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Cholesterol lipids and cholesterol-containing lipid rafts in bacteria

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Abstract

Sterols are important components of eukaryotic membranes, but rare in bacteria. Some bacteria obtain sterols from their host or environment. In some cases, these sterols form membrane domains analogous the lipid rafts proposed to exist in eukaryotic membranes. This review describes the properties and roles of sterols in *Borrelia* and *Helicobacter*.

Keywords

membrane raft; lipid raft; sterol; ordered membrane domain; biomembranes

Introduction

Sterol molecules are important structural components of eukaryotic membranes. One important ability of membrane sterols is to promote the formation of liquid ordered (Lo) domains. (1). In model membranes, co-existence of liquid ordered domains rich in sterol and sphingolipids, often called lipid rafts (2, 3), with disordered lipid domains rich in unsaturated lipids has been well-established by numerous studies (1). Lipids in the liquid ordered domains are more tight packed that those in liquid disordered domains, and exhibit slightly slower, but still considerable, lateral diffusion. The potential co-existence of ordered and disordered domains in living cells has been a subject of much interest and study. The first strong evidence for the presence of Lo domains in cells was the characterization of sphingolipid and cholesterol rich detergent (Triton X-100) resistant membrane (DRM) fractions from mammalian cells (4). It was soon shown, using model membrane lipid vesicles, that Lo domains are detergent insoluble at low temperatures, presumably because of the tight lipid packing between saturated acyl chains and cholesterol, while liquid disordered domains are not detergent insoluble, and that the insolubility of cellular glycosyl phosphatidylinositol (GPI) anchored proteins, anchored in membranes by saturated acyl chains could be replicated in model membranes (5). Because of potential perturbation by detergent, the isolation of DRM from cells is not considered sufficient to prove the presence of Lo domains. It has been proposed that under some conditions Triton X-100 could even

induce formation of ordered domains (6) although subsequent work indicates that what occurs upon introduction of Triton X-100 is coalescence of pre-existing ordered domains into larger ones (7).

Interest in membrane domains arises from their potential functions (8, 9). Domains have been proposed to participate in a variety of biological functions, with many studies of their roles in infection, and signal transduction, especially in immune cells (10, 11). As far back as three decades ago a role for sphingolipid-rich domains in membrane sorting processes was proposed (12), and consistent with this different proteins and lipids have very different affinities for ordered and disordered domains (13). In addition, protein conformations might differ in ordered and disordered domains. The long-standing question of whether lipid rafts exist in living cells remains controversial. Many studies are highly supportive of the existence of lipid raft domains in mammalian cells. However, such studies often rely on indirect detection of ordered domains, which may usually exist at the low nanometer size range, inaccessible to most techniques. As described below, it may be easier to detect ordered domains in bacteria, not thought to have such domains until recently.

Lipid composition of cholesterol-containing bacteria: *Borrelia*

Bacteria should not be good candidates for raft formation because are unable to synthesize cholesterol. However, some bacteria obtain cholesterol from host cells. At present, only a few bacteria are known to have cholesterol in their membranes. This includes bacteria in the genera *Mycoplasma, Ehrlichia, Anaplasm, Brachyspira, Helicobacter* and *Borrelia* (14–18). In some other bacterial hopanoids, which have a similar ring structure as sterols, may act as functional analogs of sterols (19). The most information about the lipids of a cholesterol-containing bacterium exists for *Borrelia*.

Borrelia burgdorferi, is a pathogenic spirochete transmitted to mammals through the bite of infected *Ixodes* ticks (20). It is the causative agent of Lyme disease (20, 21). *B. burgdorferi* is gram-negative like, with an inner and outer membrane (22). Lipid composition has been analyzed for whole *Borrelia* cells (23–26). Eleven lipids components were identified using high performance thin layer chromatography (HPTLC). Among these were two major phospholipids, phosphatidylcholine (PC) and phosphatidylglycerol (PG). Phospholipids commonly comprise over 20% of total *B. burgdorferi* lipids. *B. burgdorferi* also three glycolipids. One is mono-α-galactosyl-diacylglycerol (MGalD/BbGL-II). MGalD has two acyl chains and a galactose attached to the glycerol. A second glycolipid is cholesterol-β-D-galacto-pyranoside (CGal), which has cholesterol attached to galactose. The third is cholesteryl 6-O-acyl-β-D-galactopyranoside (ACGal/BbGL-I) which is CGal with one acyl chain attached to the 6 carbon of the galactose. The glycolipids constitute about 55–60% of the total lipids, with MGalD and ACGal being the major species. Lipids not mentioned above include minor species and free cholesterol (24).

The fatty acid composition of the major lipids has been analyzed (24, 25). The major fatty acids present are palmitic, stearic, oleic, and to a lesser extent linoleic. The fatty acid composition of the major lipids, PC, PG, ACGal, and MGalD, differ (23–26). For the phospholipids, the total saturated fatty acyl content is significantly greater than 50%. This

means, that a significant fraction of these lipids must carry two saturated fatty acyl chains, which should promote interaction with ordered membrane domains/rafts. ACGal also has a high fraction of saturated acyl chains (~70%). Since it is has only one acyl chain, this implies that most of the ACGal molecules have a strong ability to participate in ordered domain formation. MGalD has a unsaturated:saturated acyl chain ratio close to 1:1. This could give rise to MGalD molelcules with various combinations of saturated and unsaturated chains, but is consistent with most or even almost all MGalD molecules having one saturated and one unsaturated acyl chain, similar to most mammalian phospholipids.

Other *Borrelia* species, have lipid compositions similar to *B. burgdorferi*. Like *B. burgdorferi, B. garinii* and *B. afzelii* has significant amounts of ACGal (26). *B. hermsii* contains 6-O-acyl-β-glucopyranoside (ACGlc) which has glucose in place of galactose. Interestingly, this bacterium does *not* substitute glucose for galactose in MGalD (26). As in *B. burgdorferi*, ACGal/ACGlc and MGalD are the most abundant glycolipids, with ACGal or ACGlc, comprising about 25% of whole lipid extracts (24, 26).

Acquisition of cholesterol by *Borrelia*

B. burgdorferi requires cholesterol in order to grow (27). Since it cannot synthesize cholesterol, as noted above, it has to get access to the cholesterol source either from host cells or (*in vitro*) from the culture media (27). It has been observed that cholesterol exchange between *B. burgdorferi* and mammalian cells can occur by direct contact between bacteria and mammalian cells and/or through outer membrane vesicles released from the bacterium, and which then presumably come into contact with the mammalian cells, as shown by the transfer of labeled cholesterol between bacteria and mammalian cells. Labeled cholesterol transferred to bacteria could be incorporated into the cholesterol glycolipids (28). It has been proposed that these processes might somehow involve lipid rafts.

Lipid rafts in *Borrelia*

The presence of significant amounts of cholesterol glycolipids in *Borrelia* raises the question of whether *Borrelia* can form lipid rafts. Lipid microdomains in *B. burgdorferi* were directly observed by transmission electron microscope (TEM) studies using gold labeled antibodies that bind to ACGal (29). These clusters were detected at as high as 37°C, and both in bacteria from culture and *B. burgdorferi*–infected mice (29). TEM was also used to visualize domains after lipid substitution experiments in which *B. burgdorferi* were treated with methyl-beta-cyclodextrin and then incubated with various sterols (30). Substitution with sterols (ergosterol, cholesterol, dihydrocholesterol, and stigmasterol) shown to raft-promoting in model membrane studies (31–33) resulted in formation of microdomains very similar to those *B. burgdorferi* before sterol substitution. Microdomains were also observed in *B. burgdorferi* after substitution of sterols (desmosterol, lanosterol, zymosterol and cholesterol formate) having an intermediate ability to form rafts in model membranes, although a higher amount of isolated gold particles were noted in these cases. No microdomains were detected after substitution with sterols (coprostanol and androsterol) shown to be raft-inhibiting in model membrane studies (30).

Interestingly, DRM fractions could also be isolated from *Borrelia* (29). Lipid analysis of the DRM from *B. burgdorferi* gives a rough idea about the lipids most likely to contribute to raft formation. PC, PG and MGalD were found to be largely soluble in Triton X-100, while ACGal, CGal and free cholesterol predominated in the DRM fraction (24). Because detergents may perturb membranes, as noted above, such studies may not give a fully accurate picture of *Borrelia* raft composition. Nevertheless, a crucial role for ACGal is very likely. In fact, prior studies of acyl steryl glycosides, molecules very similar in structure to ACGal, have shown that acyl steryl glycosides are enriched in DRM from plants (34), and that they stabilize the formation of ordered domains in model membrane vesicles (35).

The formation of DRM from *Borrelia* lipids is consistent with the formation of ordered domains. Consistent with this, fractional insolubility of multilamellar vesicles (MLVs) made of extracted *Borrelia* lipids after treatment with Triton X-100 at room temperature was slightly less than that of liquid ordered (Lo) state vesicles composed of 2:1 sphingomyelin/cholesterol vesicles, and much greater than that of liquid disordered (Ld) state composed of 2:1 dioleoylphosphatidylcholine/cholesterol (29). In agreement, in the absence of detergent, DPH anisotropy fluorescence in MLVs from *B. burgdorferi* lipid extracts indicated a degree of ordered intermediate the between Lo state and Ld state vesicles at room temperature (29). To confirm that the high degree of order in model membranes composed of *Borrelia* lipids came come from the coexistence of ordered and disordered domains, a FRET assay was used. Domain formation in model membranes is accompanied by reduced FRET due to partial segregation of donor and acceptor into different domains. FRET in *Borrelia* MLVs was compared to those made of dioleoylphosphatidylcholine/cholesterol, which do not form ordered domains. Reduced FRET was observed over a range of temperatures for *Borrelia* MLVs, with FRET levels only approaching that in the Ld state vesicles at high temperature, This indicated temperature-dependent segregation of ordered and disordered domains over a wide range of temperatures for *Borrelia* lipid extracts (29).

Parallels to TEM results were also noted when the yield of DRM from *B. burgdorferi* was measured after sterol substitution. Highest yield of DRM, as judged by cholesterol glycolipid levels, was measured after substitution with the strongly raft supporting sterols ergosterol and cholesterol, intermediate yields were obtained after substitution with the weakly raft supporting sterols lanosterol and zymosterol, and lowest yield of DRM was obtained after substitution with the raft-formation inhibiting sterols coprostanol and androsterol (30).

The relationship between acyl chain structure and association with membrane *Borrelia* membrane domains also indicates they have raft-like properties. The lipid biotin-PEG-DPPE, which has saturated acyl chains, was observed to colocalize with, or adjacent to, ACGal containing microdomains in *B. burgdorferi* by TEM, while biotin-PEG-DOPE, which has unsaturated acyl chains, did not, suggesting that the ACGal-containing microdomains in *B. burgdorferi* preferentially accumulate saturated acyl chain lipids (30). This is exactly what is expected because saturated chain lipids are well-known to favor location in Lo domains while unsaturated acyl chains do not.

To rule out the possibility that preparation of samples for TEM analysis or detergent treatment artificially induced domain formation, FRET experiments to detect domains were carried out in living *B. burgdorferi*. FRET confirmed that the co-existing ordered and disordered domains formed in *B. burgdorferi* up to at least 35–40°C (30). Furthermore, FRET responded to sterol substitution as expected from the TEM and DRM results. Reduced FRET that increased at higher temperature was observed in the case of strongly and moderately raft-supporting sterol substitution, but coprostanol and androsterol substitution resulted in strong and temperature-independent FRET. Cholesterol lipid depletion by itself destabilized domains, as shown by their disappearance at a lower temperature than for untreated cells, but did not totally abolish their formation (30).

To summarize, TEM, FRET and DRM studies, including those involving sterol substitution, all indicate that ordered domains exist in *Borrelia*, and to all intents and purposes can be considered lipid rafts similar to those proposed in eukaryotic cells.

The function of raft domains in *Borrelia* is not known. However, sterol substitution experiments suggest that they play a role in membrane integrity. After prolonged incubation subsequent to substitution with sterols that either weakly support or inhibit raft formation *Borrelia* lost their normal wave morphology and membrane integrity as judged by permeability experiments and TEM (30). These changes were associated with increased sensitivity to osmotic pressure across the cell membranes, and were more severe after substitution with raft-inhibiting sterols than after substitution with weakly raft-supporting sterols.

Raft-associated proteins in *Borrelia*

In addition to lipids, proteins associated with lipid rafts also could play a role in their formation and stability. Recent studies have investigated the influence of *Borrelia* outer membrane lipoproteins upon lipid raft formation in *B. burgdorferi*. OspA, OspB and P66 were identified in the DRM fraction of *B. burgdorferi* (29). In a subsequent study, ordered domain formation was studied in single-gene mutants (ΔOspA, ΔOspB and ΔOspC) and a double-mutant (B313) which did not express OspA and OspB. FRET experiments in living *B. burgdorferi* indicated that single-deletion mutants formed ordered domains at similar levels and thermal stability as those in wild type *B. burgdorferi*. In contrast, B313, while exhibiting weak FRET indicative of ordered domain formation at low temperature, lost domain segregation as judged by FRET at lower temperatures that in the single-deletion mutants ΔOspA, ΔOspB, ΔOspC, or wild type bacteria. Domain segregation levels of B313 could be restored to wild type levels by transforming B313 with OspA, indicating that OspA was raft-stabilizing lipoproteins and had redundant functions with OspB for maintaining membrane domain stability. In contrast, over expression of OspC in the B313 strain did not restore the membrane order level to wild type levels, suggesting that OspC is not raft-stabilizing (36).

An analysis of DRM proteins provided a potential lipid raft proteome for *B. burgdorferi* (37). DRM and non-DRM fractions were characterized by liquid chromatography MS/MS or multidimensional protein identification technology (MudPIT) MS. DRM-associated proteins

were identified that are involved in biological functions such as motility, chemotaxis and signaling, suggesting that lipid rafts in *Borrelia* could play many important roles in its life cycle (37). Acylation was identified as one of the properties targeting proteins to lipid rafts, with the acylated proteins OspA, OspB (as also seen in previous studies), and BB_0323 being especially highly enriched in DRM. Two other Borrelia proteins, HflC and HflK have SPFH domains which have been proposed to be involved in lipid raft association (38).

Lipid composition of *Helicobacter pylori*

Helicobacter pylori is a Gram-negative spirochete. It is the primary cause of gastric ulcers, and a cause of carcinomas and lymphomas (39, 40). Several studies revealed that *H. pylori* contains cholesterol glycolipids (14, 15, 41). Three kinds of cholesterol glycolipids (CGs), designated G-1, G-2 and G-3, were detected in its lipid extracts, and their chemical structure analyzed by mass spectrometry and nuclear magnetic resonance (15). The sum of three glycolipids accounted for 25% of total lipid in a ratio 1:0.5:0.3 (wt//wt/wt) for G-1/G-2/G-3 (42). Analogous to the glycolipids in *Borrelia*, G-2 was identified as cholesterol-α-D-glucopyranoside, which contains glucose and cholesterol in a molar ratio of 1:1, while G-1 was found to be cholesteryl-6-O-tetradecanoyl-α-D-glucopyranoside (G-1) in which an acyl chain is attached to glucose. The proposed structure for G-3 is cholesterol-6-O-phosphatidyl-α-D-glucopyranoside, in which a G-2 moiety is attached to a phosphatidic acid via linkage between the phosphate and 6 carbon of glucose. A method to chemically synthesize cholesterol-6-O-phosphatidyl-α-D-glucopyranoside has been achieved by starting with D-glucose. It was used to prepare G-3 analogues with various diacylglycerols (42).

H. pylori also has a considerable amount of phospholipid. Phosphatidylethanol (PE) is the most abundant phospholipid (~58% of total phospholipid), with lesser amounts of cardiolipin (CL) 22% and PG 13%, plus even very small amounts of PS and SM (15).

In terms of fatty acyl composition G-1 contains myristic acid (C14:0) (15). The remaining lipids contain roughly 50% of the saturated fatty acids, mainly myristic, and 50% of either unsaturated or cyclopropyl-containing fatty acids (15). A typical cyclopropyl group near the middle of an acyl chain would be expected to behave similar to a double bond (15, 43). Assuming the saturated fatty acids are in position 1 of the glycerol, these lipids probably have low Tm values when formed into bilayers, and might not strongly support ordered domain formation. On the other hand, G-1 may be able to make ordered domains when mixed with the other *H. pylori* lipids given its combination of cholesterol and a saturated acyl chain (15).

Biosynthesis of *H. pylori* cholesterol lipids

A cholesterol-α-glucosyltransferase (CGT) consisting of 389 amino acids, and encoded by gene *hp0421/capj*, is responsible for synthesizing *H. pylori* cholesterol glucosides from cholesterol extracted from mammalian cells (44–46). Cholesterol-α-glucosyltransferase belongs to the glycosyltransferase family 4 (GT4), and shows sequence similarity to several members of the family with diacylglycerol glycosyltransferase activity (44). Deletion of the *hp0421* gene leads to the complete loss of the ability of *H. pylori* to synthesize CGs. CGT

catalyzes the first step in the biosynthesis of cholesterol glycolipids, transfer of glucose to C3 in cholesterol through an α-linkage. An *in vitro* study demonstrated that its gene encodes a membrane-bound, UDP-glucose-dependent, cholesterol-α-glucosyltransferase (44). The crystal structure of the catalytic domain of *H. pylori* CGT has been solved (47), and the enzymatic activity of the protein studied. Its catalytic domain is similar to that of other GT4 members.

CGT is synthesized in the cytoplasm of *H. pylori* as an inactive form and becomes activated upon association with bacterial membranes. The distribution of CGT was studied by immunogold labeling. It was found that 66% of the gold particles were located in the cytoplasm, 25% in the inner membrane, 3% in the periplasm, and 5% in the outer membrane (48).

The enzyme does not have an absolute specificity for cholesterol. The expression of *hp0421* in a double null mutant of *P. pastoris*, a fungus devoid of both steryl glucosides and glucosyl ceramides, resulted in the biosynthesis of ergosteryl-α-glucoside (44). In addition, GT expressed in *E. coli* could use various sterols as sugar acceptors including cholesterol, ergosterol, β-sitosterol, stigmasterol, and campesterol. However, cholesterol had the greatest activity. Other sterols gave at most 13% as much incorporation into glycolipids as cholesterol (44). Ceramide and diacylglcerol did not function as cholesterol acceptors (44).

Effect of sterols and steroids upon *H. pylori*

The effect of the various sterols noted above upon *H. pylori* has not been studied. However, the effect of the cholesterol biosynthetic precursor 7-dehydrocholesterol (7DHC) has been studied. It was found to be fatal to *H. pylori* (49). Glucosylation detoxified 7DHC, as indicated by the observation that mutant *H. pylori* lacking the ability to glucosylate cholesterol had higher 7DHC susceptibility (49). The effect of other steroids upon *H. pylori* has also been studied. Some steroids inhibit the *H. pylori* growth. Estradiol, androstenedione, and progesterone fall into this category (50). 17α-hydroxy progesterone caproate, a synthetic progesterone derivative, had a stronger anti-*H. pylori* action than progesterone, while 17α-hydroxy progesterone had no effect on *H. pylori* growth. Progesterone and 17α-hydroxy progesterone caproate damaged the *H. pylori* cell membrane, inducing prompt cell lysis and leakage of intracellular proteins from the cells (50). Pre-culturing *H. pylori* with progesterone decreased cholesterol uptake, possibly indicating that progesterone obstructs cholesterol assimilation by competitively inhibiting cell surface binding. Surprisingly however, progesterone did not decrease CG level (50).

Acquisition of cholesterol by *H. pylori*

H. pylori, which like other bacteria cannot synthesize cholesterol, can sense cholesterol in its environment and move to a high concentration cholesterol nutrient source. It has been found to assimilate cholesterol during infection from the plasma membrane of epithelial cells (46). Studies using gastric cancer (AGS) cells showed that cholesterol acquisition by *H. pylori* was associated with cholesterol-rich membranes in AGS cells (45, 46), and led to the destruction of lipid rafts in AGS cells (46). Experiments in which cholesterol levels in AGS

cells were decreased by treatment with docosahexaenoic acid (DHA) resulted in a decrease of *H. pylori* synthesis of cholesterol glycolipids and decreased *H. pylori* growth (51). DHA also has a direct inhibitory effect on *H. pylori* growth (52). Cholesterol from host cells alleviated this growth inhibition (51). Interestingly, in mice administration of DHA inhibits *H. pylori* gastric colonization as well as decreased gastric mucosa inflammation (52).

As in *Borrelia*, lipid exchange appears to be bidirectional. When NBD-cholesterol-loaded AGS cells were infected with wild type *H. pylori* before lysis by Triton X-100, NBD-cholesterol glycolipids were detected in the DRMs fraction of the cells. It was not detected upon infection with a mutant *H. pylori* lacking the cholesterol-α-glucosyltransferase (45). It should be noted, that if *H. pylori* has rafts, and a significant number of bacteria were present, bacterial DRM might have been present in this material.

Functional effects of cholesterol glycolipids in *Helicobacter*

An important question is whether cholesterol or cholesterol glucosylation plays a role in *H. pylori* infections. *H. pylori* has been cultured in media with or without cholesterol, and then exposed to a series of antibiotics, antifungals, and antimicrobial peptides. *H. pylori* grown in the presence of cholesterol was more resistant to eight antibiotics, and the antibiotic peptide LL-37 (53). However, cholesterol did not alter the susceptibility of *H. pylori* to antifungal drugs (53). The presence of cholesterol in the bacterial growth medium is essential for gastric colonization of gerbils by *H. pylori* (54). It should be noted that cholesterol depletion alters other *H. pylori* lipids. Cholesterol depletion was associated with additional LPS bands, while use of a cholesterol-containing medium increased expression of Lewis X and Lewis Y antigens (54).

Glucosylation of cholesterol also affects *Helicobacter* infections. CGT-lacking *H.pylori* exhibited reduced colonization in gerbils (53). A CGT-lacking mutant of *H. pylori* maintained cholesterol-dependent resistance to most antimicrobials, except to colistin (53). Colonization by *H. hepaticus* was studied in male A/JCr mice. An inability to colonize the intestine and liver was observed using CGT-deficient *H. hepaticus* also suggesting that CGT plays a key role during infection (54). In addition, a study of the internalization of *H. pylori* into macrophages suggested that cholesterol glucosylation of *H. pylori* regulated its macrophage lipid raft-dependent endocytosis (55). Of course, the observation that mammalian cell lipid rafts are involved with cholesterol uptake into *H. pylori*, does not say anything about the existence lipid rafts in *H. pylori* itself. Nevertheless, the potential presence of rafts in *H. pylori* is an obvious question for future studies.

Lipid rafts in bacteria lacking cholesterol

For *B. subtilis* and *S. aureus*, membrane domains have also been observed, but their origin is less clear (56). *B. subtitlis* has no sterols, but polyisoprene synthesis blocking inhibitors have hinted at sterol analogs (56). (Sterols, only made in eukaryotes, are products of polyisoprene processing.) Interestingly, the lipid giving the Gram-positive bacterium *S. aureus* its color, staphyloxanthin, a virulence factor in infection, is analogous to raft-forming lipid ACGal, but with an unusually rigid polyisoprene in place of sterol. *S. aureus* also has lipids with

saturated acyl chains (although often branched near the end of the chain) (57). These should have relatively high Tm values based on literature values for saturated and branched lipids (43), and so tend to form ordered domains. Interestingly, cholesterol has also been reported to enhance *S. aureus* growth (58). This might just reflect a nutritional use of cholesterol (e.g. as a carbon source) but it has been reported in one study that *S. aureus*-associated cholesterol can comprise 15% of total lipid (14). Further work is needed to clarify the physical and chemical basis of domain formation in these bacteria.

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