case of the FtsH protease complex[61], the Sec protein secretion machinery[62] and several ABC transporters[63]; this is consistent with the idea that FMM catalyzes efficient protein-protein interactions and complex assembly. Flotillin-defective *B. subtilis* mutants show altered oligomerization of several sensor kinases (e.g., PhoR, ResE and KinC) and thus, inhibited signaling transduction cascades[16,24]. In addition, the multimeric protease FtsH and the Sec protein secretion machinery interact physically with flotillins in *B. subtilis* in an interaction important for their respective protease and protein secretion activities[22,23,55] (Fig. 2).

It would be of interest to determine how a particular pool of proteins associates with FMM to the exclusion of other membrane proteins. Lipid-protein interactions probably have a role, similar to the glycosylphosphatidylinositol proteins associated with lipid rafts in eukaryotic cells[64] (Fig. 1). Although this type of post-translational modification is not described in prokaryotes, the association of protein cargo with FMM is likely to rely on molecular mechanisms yet to be described.

Molecular approaches to the study of FMM

FMM are compact hydrophobic membrane platforms, making them relatively resistant to disruption by detergent treatment. Detergent insoluble fragments can be separated in a sucrose gradient and analyzed independently[65]. Although this technique is an excellent

starting point from which to produce a fraction enriched in FMM proteins, it should not be assumed that any protein associated with the DRM fraction equates to a FMM protein[66]. Additional experiments, such as protein-protein interaction analyses with flotillin, are necessary to test whether a DRM protein is part of the FMM. New high-resolution microscopy techniques such as STED, SIM, PALM and dSTORM are becoming very useful for elucidating the structure and function of nanoscopic assemblies within living cells. Combining these approaches with new dyes allows precise description of the physicochemical properties of membrane microdomains. The dye C-laurdan distributes evenly into cell membranes, but alters its emission spectra in response to hydrophobic changes, with a blue-shift emission spectrum in extreme hydrophobic environments such as lipid rafts, and a red-shift spectrum in the phospholipid membrane[19,67–70]. Despite remarkable progress in the methods used to study membrane microdomains, we still lack robust quantitative lipid profiling approaches that precisely identify and describe the constituent lipids of FMM. Technological advances in lipid identification and profiling have nonetheless increased in recent years[71] and we are optimistic that these questions will be resolved in the near future.

Biological significance of FMM

Gram-positive bacteria

B. subtilis was the first bacterial system in which FMM's were studied. In this organism, FMM's are known to promote interactions among a diverse group of effector proteins that include sensor kinase and FtsH[72,73]. Efficient FtsH oligomerization in *B. subtilis* requires FloA and FloT scaffold activity[23]. As a result, flotillin-defective mutants show reduced FtsH activity[23], and FtsH-related cell processes such as biofilm formation and sporulation are impaired[72,74]. *B. subtilis* was also the subject organism in studies that demonstrated the association of the Sec secretion system with FMM[19]. Pull-down assays indicate that the channel subunit SecY and the signal peptidase SppA interact physically with FloT[19], consistent with the defect in Sec-mediated protein secretion detected in flotillin-defective mutants[22,23,55]. It was recently suggested that a heterogeneous FMM population coexists in *B. subtilis* cell membranes[17]. Uneven FloA and FloT distribution diversified two FMM subpopulations, one containing only FloA and another with both FloA and FloT. Blue native polyacrylamide gel electrophoresis (BN-PAGE) coupled to immunoblot experiments show that FloA-containing FMM are enriched in proteins associated with cell wall turnover (e.g., PhoR sensor kinase), and cell wall turnover is thus partly affected in the *floA* mutant. In contrast, FloA/FloT-containing FMM are enriched in stationary phase-related proteins (e.g., ResE sensor kinase), and mutants lacking FloT are thus partly affected in their adaptation to oxygen-limiting conditions, siderophores and antibiotics production. It is possible that temporal and/or spatial diversification of FMMs regulates mechanisms that bring about temporal and/or spatial control of signal transduction and other cellular processes in bacteria[75,76].

Ongoing research is exploring FMM in distinct bacterial species. FMM have been shown in the pathogen *B. anthracis*[77]. Although this species appears to lack a FloA homolog, it does express FloP, a FloT homolog whose structure and subcellular distribution resemble that of

B. subtilis FloT (~65% identity). FloTP expression and subcellular localization were altered in mutant cells with perturbed membrane lipid production. Alteration of FloTP distribution was concomitant with increased membrane fluidity and decreased toxin secretion. FMM organization in other gram-positive pathogens, such as *S. aureus* or *Mycobacterium tuberculosis*, is also under investigation. *Staphylococcus aureus* expresses a flotillin protein similar to *B. subtilis* FloA (~90% identity). In *M. tuberculosis*, indirect analyses show an intriguing correlation between FMM protein content and extracellular vesicles[78]. *Mycobacterium tuberculosis* flotillin (Rv1488 protein) is highly concentrated in extracellular vesicles, with other FMM-associated proteins such as the Sec machinery, ABC transporters and enolase[78]. FloTillin from *M. tuberculosis* is strongly immunoreactive, a feature probably associated with the role of extracellular vesicles in host-pathogen interactions, and might thus be a good marker for *M. tuberculosis* immunodiagnosis[79]. Consistent with this, *M. tuberculosis* flotillin is an epitope for T cell recognition and activation of an adaptive antibody response in tuberculosis patients[80].

Gram-negative bacteria

Gram-negative bacteria are appealing models for the study of FMM, as their cell envelope has an inner and an outer cell membrane. In its outer membrane, *Methylobacterium extorquens* organizes microdomains constituted by hopanoid aggregation and interaction with lipid A[33]. Alteration of hopanoid production causes a defect in multidrug efflux pumps and generates greater antibiotic sensitivity. DRM-associated proteins of the pathogen *B. burgdorferi*, such as OspB, OspA, and P66, are likewise outer membrane lipoproteins needed to maintain cell morphology and membrane permeability in this bacterium[60,81,82]. Ultrastructural analyses confirmed assembly in the *B. burgdorferi* outer membrane of microdomains enriched in cholesterol and OspB, OspA, and P66[82].

Another interesting line of FMM research is being developed in the pathogen *Campylobacter jejuni*, which invades intestinal epithelial cells and causes infectious gastroenteritis. A transposon mutagenesis screen identified a flotillin-defective mutant that is unable to adhere to and be internalized by epithelial cells, resulting in impaired virulence [83]. *In vivo* experiments in a murine infection model showed that the flotillin-defective mutant did not develop campylobacteriosis[84]. An independent study identified flotillin as a component of the *C. jejuni* flagellar systems, which suggested that flagellum structure was partly compromised in the absence of flotillin; immunoprecipitation analyses showed that flotillin interacts with components of the flagellar system[85]. These results are consistent with the current hypothesis that flotillins act as scaffold proteins for efficient multiprotein complex assembly in FMM, and could explain the virulence defect of the flotillin-deficient mutant, since non-motile *C. jejuni* mutants show an acute decrease in cell-invasive ability[86–88].

In the cyanobacterium *Synechocystis sp.*, flotillin oligomerizes in the cellular and thylakoid membranes and has a role in assembly of the photosynthetic apparatus. Motility is impaired in a flotillin-truncated mutant, but no effect was detected in the photosystem complex[89]. Further studies using a flotillin deletion mutant showed a severe defect in thylakoid biogenesis and structural integrity[90]. In this mutant, defective thylakoids are smaller and

lack typical internal membrane structure. Defective thylakoids showed decreased chlorophyll density and, although photosynthetic efficiency was unaffected, mutant viability was light intensity-dependent[90].

Archaea

The flotillin core domain (residues 56-234) resolved by NMR from the hyperthermophilic archaeon *Pyrococcus horikoshii* shows considerable similarity to eukaryotic flotillins[91,92]. Moreover, flotillin and its operon protein partner NfeD have been resolved as oligomerz[93]. Archaeon NfeD has an oligosaccharide/oligonucleotide-binding fold (OB-fold) domain that lacks residues found to be critical for its OB-fold activity in eukaryotic NfeD. This protein could thus play a role in binding small molecules or ligands that might regulate the activity of spatially proximal proteins[94].

Conclusions

The existence of FMM in bacteria demonstrates programs of membrane compartmentalization in prokaryotes, comparable in their sophistication to those of their eukaryotic counterparts. Indeed, FMM and lipid rafts might be two structurally related membrane platforms with distinct biological roles. The FMM appears to facilitate efficient oligomerization of FMM-associated protein complexes (i.e., oligomerization factories), whereas lipid rafts specifically harbor proteins associated with signal transduction and membrane trafficking. Outstanding questions nonetheless remain unanswered, such as how flotillin scaffold activity influences interacting protein partners, how lipids organize in membrane microdomains, and the relevance of these domains for cell physiology. New and interesting contributions to this growing field will emerge in coming years and help to clarify the importance of FMM in bacterial physiology.

Acknowledgements

This work was funded by ERC Starting Grant ERC335568 (European Union) and BFU2014-55601-P (MINECO, Spain) to DL.

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(*) Papers of special interest

(**) Papers of outstanding interest

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Highlights

- Functional membrane microdomains promote efficient oligomerization of protein partners
- They form by colocalization of specific lipids and scaffold proteins termed flotillins
- These domains are found in Gram-positive and -negative bacteria, and also archaea
- Membrane microdomain integrity is important for associated oligomer activity

Figure 1. Structural similarities and differences between lipid rafts and functional membrane microdomains.

Scheme of the molecular organization of lipid rafts (LR) (**A**) and functional membrane microdomains (FMM) (**B**). The architecture of these two membrane microdomains shows a number of similarities and certain structural differences. For instance, proteins associate with LR using different molecular mechanisms than those associated with FMM. Most FMM proteins are multimeric complexes, whereas LR can contain monomeric proteins. The nature of the FMM constituent lipids represented in this figure is speculative, based on current working hypotheses. LR are composed of cholesterol and sphingolipids, and FMM are probably enriched in farnesol, hopanoids and other polyisoprenoid lipids.

Figure 2. The scaffold activity of flotillin tethers interaction partners and increases the efficiency of biochemical reactions.

(A) Flotillin stabilizes interacting partners. The scaffold activity of flotillin benefits biochemical reactions and prevents the formation of non-specific aggregates. (B) The scaffold activity of flotillin protein has certain limitations. A low flotillin concentration does not promote efficient interaction of protein partners. A medium flotillin concentration is optimal for oligomerization of interacting partners. High flotillin concentration might titrate

protein components away from each other and thus reduce efficient activation of the biochemical reaction. Adapted from Good *et al.* [58].

Table 1
Most-represented FMM-associated proteins in several bacterial species

<i>Bacillus subtilis</i>		<i>Staphylococcus aureus</i>		<i>Borrelia burgdorferi</i>	
Name	Function	Name	Function	Name	Function
FloA	Scaffold protein	FloA	Scaffold protein	HflK	Scaffold protein
FloT	Scaffold protein	Rny	RNA degradation	HflC	Scaffold protein
KinC	Sensor kinase	FtsH	AAA-protease	HtrA	Serine protease
PhoR	Sensor kinase	EbpS	Cell adhesion	FtsH	AAA-protease
ResE	Sensor kinase	WalK	Sensor kinase	SecD	Protein secretion
SecY	Protein secretion	SecA	Protein secretion	FlaA,B	Flagellar filament
FtsH	AAA-protease	TcaA	Antibiotic resistance	FlgE	Flagellar hook
PrsA	Protein secretion	PrsA	Protein secretion	P83/100	Virulence
TagU	Cell wall metabolism	KdpB,C,A	K ⁺ transporter	OspA,B	Gut colonization
PBP1A	Cell wall metabolism	SirB	ABC transporter	Mcp-2	Chemotaxis
McpA,B,C	Chemotaxis	SA1909-11	ATP synthase		
AtpD,G	ATP synthase	Sip1B	Protein secretion		
QoxA	Energy metabolism	SA1283	Cell wall metabolism		
SdhA	Succinate dehydrog.				
OppA	Peptide transport				
OpuAC	Peptide transport				
FhuD	Siderophore uptake				