

Novel sterol binding domains in bacteria

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Abstract

Sterol lipids are widely present in eukaryotes and play essential roles in signaling and modulating membrane fluidity. Although rare, some bacteria also produce sterols, but their function in bacteria is not known. Moreover, many more species, including pathogens and commensal microbes, acquire or modify sterols from eukaryotic hosts through poorly understood molecular mechanisms. The aerobic methanotroph *Methylococcus capsulatus* was the first bacterium shown to synthesize sterols, producing a mixture of C-4 methylated sterols that are distinct from those observed in eukaryotes. C-4 methylated sterols are synthesized in the cytosol and localized to the outer membrane, suggesting that a bacterial sterol transport machinery exists. Until now, the identity of such machinery remained a mystery. In this study, we identified three novel proteins that may be the first examples of transporters for bacterial sterol lipids. The proteins, which all belong to well-studied families of bacterial metabolite transporters, are predicted to reside in the inner membrane, periplasm, and outer membrane of *M. capsulatus*, and may work as a conduit to move modified sterols to the outer membrane. Quantitative analysis of ligand binding revealed their remarkable specificity for 4-methylsterols, and crystallographic structures coupled with docking and molecular dynamics simulations revealed the structural bases for substrate binding by two of the putative transporters. Their striking structural divergence from eukaryotic sterol transporters signals that they form a distinct sterol transport system within the bacterial domain. Finally, bioinformatics revealed the widespread presence of similar transporters in bacterial genomes, including in some pathogens that use host sterol lipids to construct their cell envelopes. The unique folds of these bacterial sterol binding proteins should now guide the discovery of other proteins that handle this essential metabolite.

eLife assessment

This is a **valuable** contribution to our understanding of how some bacteria can transport sterols from the cytoplasm to the outer membrane. Though much remains to be tested and explored, the data and analyses presented here provide **solid** evidence for the genetic and physical interaction of BstA/B/C with bacterially-produced sterols. The manuscript will be of interest to scientists focusing on the characterization of novel bacterial proteins and those studying lipid transport and acquisition in bacterial pathogens.

Introduction

Sterol lipids are ubiquitous and essential components of eukaryotic life, playing vital roles in intra- and intercellular signaling, stress tolerance, and maintaining cell membrane integrity (Bi and Liao, 2010; Bloch, 1991; Huang et al., 2016; Miao et al., 2002). Although sterol synthesis is often considered to be a strictly eukaryotic feature, several bacteria have been shown to also produce sterols (Bloch, 1991; Ourisson et al., 1987). Bacterial sterols were first discovered in *Methylococcus capsulatus* more than 40 years ago (BIRD et al., 1971) and initially, were only observed in a few isolated aerobic methanotrophs (ψ -Proteobacteria) and a few myxobacteria (δ -Proteobacteria) (Bode et al., 2003; Kohl et al., 1983; Patt and Hanson, 1978; Schouten et al., 2000). Subsequent comparative genomics analyses have revealed the potential to produce sterol in a variety of bacterial groups including Planctomycetes, α -Proteobacteria, and Bacteroidetes (Bode et al., 2003; Pearson et al., 2003; Wei et al., 2016). Sterols produced by bacteria, however, tend to differ from eukaryotic sterols in both structure and in their biosynthetic pathways.

Sterol synthesis in both bacteria and eukaryotes requires the cyclization of the linear substrate oxidosqualene by an oxidosqualene cyclase (Osc) to generate either lanosterol or cycloartenol (Abe, 2014). However, fungi, vertebrates, and plants further modify these initial cyclization products to synthesize ergosterol, cholesterol, and stigmasterol, respectively (Desmond and Gribaldo, 2009). These biochemical transformations include demethylations, isomerizations, saturations, and desaturations that are essential for sterols to function properly in eukaryotes (Nes et al., 1993; Xu et al., 2005). Although bacterial production of fully modified sterols such as cholesterol is rare (Lee et al., 2023), some bacteria, including several aerobic methanotrophs and myxobacteria, do modify their sterols. For example, *M. capsulatus* produces sterols that are demethylated once at the C-4 and C-14 positions and contain a unique desaturation between C-8 and C-14 (Bouvier et al., 1976) (**Fig. 1A**). In addition, studies have revealed that bacterial proteins required to modify sterols can differ from the canonical eukaryotic sterol modifying proteins. Examples are the recently identified sterol demethylase proteins, SdmA and SdmB, in *M. capsulatus* that use O₂ to remove one methyl group at the C-4 position; the enzymes are mechanistically distinct from the eukaryotic C-4 demethylase enzymes (Gachotte et al., 1998; Lee et al., 2018; Rahier, 2011).

Although we have a better grasp of the taxonomic distribution of sterol synthesis in bacteria and of the distinct bacterial proteins involved in their biosynthesis, the function of these lipids in bacteria remains a mystery. It has been posited that bacterial sterols play a role in modulating the fluidic properties of the cytoplasmic membrane, similar to what is observed for cholesterol and ergosterol in eukaryotic cells (Miao et al., 2002; Parks et al., 1995; Summons et al., 2006). However, studies in *M. capsulatus* have demonstrated that the distribution of sterols differs considerably from what occurs in eukaryotes. Approximately 90% of the free sterol pool in eukaryotic cells is found in the cytoplasmic membrane (Jacquier and Schneider, 2012) while in *M. capsulatus*, 75% of sterols are localized to the outer membrane (JAHNKE et al., 1992). The outer membrane is the external asymmetric membrane of Gram-negative bacteria and differs from the cytoplasmic membrane in that its outer leaflet is composed of lipopolysaccharides (LPS) in addition to phospholipids (Ruiz et al., 2006). Additionally, sterols that differ by only a single methylation in the core structure can produce significantly different effects in terms of membrane fluidity and stability (Bacia et al., 2005). Given that *M. capsulatus* primarily produces sterols with one or two C-4 methyl groups, any sterol-membrane interactions in *M. capsulatus* could differ significantly from what is observed in eukaryotic membranes with cholesterol and ergosterol, which lack methyl groups at the C-4 position.

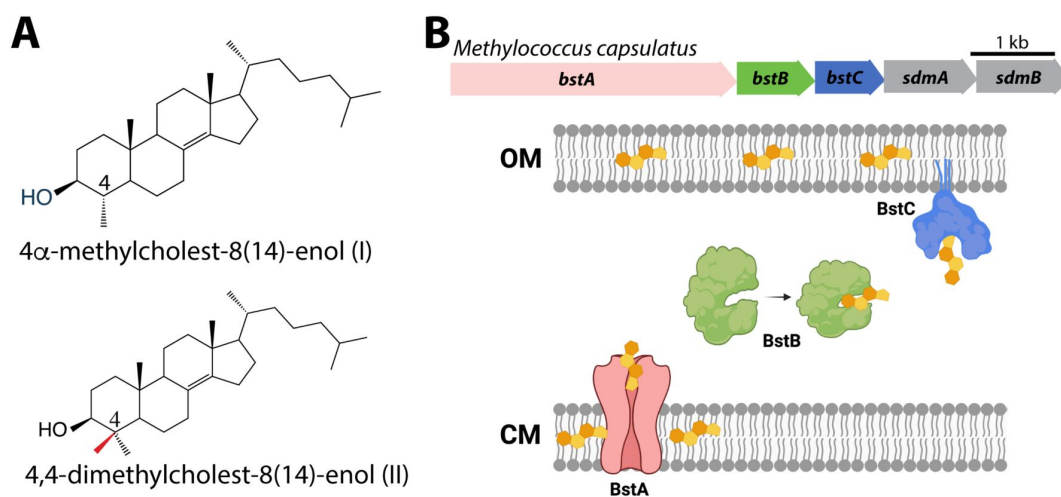


Figure 1.

(A) Structures of C-4 methylated sterols synthesized by *Methylococcus capsulatus*. (B) Operon of sterol biosynthesis and transport genes and (top) and schematic description of their localization. OM: outer membrane; CM: cytoplasmic membrane.

Interestingly, the 4,4-dimethyl sterols and 4-monomethyl sterols found in *M. capsulatus* also exist as precursors of pathway end-products in eukaryotes and accumulation of C-4 methylated sterols has been associated with a variety of eukaryotic processes. The accumulation of C-4 methylated intermediates is considered to be the cause of human genetic diseases known as sterolosis (He et al., 2011 [↗](#); König et al., 2000 [↗](#); McLaren et al., 2010 [↗](#)) and 4,4-dimethyl sterols such as lanosterol in the brain are implicated in Parkinson's disease (Lim et al., 2012 [↗](#)). In a variety of yeast species, C-4 methylated intermediates can regulate cellular processes associated with hypoxia and other conditions of cellular stress (Hughes et al., 2007 [↗](#); Serratore et al., 2017 [↗](#); Todd et al., 2006 [↗](#)). Although the function of 4,4-dimethyl sterols and 4-monomethyl sterols in bacteria is still elusive, a functional role for these specific sterols beyond maintaining membrane fluidity and integrity seems plausible.

To better understand the significance of sterol utilization in the bacterial domain, a fuller understanding of the molecular mechanisms controlling sterol production and localization is needed. Given the observed distribution of sterols in the outer membrane of *M. capsulatus*, we hypothesized that transporters specific for C-4 methylated sterols must exist that can shuttle these substrates to the outer membrane. Trafficking of sterols to various organelles in eukaryotic cells is not fully understood (Jacquier and Schneider, 2012 [↗](#)) and impaired sterol transport is related to a variety of defects including lysosomal storage diseases (Vance and Peake, 2011 [↗](#)). Identifying and characterizing bacterial proteins that transport sterol could reveal novel sterol binding motifs and folds and will provide meaningful insights into protein-sterol interactions and lipid trafficking more broadly.

In this study, we present three putative bacterial sterol transport proteins in *M. capsulatus* that exhibit remarkable specificity for C-4 methylated sterols. We first used bioinformatics to identify a cytoplasmic membrane protein (BstA), periplasmic protein (BstB), and outer membrane associated protein (BstC). Their ability to recognize and bind sterols was confirmed using protein-lipid pull down assays, where they showed selective binding to C-4 methylated sterols when in the presence of total lipid extracts from *M. capsulatus*. Quantitative assessment of ligand binding using Microscale Thermophoresis (MST) confirmed their preference for 4-monomethyl sterol, which they bind with equilibrium dissociation constants that are 30-90-fold lower than the 4,4-dimethyl sterol. High-resolution crystallographic structures of BstB and BstC reveal their pronounced divergence from eukaryotic sterol transporters. Docking studies and molecular dynamics simulations with sterol substrates reveal putative substrate binding sites and recognition mechanisms. Collectively, these data provide evidence for a novel system for sterol binding and possibly transport within the bacterial domain and advances our understanding of bacterial sterol lipids.

Results

Bioinformatic identification of sterol transport proteins in *M. capsulatus*

In a previous study, we used the Joint Genome Institute (JGI) Integrated Microbial Genomes (IMG) Phylogenetic Profiler to identify seventeen candidate genes in *M. capsulatus* that are unique to C-4 demethylating methanotrophs (Lee et al., 2018 [↗](#)). Among them, a putative operon that contains five genes was found to localize next to three sterol biosynthesis genes. Two (*sdmA/sdmB*) have been characterized as demethylases involved in sterol demethylation at the C-4 position. Homologs of the other three hypothetical genes we have named *bstA*, *bstB* and *bstC* (bacterial sterol transporter), are found in all other aerobic methanotroph genomes that contain *sdmA/sdmB*. In some of these species, these three genes are also seen to be adjacent to other sterol synthesis genes such as the oxidosqualene cyclase (*osc*) and squalene monooxygenase (*smo*), implying they may be functionally related to sterol physiology (Fig. 1B [↗](#)).

To obtain additional insights into the proteins encoded by these genes, we performed more refined bioinformatic analyses including protein sequence similarity network (SSN) (Atkinson et al., 2009 [\[4\]](#)) and structure prediction with Phyre2 (Kelley et al., 2015 [\[5\]](#)) and I-TASSER (Yang et al., 2015 [\[6\]](#)). BstA is predicted to belong to the Resistance Nodulation Division (RND) superfamily of transporters, which comprises inner membrane proteins that facilitate drug and heavy metal efflux, and protein secretion, amongst others. They are widespread in gram negative bacteria, but homologs are also found in archaea and eukaryotes. Interestingly, the human Newman Pick Type C1, a transporter of sterols, also contains a transmembrane RND domain (Nikaido, 2018 [\[7\]](#)). The SSN of this superfamily shows that BstA is grouped into a single cluster containing 5 nodes (where sequences with >50% identity are merged into a single node). Structural prediction shows that BstA shares a high homology with a membrane-bound hopanoid transporter, HpnN (PDB 5KHS, *Burkholderia multivorans*), a RND transporter that moves hopanoids from the cytoplasm or cytoplasmic membrane (Doughty et al., 2011 [\[8\]](#)). In the SSN, HpnN is clustered separate from BstA, suggesting a possible functional divergence (**Fig. 2A** [\[4\]](#), Table S1).

BstB belongs to a family of periplasmic/substrate binding proteins (SBPs/PBPs) known to traffic bacterial metabolites such as phosphonate. BstB proteins are grouped into a single cluster consisting of 16 sequences, with most of the sequences belonging to the *Methylococcaceae* family. Notably, the BstB proteins cluster separately even under stringent cutoffs of sequence identity (as low as 20%), hinting at a distinction of function compared with homologs (**Fig. 2B** [\[4\]](#), Table S1). Structure prediction of BstB shows homology to PhnD (PDB 3QK6), a periplasmic solute-binding protein of the phosphonate uptake system in *E. coli* (Alicea et al., 2011 [\[9\]](#)). All structurally characterized PhnD homologs are distributed into different clusters in the SSN.

Finally, BstC is predicted to be a T-component of the tripartite ATP-independent periplasmic component superfamily, which comprises periplasmic lipoproteins implicated in the cytoplasmic import of small molecules. The SSN of BstC reveals that the protein and its closest homologs are also grouped into a separate cluster even under a sequence identity cutoff of 20%. The cluster consists of 13 sequences, with most from *Methylococcaceae* and *Deltaproteobacteria* families. At the time of analysis, only one protein in the entire superfamily had structural or biochemical information: Tp0956 from *Treponema pallidum* (PDB 3U64) is found in the second largest cluster of the network (**Fig. 2C** [\[4\]](#), Table S1). Compared with the SSNs of BstA and BstB, that of BstC shows the family is predominantly bacterial, with only 1 and 5 sequences from eukaryotes and archaea, respectively (**Fig. 2A-C** [\[4\]](#)).

In summary, sequence and predicted structural homology analyses reveal BstA, BstB, and BstC to be highly similar to transporters involved in disparate bacterial transport systems. However, their separation into distinct clusters in the SSNs imply their functions, including potential substrates, are not identical to their homologs. Because the Bst genes always co-localize with sterol synthesis genes (*osc*, *sdmA* and *sdmB*) in methanotrophs that produce sterols, we hypothesize that BstA/BstB/BstC represent a novel transport system for sterols in bacteria (**Fig. 1B** [\[4\]](#) and **Fig. 2D** [\[4\]](#)).

Sterol interaction with transporters

To determine whether BstA, BstB, and BstC are sterol transporters, we first attempted gene deletion in *M. capsulatus* (data not shown). The deletion of these genes, along with others that are required for sterol synthesis (e.g., *osc*) were not successful, hinting that sterol synthesis and proper localization might be essential in this organism. Because these proteins share homology with well-known bacterial transporters, we reasoned that they are transporters and focused on determining their substrate preference by conducting pull-down assays to assess the binding of sterols from *M. capsulatus* lipid extracts. In these studies, an excess of recombinantly produced pure proteins was incubated with either the total lipid extract (TLE) from *M. capsulatus* or a specific polar fraction enriched for native hydroxy-lipids/sterols (HS). Using the HisPur Ni-NTA resin, proteins were isolated from the protein-lipid mixtures, and the protein-bound lipids were extracted and identified by GC-MS. In the presence of the TLE and HS fraction, BstA^{PD} (the periplasmic domain of

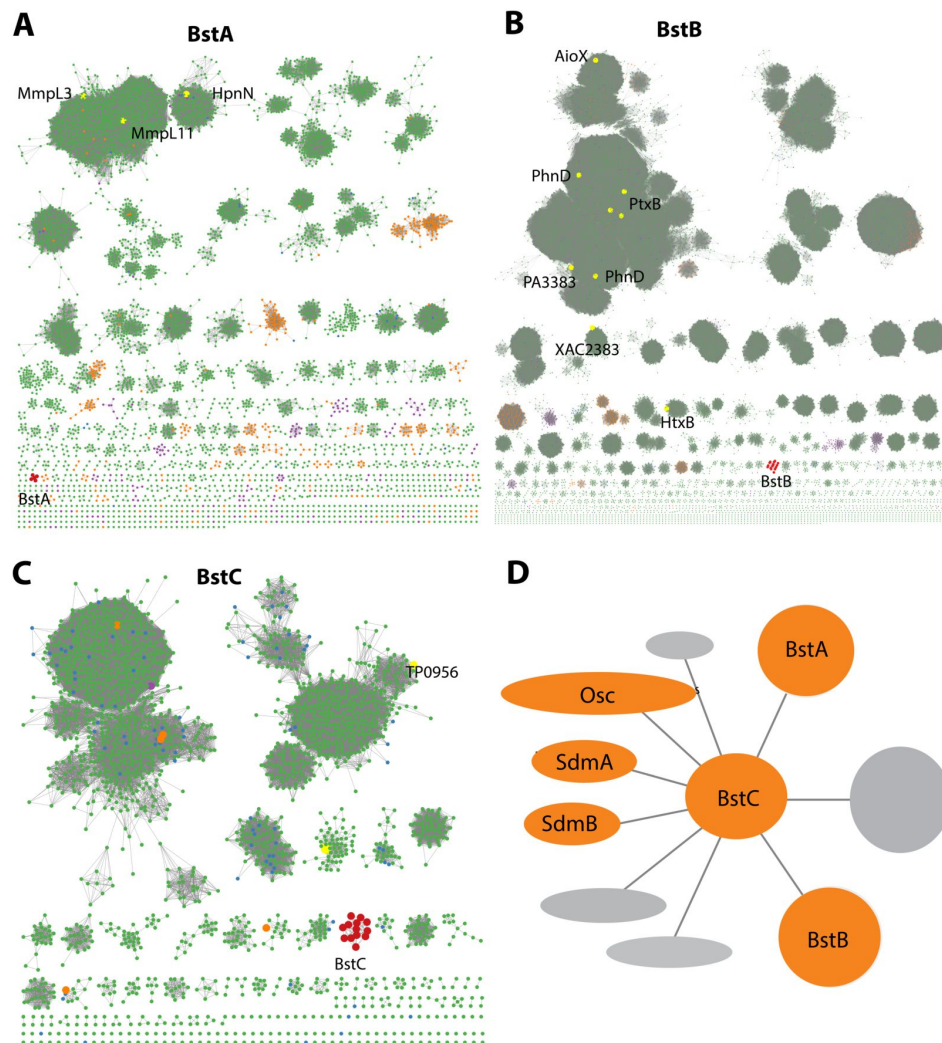


Figure 2.

Bioinformatic analysis of Bst proteins. **(A-C)**: Protein sequence similarity networks (SSN) of BstA, BstB and BstC with the cutoff of 35%, 40%, and 40% sequence identity, respectively. Taxonomic distribution of Bst protein families showing their presence in bacterial (green), eukaryotic (purple), and archaeal (orange) genomes. **(D)** Genome neighborhood network of Bst proteins. Shown in circles and ovals are genes within 10 genes of BstC; colored in orange are those with identified or predicted roles in sterol synthesis and trafficking.

BstA; details in method), BstB, and BstC bound to 4-monomethyl and 4,4-dimethyl sterols (Fig. 3A, Fig. S1). In contrast, neither the 4-monomethyl sterol, the 4,4-dimethyl sterol, nor any other lipids were present when the proteins were incubated with the DMSO negative control, suggesting that the recombinantly produced proteins did not co-purify with any lipids from the expression host. These results indicate that BstA^{PD}, BstB, and BstC preferentially bind C-4 methylated sterols in a mixture of native *M. capsulatus* sterols, hopanoids, and fatty acids.

Quantitative analyses of protein-sterol interactions

The data from the pull-down assays demonstrated that C-4 methylated sterols have affinity for these putative sterol transporters. We next used microscale thermophoresis (MST) to determine the equilibrium binding affinities for these substrates. Binding curves were generated upon titration of the labeled proteins with serially diluted sterol substrates, and the equilibrium dissociation constants were calculated by fitting the curves with different kinetic models (Fig. 3B, methods). The “ K_d ” model applies to binding events with a single binding site or multiple independent binding sites, while the Hill model is invoked in instances where multiple binding sites that exhibit cooperativity are present, with Hill coefficients (n_H) greater than 1 signaling positive cooperativity. The average equilibrium dissociation (K_d) constant for BstA^{PD} binding to 4-monomethyl sterol was determined to be $4.41 \pm 0.14 \mu\text{M}$ ($n_H = 4.0$) and $\geq 203 \pm 155 \mu\text{M}$ for the interaction with 4,4-dimethyl sterol. For BstB, the K_d value of $2.43 \pm 0.13 \mu\text{M}$ (n_H of 2.4) was determined for 4-monomethyl sterol; and $\geq 232 \pm 84.3 \mu\text{M}$ for 4,4-dimethyl sterol. With BstC, the determined values were $2.50 \pm 0.39 \mu\text{M}$ and $\geq 80.9 \pm 14.2 \mu\text{M}$ for 4-monomethyl sterol and 4,4-dimethyl sterol, respectively. All K_d values for the 4,4-dimethyl sterol could not be accurately determined due to large errors caused by non-saturation even at the highest substrate concentration. In all three proteins, we measured clear binding to C-4 methylated sterols, with marked preference for the 4-monomethyl substrate. Additionally, BstA^{PD} and BstB exhibit cooperativity for binding to 4-monomethyl substrate, indicating that multiple binding sites are plausible.

To further define the specificity of Bst proteins for sterols, we again used MST to detect their interactions with cholesterol and lanosterol, a lipid and precursor that differ in their methylation at the C-4 and C-14 positions, as well as their unsaturation patterns in the core ring structure. The MST results revealed no interaction between BstA^{PD} and BstB with either cholesterol or lanosterol. However, BstC did bind to cholesterol with a K_d value of $3.6 \pm 0.64 \mu\text{M}$ and to lanosterol with a value of $17.3 \pm 3.92 \mu\text{M}$ (Fig. S2). These data hint that BstC is more tolerant to non-native substrates.

Crystallographic structure of BstB

To understand the molecular details that govern sterol recognition and binding, we crystallized BstB and obtained crystals of the apo form (Table S2). The 1.6 Å-resolution structure of was determined by experimental phasing, producing electron density that allowed the unambiguous building of a model of BstB (except for one disordered loop spanning residues 202-204). The structure comprises two globular α/β domains (Fig. 4A, domains A and B) that form a cleft at the middle. Domain A contains six β -strands while domain B contains five. All the β -strands are flanked by α -helices. The domains are connected by two loops: one 9-residue loop that runs from $\beta 4$ in domain A to $\beta 5$ in domain B, and a second 8-residue loop that connects $\beta 9$ in domain B and $\beta 10$ in domain A. These two long loops could work as a hinge to allow a bending motion of two domains to induce a conformational change upon substrate binding that is often observed with SBPs/PBPs (Boer et al., 2019). In addition, an α -helix formed by the C-terminal residues lies behind the cleft and may also allow flexibility of the two domains. Several extend loops are found on the domain-domain interface. The loops (residues 34-38 and residues 171-177) exhibit poor electron densities, suggesting conformational sampling of more than one state (Fig. S3A).

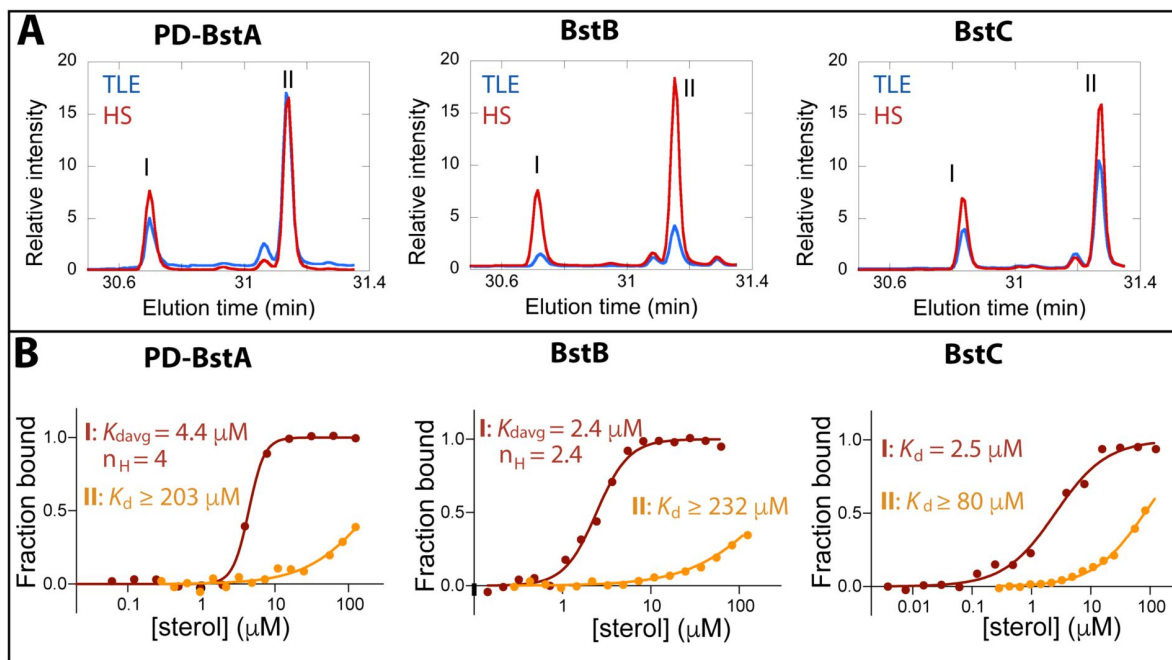


Figure 3.

Binding of C-4 methylated sterols to transporter proteins.

(A) GC chromatograms of sterols bound to purified (40 μM) *M. capsulatus* BstA^{PD}, BstB, and BstC during protein-lipid pull-down assays. The proteins preferentially bind 4-monomethyl (I) and 4,4-dimethyl (II) sterols when incubated with the total lipid extract (TLE) and hydroxy lipid (HS) fraction from *M. capsulatus*. (B) MST analysis of the proteins (50 nM) binding to C-4 methylated sterols (3.8 nM -1125 mM). Equilibrium dissociation constants (K_d) and Hill coefficients (n_H) are reported on the plots.

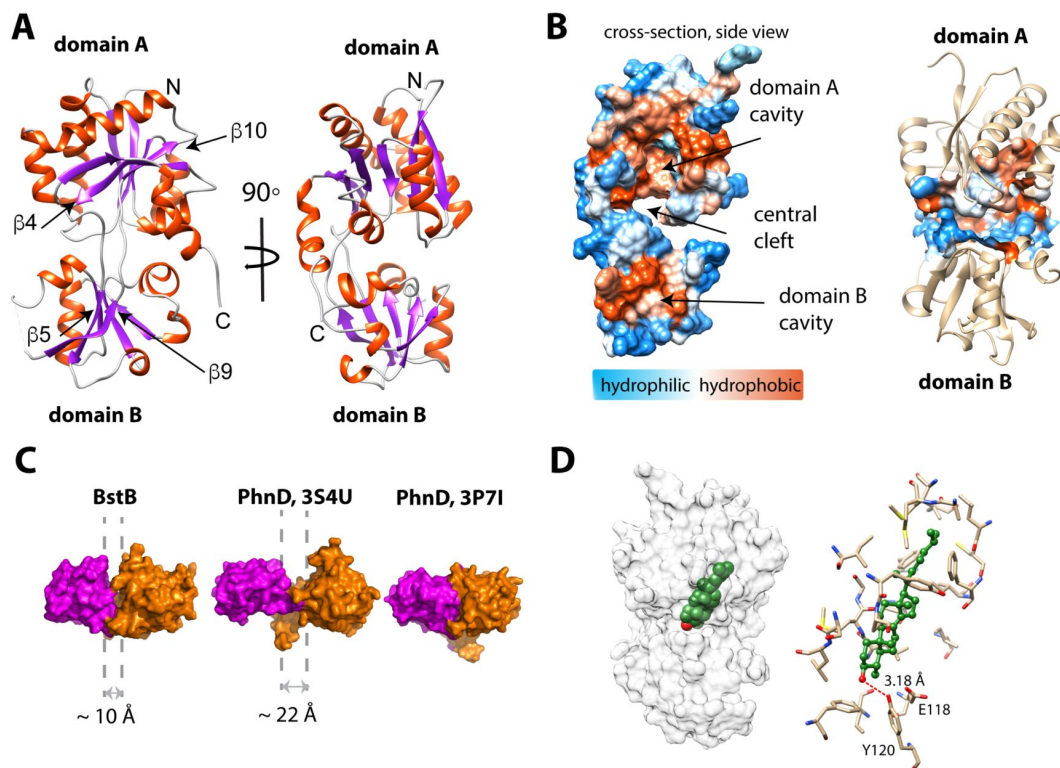


Figure 4.

Structure of BstB.

(A) Cartoon representation of BstB with the α -helices and β -strands colored in red and magenta, respectively. (B) Hydrophobicity representations of BstB show a cleft in the middle and a cavity in domain A. The cavity is dominantly hydrophobic and open only to one side. (C) Comparison of the cleft between the two domains of BstB, apo-PhnD (3S4U), and liganded PhnD (3P71). BstB displays a similar but narrower opening (~ 10 Å) compared to non-ligand PhnD (~ 22 Å). (D) Docking model of the structures of BstB with 4-monomethyl sterol. **Left**, the docking model is shown in surface representation with 80% transparency. Sterol is shown in sphere and colored in green with the hydroxyl group is colored in red; **Right**, docking complex of *Mono*_A. The surrounding residues are shown in stick and ball. Hydrogen bonds between the hydroxyl group of the sterol and Tyr120 is shown in the red dashed line.

The cleft between domains A and B forms a bulky cavity with an area of $\sim 1179.5 \text{ \AA}^2$ and a volume of $\sim 1009.6 \text{ \AA}^3$ that extends into domain A and is only accessible to solvent from one side of the structure. The cavity is predominantly hydrophobic, suggesting large and hydrophobic substrates like sterols are favorable for binding (Fig. 4B, Table S4). Additionally, a second pocket (area of $\sim 172 \text{ \AA}^2$; volume of $\sim 117 \text{ \AA}^3$) is found in domain B and close to the central cleft. This pocket is localized behind the unmodeled loop (residues 202-204), with the extremely poor density in this part implying high flexibility. This pocket is highly hydrophobic, but its role (if any) in ligand binding is unexplored (Fig. 4B; Fig. S4). Other separate hydrophobic areas are also observed on the protein surface and a new hydrophobic interface could be formed by two hydrophobic areas across the cleft upon conformational change induced by substrate binding (Fig. S4). These areas may be helpful for mediating the protein-protein interactions with BstA or BstC to facilitate the substrate transfer.

A DALI structural homology search (Holm and Rosenström, 2010) suggests the structure of BstB closely resembles that of PBPs that share a bi-lobe architecture and undergo conformational change upon substrate binding to the central cavity (Quiocho and Ledvina, 1996). A comparison of the BstB structure with the phosphonate-binding protein PhnD from *E. coli* with and without its substrate (2-aminoethyl phosphonate, 2AEP, PDB 3P7I and 3S4U) was performed (Alicea et al., 2011). The comparison revealed that BstB adopts an intermediate conformation between the unliganded PhnD (3S4U) and liganded one (3P7I, Fig. 4C). The cleft in BstB is less open ($\sim 10 \text{ \AA}$) than that in apo-PhnD ($\sim 22 \text{ \AA}$). There are several possible explanations for this: 1) compared with apo-PhnD structure, the cleft of BstB is deeper and more hydrophobic. Given that BstB binds to sterols (which have a nearly planar architecture and are more hydrophobic than phosphonates), the different architecture of the cleft in BstB may be related to its functional role; 2) it is known that binding of non-substrate ligands can at times trigger a conformational change in PBPs (Boer et al., 2019). Although no obvious ligand density is observed in the BstB structure, weak unmodeled densities were observed inside the central cavity. Most of these are small patches that cannot be satisfactorily modeled with water and do not closely correspond to any reagent used for crystallization. One of these patches in the central cleft could be modeled with a chloride ion engaging in polar interactions with the surrounding Glu118, Tyr120 and Phe150. Additionally, more unmodeled positive density is observed in further into the cavity in domain A (Fig. S4B-C). It is plausible that the binding of ligands co-purified with the protein triggered a conformational change leading to a “partially-open” state. A third possibility is that BstB is structurally distinct from the PhnD homologs in that its cleft is much smaller.

Substrate docking and molecular dynamics simulation of BstB

To obtain greater insights into the mechanism of substrate recognition and binding in the absence of a substrate-bound structure, we used ICM-Pro (Abagyan et al., 1994; Abagyan and Totrov, 1994) to generate docking models of C-4 methylated sterol binding to BstB. A three-dimensional model of 4 α -monomethyl sterol was constructed from the structure of cholesterol using the Molecular Editor in ICM-Pro and then docked to the BstB structure. In the first iteration, the protein was held rigid, and the ligand allowed to dock flexibly. In this docking model, the sterol was positioned in the hydrophobic cavity in domain A with the polar head pointing toward the central cleft and domain B, adjacent to three residues: Glu118, Tyr120 and Asn192. In subsequent replicate docking iterations, the side chains of these three residues were allowed to be flexible along with the ligand. There are three energetically favored poses: two (*Mono_A* and *Mono_B*) form a single hydrogen bond to Tyr120, while pose *Mono_C* where the hydroxyl makes two hydrogen bonds to Tyr120 and Asn192 (Fig. 4D and Figs. S5A-C, Table S3).

To probe the dynamics of the BstB structure during substrate binding, we performed multiple 20 ns molecular dynamics (MD) simulations using Desmond (Schrodinger) on each docked poses for monomethyl sterol from ICM-Pro. In all cases, analysis of the resultant MD trajectories indicated that the simulations became stable after the first few picoseconds. In the three poses where there was initially an interaction with Tyr120, that interaction is lost and replaced with a new hydrogen

bond with Glu118, which persists for 85%-95% of the simulation. Pose *Mono_C* also has an intermittent interaction with Asn192 for approximately 11% of the simulation. In all simulations, whether there is a hydrogen bonding interaction with the ligand or not, the amide group of Asn192 is flipped.

To better probe the protein's substrate preference, docking and MD simulations with the 4,4-dimethyl sterol were also generated. In these models, the 4,4-dimethyl sterol also docks to the cavity in domain A with three poses of roughly equal energies (Figs. S5D-F, Table S3). In one (pose *Di_A*), hydrogen bonds to Glu118, Tyr120 and Asn192 are possible; in a second (*Di_B*), hydrogen bonds to Glu118 and Tyr120 are possible; the third (*Di_C*) has a hydrogen bond to Tyr120 only. The 4,4-dimethyl sterol showed interesting behavior during the 20 ns simulations. Pose *Di_A*, which had three hydrogen bonds after ICM-Pro docking, retained all three interactions (although those with Glu118 and Tyr120 are intermittent and persist for only 12% of the simulation). Pose *Di_B* retained both interactions from the docking model, although the hydrogen bond to Tyr120 was only present for 10% of the simulation. The third pose, *Di_C*, showed the largest structural changes, losing the one interaction it had with Tyr120. In this pose, the head group of the sterol moved out of the binding pocket delineated by Glu118, Tyr120 and Asn192 and a new hydrogen bond with the carbonyl oxygen of Pro34 formed, persisting for the duration of the simulation (Table S3). It is possible that these differences, although subtle, explain the reduction in affinity for the dimethyl sterol substrate.

The aliphatic tails of all docked sterols reside in a hydrophobic cavity in domain A lined by the side chains of Tyr33, Pro34, Ala46, Met47, Met50, Ile89, Ser91, Leu92, Pro110, Ile112, Leu219, Met220, Val222, Leu244, Leu253, Cys254, Phe257, Ile259 and Phe262 in domain A. Sequence alignment shows that these residues are highly conserved between proteins in the same SSN cluster, implying their significance in forming the substrate binding pocket. Mutagenesis to remove or reduce the potential interactions with the polar head and the aliphatic tail showed subtle influences (<2-fold) on the sterol binding affinity, indicating that the interaction between the protein and sterol is extensive and principally depends on hydrophobic interactions that are not easily disrupted (Fig. S7). Moreover, the conformational dynamics of PBPs could mask the identity of additional residues that might be involved substrate binding and stabilization. Regardless, the determination of the sterol binding site in BstB remains to be experimentally verified.

To investigate whether BstB can undergo conformational change (as most PBPs do upon substrate binding), a 150 ns molecular dynamics simulation on apo-BstB was performed. Plotting the *RMSD* of the simulated structure against the initial crystal structure at 25 ps intervals during the MD trajectory shows that for the first ~90 ns the protein showed some degree of flexibility (Fig. S8A). During this part of the trajectory, the *RMSD* oscillates between 2.0 to 3.1 Å (average *RMSD* = 2.6 Å). At ~90 ns, there was a distinct conformational change in the structure giving rise to conformations with *RMSDs* ranging from 2.7 to 4.5 (average *RMSD* = 3.3 Å). Visual analysis of the MD trajectory shows that at ~90 ns the two domains open by approximately 20° (Fig. S8B, Movie 1). In contrast, the simulation (150 ns) on the *Mono_C* docked complex which had a *RMSD* of 2.6 Å for the entire length of the trajectory and did not show the same conformational change as the apo-BstB simulation, which supports the idea that the apo-BstB structure represents a “partially-open” form of the protein (Movie 2).

Crystallographic structure of BstC

The structure of BstC was also determined by experimental phasing to a resolution of 1.9 Å. Two monomers (*RMSD* of 0.656 Å for all Cαs) in the asymmetric unit form a dimer with 180° rotational symmetry. Each monomer adopts an all-α-helical structure with 12 α-helices in total, designated with H1 to H12; 9 antiparallel arranged helices (H4-H12) form an irregular ring with a pore

running through the center. The H4, H6, H8, H10, H12 constitute the inside wall of the pore, while the others form the outside wall. Two helices (H1-H2) at the N-terminus sit on the side-top of the ring with a short helical turn (H3) connecting the two (**Fig. 5A** [↗](#)).

Hydrophobicity analysis shows that BstC has a predominantly hydrophilic exterior surface (Fig. S9A). Three cavities are found in the center of the structure. We annotated them as Tunnel 1 (TN1), Cavity 1 (CA1) and Cavity 2 (CA2), with total area of $\sim 597.1 \text{ \AA}^2$ and total volume of $\sim 447 \text{ \AA}^3$. TN1 is closer to the N-terminal face of the structure, while CA1 and CA2 are proximal to the C-terminal face and form two open hydrophobic pockets; TN1 exhibits a mixture of hydrophobic and hydrophilic amino acids (**Fig. 5B** [↗](#) and Fig. S8B, Table S4). The extensive hydrophobic environment inside the BstC structure suggests it can accommodate large hydrophobic substrates. Notably, electrostatic calculation shows that most of the surface of BstC, especially the C-terminal face, exhibits strong negative charge (Fig. S9). These charged surfaces may mediate protein-protein interactions, perhaps with BstB.

A structure similarity search using the DALI server revealed high homology to Tp0956 (PDB 3U64, *Treponema pallidum* strain Nichols). Tp0956 is the T-component protein of a subfamily of TRAP (tripartite ATP-independent periplasmic) transporters known as TPATs (tetratricopeptide repeat-protein associated TRAP transporters), which are implicated in the transport of organic acid ligands in bacteria (Brautigam et al., 2012 [↗](#)). The true physiological substrate of Tp0956 has not been determined, but *Treponema* is a spirochete known to rely on import of host lipids and fatty acids to construct its cell envelope (Radolf et al., 2016 [↗](#)). Tp0956 contains four helical hairpins that are similar to tetratricopeptide repeat (TPR) motifs, which are hallmarks of proteins that are involved in protein-protein interactions (D'Andrea and Regan, 2003 [↗](#)). Comparison of the structures show that two antiparallel α -helices exist in Tp0956 are missing in the structure of BstC (between H5-H6), as well as a shorter H4 and H5. But the TPR-like substructure is also observed in BstC and forms part of the pore and lateral surface of the protein, hinting that BstC might engage with itself or other proteins (Fig. S10). Size-exclusion chromatography coupled with multiangle light scattering (SEC-MALS) analysis shows that indeed the purified BstC exists both as a monomer and a dimer in solution with a ratio $\sim 3:1$ (Fig. S11). However, the role of the dimerization in this protein is not clear and its determination will require additional studies.

Substrate docking and molecular dynamics simulations with BstC

To assess substrate-binding, we performed the docking of 4-monomethyl sterol and 4,4-dimethyl sterol into the BstC structure using ICM-Pro. The final docking model was obtained by choosing the one with the most negative ICM-Pro score. All the sterols were found embedded in the hydrophobic cavity (CA1) lined by Gly128, Val131, Arg132, Met135, Ala138, Leu139, Leu142, Leu145, Leu167, Met170, Leu171, Lys174, Ala175, Pro176 and Phe208, with the hydroxyl group pointing inwards and the aliphatic tail pointed out (**Fig. 5C** [↗](#) and Fig. S12A-B). Residues involved in sterol interaction are located on H6, H7, H8 and H10 and are all highly conserved in a sequence alignment of the closest BstC homologs. No hydrogen bond is anticipated between the sterol and protein in the docking models.

We then performed multiple 20 ns molecular dynamics simulations on the docked models of 4-monomethyl sterol from ICM-Pro. The simulations reveal that the substrate moves approximately 8-9 Å further into the binding site and the polar head makes a hydrogen bonding contact with the side chain of Glu86 (**Fig. 5C** [↗](#)). This movement takes place almost immediately and the hydrogen bond persists for 94% of the total time. In both the docking models and MD simulations, the sterol is positioned near the entrance of CA1 from the C-terminal side. Next to the entrance is an extended loop that consists of highly conserved hydrophobic and neutral residues (residues 175-184).

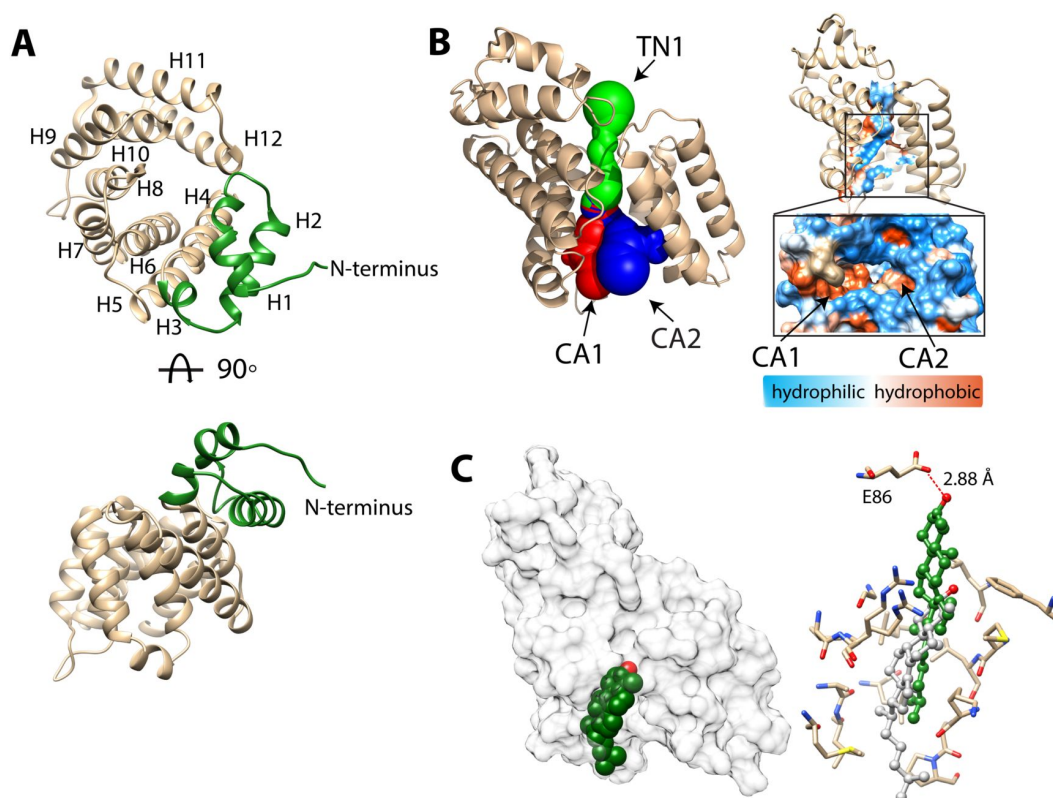


Figure 5.

Structure of BstC.

(A) Cartoon representation of BstC. The two α helices at the N-terminus are colored in green. Two monomers (RMSD of 0.656 Å for all C α s) with 180° rotational symmetry exist in the asymmetric unit. (B) A representation of cavities BstC (left, with Tunnel 1 colored in green, Cavity 1 in red, and Cavity 2 in blue) alongside hydrophobicity representation of the cavities (right). (C) Docking model of the structures of BstC with 4-monomethyl sterol. **Left**, the docking model is shown in surface representation with 80% transparency. Sterol is shown in sphere and colored in green with the hydroxyl group is colored in red; **Right**, docking complex before and after MD simulation. The surrounding residues are shown in stick and ball. The sterol prior to the MD simulation is colored in grey and while green shows its position after the simulation. A hydrogen bond between the hydroxyl group of the sterol and Glu86 is shown as a red dashed line.

Intriguingly, simulations with the 4,4-dimethyl sterol revealed that it behaves differently in that it moves slowly inwards and does not get as deep (~ 2.6 Å) into the pocket as the monomethyl sterol (Fig. S12C). A water-mediated contact is formed between the polar head and the side chain of Glu86 but persist for only 2% of the time. This lack of additional movement and H-bonding interaction may explain the binding data, where the protein has a K_d for the monomethyl sterol that is $\sim 30\times$ lower than for the dimethyl sterol substrate.

Mutagenesis to convert Glu86 to Gly and Trp to remove or reduce the potential interactions with the polar head of the sterols leads to ~ 4.5 - and 100-fold decrease in the equilibrium affinity for 4-monomethyl sterol (Fig. S143A). Meanwhile, the mutagenesis eliminates the binding to 4,4-dimethyl sterol, cholesterol and lanosterol (Fig. S13B,C). These findings indicate that Glu86 plays a critical role in interacting with and stabilizing the sterols.

Comparison to eukaryotic sterol transporters

In most eukaryotic cells, lipid transfer proteins (LTPs) are used to transfer sterols between subcellular membranes by non-vesicular transport to maintain sterol homeostasis in different subcellular organelles. (Baumann et al., 2005 [DOI](#)) The most-studied family in mammals is the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain (STARD) proteins. All fifteen STARD proteins identified to date possess lipid-harboring START domains that can be divided into six subfamilies based on sequence similarity and ligand specificity. In addition to STARD proteins, a novel yet evolutionarily conserved family that contain the START-like domains called the GRAMD1s/Lam/Ltc family (GRAMD1s in mammals and Lam/Ltc in yeast) was shown to mediate non-vesicular sterol transport from the plasma membrane to the endoplasmic reticulum to maintain sterol homeostasis (Gatta et al., 2015 [DOI](#)). A third family consists of the Osh/ORP/OSBP proteins (Oxysterol-binding homology in yeast and Oxysterol-binding Protein/OSBP-Related Protein in mammals), which are considered to be either sterol-sensing and/or sterol-transfer proteins (Raychaudhuri and Prinz, 2010 [DOI](#)). Besides these, two Niemann-Pick type C proteins, NPC1 and NPC2, were also reported to directly bind and transport cholesterol and various oxysterols; defective NPC proteins causes NPC disease, a fetal neurodegenerative disorder where sterols aberrantly accumulate in the lysosome (Carstea et al., 1997 [DOI](#)).

To better understand the differences between eukaryote and bacteria sterol transport, we compared the structures of BstB and BstC with well-studied eukaryotic sterol transporters from different families. Human STARD4 (PDB 6L1D), yeast Lam4 (PDB 6BYM), yeast Osh4 (PDB 1ZI7 and 1ZHY), yeast NPC2 (PDB 6R4M and 6R4N), as well as a non-specific cytosolic sterol carrier protein, rabbit SCP2 (PDB 1C44) were chosen for the comparison. The crystal structures of STARD4, Lam4 and Osh4 show that they share a similar fold in that they are dominated by β -sheet motifs, which wrap around the longest α -helix and fold into a half-barrel/groove structure with the other side (or two) packed with several α -helices (Fig. S14A-D). A central hydrophobic cavity can accommodate sterols. In most cases, the sterols are positioned proximal to the tunnel entrance with the hydroxyl end oriented towards the inside of the tunnel. Also, a “lid” comprising a flexible “Q1” loop is observed near the entrance of tunnel in STARD4 and sterol-bound Lam4. This loop is replaced by an N-terminal α -helix in Osh4, which serves a similar function. The SCP2 structure also exhibits an α/β -fold with a “lid”-like α -helix observed close to the N-terminus (Fig. S14E). The sterol-bound NPC2 adopts a different immunoglobulin-like β -sandwich fold with seven strands arranged into two β -sheets with a loosely packed hydrophobic cavity formed between the two sheets. The sterol is positioned at the entrance of the cavity with the hydroxyl end pointing out; no “lid”-like structure is observed in NPC2 structure (Fig. S14F-G).

Our comparative analysis shows that the eukaryotic sterol transporters share very little resemblance with BstB and BstC. The bilobed architecture of BstB is pronounced in its divergence from eukaryotic sterol transporters, suggesting that this protein evolved separately and may work in a distinct manner. For the all-helical BstC, the only similarities are the presence of a

hydrophobic tunnel formed in the center of the protein that can accommodate sterols, and a loop next to the tunnel's entrance that may also be used as a “lid” to restrict access to the substrate binding site (Fig. S14H).

Discussion

Since their discovery more than 40 years ago, the function of bacterial sterols has remained a mystery. Recent identification of bacterial sterol demethylase enzymes that diverge from their eukaryotic counterparts provided renewed enthusiasm for deciphering the molecular machinery involved in bacterial sterol synthesis and trafficking (Lee et al., 2018 [DOI](#)). The aerobic methanotroph *M. capsulatus* is known to synthesize 4-monomethyl sterol from 4,4-dimethyl sterol and to accumulate these sterols in its outer membrane. Until now, the transport process remained elusive owing to lack of knowledge of transporters that facilitate the trafficking. We now fill this gap with the identification of potential transporters of C-4 methyl sterols in *M. capsulatus*: BstA, BstB and BstC.

Bioinformatic analyses revealed that BstA, BstB, and BstC belong to well-studied families of bacterial metabolite transporters. High resolution structures of BstB and BstC show that their large ligand binding sites display a dichotomy of hydrophobic and hydrophilic amino acids whose sidechains interact with the sterol hydrophobic tail and hydrophilic head. For BstC, this is especially interesting, as the only other family member characterized, Tp0956 from *Treponema pallidum*, displays a similar dichotomous ligand site. *Treponema pallidum*, the causative agent of syphilis, is a spirochete known to acquire and use host lipids— it is possible that Tp0956, and perhaps other T-component proteins, are involved in lipid trafficking.

In summary, the Bst proteins share sequence and structural homology with bacterial transporters involved in the trafficking of essential nutrients such as sugars, amino acids, vitamins, solutes, and metal ions (Davidson et al., 2008 [DOI](#); Higgins, 1992 [DOI](#)). Their implication in the transport of sterols expands the substrate repertoire for their respective families. The substrate binding sites detected in BstB and BstC should now enable the curation of functionally homologous sites in bacterial proteins, including in those from human pathogens known to traffic host lipids.

Materials and methods

Detailed materials and methods describing bioinformatic analyses are provided in an extended materials and methods section of the Supporting Information. Also provided are methods for recombinant protein production and purification, lipid extraction from *M. capsulatus*, protein-lipid pull-down assays and chromatographic analyses, MST experiments, macromolecular crystallography, molecular docking, and simulations.

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References

- Abagyan R, Totrov M (1994) **Biased Probability Monte Carlo Conformational Searches and Electrostatic Calculations for Peptides and Proteins** *J Mol Biol* **235**:983–1002 <https://doi.org/10.1006/jmbi.1994.1052>
- Abagyan R, Totrov M, Kuznetsov D (1994) **ICM—A new method for protein modeling and design: Applications to docking and structure prediction from the distorted native conformation** *J Comput Chem* **15**:488–506 <https://doi.org/10.1002/jcc.540150503>
- Abe I (2014) **Natural Products: Discourse Diversity, and Design** :293–316 <https://doi.org/10.1002/9781118794623.ch16>
- Alicea I, Marvin JS, Miklos AE, Ellington AD, Looger LL, Schreiter ER (2011) **Structure of the Escherichia coli Phosphonate Binding Protein PhnD and Rationally Optimized Phosphonate Biosensors** *J Mol Biol* **414**:356–369 <https://doi.org/10.1016/j.jmb.2011.09.047>
- Atkinson HJ, Morris JH, Ferrin TE, Babbitt PC (2009) **Using Sequence Similarity Networks for Visualization of Relationships Across Diverse Protein Superfamilies** *Plos One* **4** <https://doi.org/10.1371/journal.pone.0004345>
- Bacia K, Schwille P, Kurzchalia T (2005) **Sterol structure determines the separation of phases and the curvature of the liquid-ordered phase in model membranes** *P Natl Acad Sci Usa* **102**:3272–3277 <https://doi.org/10.1073/pnas.0408215102>
- Baumann NA, Sullivan DP, Ohvo-Rekilä H, Simonot C, Pottekat A, Klaassen Z, Beh CT, Menon AK (2005) **Transport of Newly Synthesized Sterol to the Sterol-Enriched Plasma Membrane Occurs via Nonvesicular Equilibration** *Biochemistry-us* **44**:5816–5826 <https://doi.org/10.1021/bi048296z>
- Bi X, Liao G (2010) **Cholesterol in Niemann-Pick Type C disease** *Sub-cell Biochem* **51**:319–35 https://doi.org/10.1007/978-90-481-8622-8_11
- Bird CW, Lynch JM, Pirt FJ, Reid WW, Brooks CJW, Middleditch BS (1971) **Steroids and Squalene in Methylococcus capsulatus grown on Methane** *Nature* **230**:473–474 <https://doi.org/10.1038/230473a0>
- Bloch K (1991) **Chapter 12 Cholesterol: evolution of structure and function** *New Compr Biochem* **20**:363–381 [https://doi.org/10.1016/s0167-7306\(08\)60340-3](https://doi.org/10.1016/s0167-7306(08)60340-3)
- Bode HB, Zeggel B, Silakowski B, Wenzel SC, Reichenbach H, Müller R (2003) **Steroid biosynthesis in prokaryotes: identification of myxobacterial steroids and cloning of the first bacterial 2,3(S)-oxidosqualene cyclase from the myxobacterium Stigmatella aurantiaca** *Mol Microbiol* **47**:471–481 <https://doi.org/10.1046/j.1365-2958.2003.03309.x>
- Boer M, de Gouridis G, Vietrov R, Begg SL, Schuurman-Wolters GK, Husada F, Eleftheriadis N, Poolman B, McDevitt CA, Cordes T. (2019) **Conformational and dynamic plasticity in substrate-binding proteins underlies selective transport in ABC importers** *Elife* **8** <https://doi.org/10.7554/elife.44652>

- Bouvier P, Rohmer M, Benveniste P, Ourisson G (1976) **$\Delta 8(14)$ -steroids in the bacterium *Methylococcus capsulatus*** *Biochem J* **159**:267–271 <https://doi.org/10.1042/bj1590267>
- Brautigam CA, Deka RK, Schuck P, Tomchick DR, Norgard MV (2012) **Structural and Thermodynamic Characterization of the Interaction between Two Periplasmic *Treponema pallidum* Lipoproteins that are Components of a TPR-Protein-Associated TRAP Transporter (TPAT)** *J Mol Biol* **420**:70–86 <https://doi.org/10.1016/j.jmb.2012.04.001>
- Carstea ED *et al.* (1997) **Niemann-Pick C1 Disease Gene: Homology to Mediators of Cholesterol Homeostasis** *Science* **277**:228–231 <https://doi.org/10.1126/science.277.5323.228>
- D’Andrea LD, Regan L (2003) **TPR proteins: the versatile helix** *Trends Biochem Sci* **28**:655–662 <https://doi.org/10.1016/j.tibs.2003.10.007>
- Davidson AL, Dassa E, Orelle C, Chen J (2008) **Structure Function, and Evolution of Bacterial ATP-Binding Cassette Systems.** *Microbiol Mol Biol R* **72**:317–364 <https://doi.org/10.1128/mmmbr.00031-07>
- Desmond E, Gribaldo S (2009) **Phylogenomics of Sterol Synthesis: Insights into the Origin, Evolution, and Diversity of a Key Eukaryotic Feature** *Genome Biol Evol* **1**:364–381 <https://doi.org/10.1093/gbe/evp036>
- Doughty DM, Coleman ML, Hunter RC, Sessions AL, Summons RE, Newman DK (2011) **The RND-family transporter, HpnN, is required for hopanoid localization to the outer membrane of *Rhodopseudomonas palustris* TIE-1** *P Natl Acad Sci Usa* **108**:E1045–51 <https://doi.org/10.1073/pnas.1104209108>
- Gachotte D, Barbuch R, Gaylor J, Nickel E, Bard M (1998) **Characterization of the *Saccharomyces cerevisiae* ERG26 gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) involved in sterol biosynthesis** *Proc National Acad Sci* **95**:13794–13799 <https://doi.org/10.1073/pnas.95.23.13794>
- Gatta AT, Wong LH, Sere YY, Calderón-Noreña DM, Cockcroft S, Menon AK, Levine TP (2015) **A new family of StART domain proteins at membrane contact sites has a role in ER-PM sterol transport** *Elife* **4** <https://doi.org/10.7554/elife.07253>
- He M *et al.* (2011) **Mutations in the human SC4MOL gene encoding a methyl sterol oxidase cause psoriasiform dermatitis, microcephaly, and developmental delay** *J Clin Invest* **121**:976–984 <https://doi.org/10.1172/jci42650>
- Higgins CF (1992) **ABC Transporters: From Microorganisms to Man** *Annu Rev Cell Biol* **8**:67–113 <https://doi.org/10.1146/annurev.cb.08.110192.000435>
- Holm L, Rosenström P (2010) **Dali server: conservation mapping in 3D** *Nucleic Acids Res* **38**:W545–W549 <https://doi.org/10.1093/nar/gkq366>
- Huang P, Nedelcu D, Watanabe M, Jao C, Kim Y, Liu J, Salic A (2016) **Cellular Cholesterol Directly Activates Smoothened in Hedgehog Signaling** *Cell* **166**:1176–1187 <https://doi.org/10.1016/j.cell.2016.08.003>
- Hughes AL, Lee C-YS, Bien CM, Espenshade PJ (2007) **4-Methyl Sterols Regulate Fission Yeast SREBP-Scap under Low Oxygen and Cell Stress*** *J Biol Chem* **282**:24388–24396 <https://doi.org/10.1074/jbc.m701326200>

- Jacquier N, Schneiter R (2012) **Mechanisms of sterol uptake and transport in yeast** *J Steroid Biochem Mol Biology* **129**:70–78 <https://doi.org/10.1016/j.jsbmb.2010.11.014>
- Jahnke LL, Stan-Lotter H, Kato K, Hochstein LI (1992) **Presence of methyl sterol and bacteriohopanepolyol in an outer-membrane preparation from *Methylococcus capsulatus* (Bath)** *Microbiology+* **138**:1759–1766 <https://doi.org/10.1099/00221287-138-8-1759>
- Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE (2015) **The Pyre2 web portal for protein modeling, prediction and analysis** *Nat Protoc* **10**:845–858 <https://doi.org/10.1038/nprot.2015.053>
- Kohl W, Gloe A, Reichenbach H (1983) **Steroids from the Myxobacterium *Nannocystis exedens*** *Microbiology+* **129**:1629–1635 <https://doi.org/10.1099/00221287-129-6-1629>
- König A, Happel R, Bornholdt D, Engel H, Grzeschik K (2000) **Mutations in the NSDHL gene, encoding a 3 β -hydroxysteroid dehydrogenase, cause CHILD syndrome** *Am J Med Genet* **90**:339–346 [https://doi.org/10.1002/\(sici\)1096-8628\(20000214\)90:4<339::aid-ajmg15>3.0.co;2-5](https://doi.org/10.1002/(sici)1096-8628(20000214)90:4<339::aid-ajmg15>3.0.co;2-5)
- Lee AK, Banta AB, Wei JH, Kiemle DJ, Feng J, Giner J-L, Welander PV (2018) **C-4 sterol demethylation enzymes distinguish bacterial and eukaryotic sterol synthesis** *Proc National Acad Sci* **115** <https://doi.org/10.1073/pnas.1802930115>
- Lee AK, Wei JH, Welander PV (2023) **De novo cholesterol biosynthesis in bacteria** *Nat Commun* **14** <https://doi.org/10.1038/s41467-023-38638-8>
- Lim L, Jackson-Lewis V, Wong LC, Shui GH, Goh AXH, Kesavapany S, Jenner AM, Fivaz M, Przedborski S, Wenk MR (2012) **Lanosterol induces mitochondrial uncoupling and protects dopaminergic neurons from cell death in a model for Parkinson's disease** *Cell Death Differ* **19**:416–427 <https://doi.org/10.1038/cdd.2011.105>
- McLarren KW *et al.* (2010) **Hypomorphic Temperature-Sensitive Alleles of NSDHL Cause CK Syndrome** *Am J Hum Genetics* **87**:905–914 <https://doi.org/10.1016/j.ajhg.2010.11.004>
- Miao L, Nielsen M, Thewalt J, Ipsen JH, Bloom M, Zuckermann MJ, Mouritsen OG (2002) **From Lanosterol to Cholesterol: Structural Evolution and Differential Effects on Lipid Bilayers** *Biophys J* **82**:1429–1444 [https://doi.org/10.1016/s0006-3495\(02\)75497-0](https://doi.org/10.1016/s0006-3495(02)75497-0)
- Nes WD, Janssen GG, Crumley FG, Kalinowska M, Akihisa T (1993) **The Structural Requirements of Sterols for Membrane Function in *Saccharomyces cerevisiae*** *Arch Biochem Biophys* **300**:724–733 <https://doi.org/10.1006/abbi.1993.1100>
- Nikaido H (2018) **RND transporters in the living world** *Res Microbiol* **169**:363–371 <https://doi.org/10.1016/j.resmic.2018.03.001>
- Ourisson G, Rohmer M, Poralla K (1987) **Prokaryotic Hopanoids and other Polyterpenoid Sterol Surrogates** *Annu Rev Microbiol* **41**:301–333 <https://doi.org/10.1146/annurev.mi.41.100187.001505>
- Parks LW, Smith SJ, Crowley JH (1995) **Biochemical and physiological effects of sterol alterations in yeast—A review** *Lipids* **30**:227–230 <https://doi.org/10.1007/bf02537825>
- Patt TE, Hanson RS (1978) **Intracytoplasmic membrane, phospholipid, and sterol content of *Methylobacterium organophilum* cells grown under different conditions** *J Bacteriol* **134**:636–644 <https://doi.org/10.1128/jb.134.2.636-644.1978>

- Pearson A, Budin M, Brocks JJ (2003) **Phylogenetic and biochemical evidence for sterol synthesis in the bacterium *Gemmata obscuriglobus*** *Proc National Acad Sci* **100**:15352–15357 <https://doi.org/10.1073/pnas.2536559100>
- Quioco FA, Ledvina PS (1996) **Atomic structure and specificity of bacterial periplasmic receptors for active transport and chemotaxis: variation of common themes** *Mol Microbiol* **20**:17–25 <https://doi.org/10.1111/j.1365-2958.1996.tb02484.x>
- Radolf JD, Deka RK, Anand A, Šmajs D, Norgard MV, Yang XF (2016) **Treponema pallidum, the syphilis spirochete: making a living as a stealth pathogen** *Nat Rev Microbiol* **14**:744–759 <https://doi.org/10.1038/nrmicro.2016.141>
- Rahier A (2011) **Dissecting the sterol C-4 demethylation process in higher plants. From structures and genes to catalytic mechanism** *Steroids* **76**:340–352 <https://doi.org/10.1016/j.steroids.2010.11.011>
- Raychaudhuri S, Prinz WA (2010) **The Diverse Functions of Oxysterol-Binding Proteins** *Annu Rev Cell Dev Bi* **26**:157–177 <https://doi.org/10.1146/annurev.cellbio.042308.113334>
- Ruiz N, Wu T, Kahne D, Silhavy TJ (2006) **Probing the Barrier Function of the Outer Membrane with Chemical Conditionality** *Acs Chem Biol* **1**:385–395 <https://doi.org/10.1021/cb600128v>
- Schouten S, Bowman JP, Rijpstra WIC, Damsté JSS (2000) **Sterols in a psychrophilic methanotroph, *Methylosphaera hansonii*** *Fems Microbiol Lett* **186**:193–195 <https://doi.org/10.1111/j.1574-6968.2000.tb09103.x>
- Serratore ND, Baker KM, Macadlo LA, Gress AR, Powers BL, Atallah N, Westerhouse KM, Hall MC, Weake VM, Briggs SD (2017) **A Novel Sterol-Signaling Pathway Governs Azole Antifungal Drug Resistance and Hypoxic Gene Repression in *Saccharomyces cerevisiae*** *Genetics* **208** <https://doi.org/10.1534/genetics.117.300554>
- Summons RE, Bradley AS, Jahnke LL, Waldbauer JR (2006) **Steroids, triterpenoids and molecular oxygen** *Philosophical Transactions Royal Soc B Biological Sci* **361**:951–968 <https://doi.org/10.1098/rstb.2006.1837>
- Todd BL, Stewart EV, Burg JS, Hughes AL, Espenshade PJ (2006) **Sterol Regulatory Element Binding Protein Is a Principal Regulator of Anaerobic Gene Expression in Fission Yeast†** *Mol Cell Biol* **26**:2817–2831 <https://doi.org/10.1128/mcb.26.7.2817-2831.2006>
- Vance JE, Peake KB (2011) **Function of the Niemann–Pick type C proteins and their bypass by cyclodextrin** *Curr Opin Lipidol* **22**:204–209 <https://doi.org/10.1097/mol.0b013e3283453e69>
- Wei JH, Yin X, Welander PV (2016) **Sterol Synthesis in Diverse Bacteria** *Front Microbiol* **7** <https://doi.org/10.3389/fmicb.2016.00990>
- Xu F, Rychnovsky SD, Belani JD, Hobbs HH, Cohen JC, Rawson RB (2005) **Dual roles for cholesterol in mammalian cells** *Proc National Acad Sci* **102**:14551–14556 <https://doi.org/10.1073/pnas.0503590102>
- Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y (2015) **The I-TASSER Suite: protein structure and function prediction** *Nat Methods* **12**:7–8 <https://doi.org/10.1038/nmeth.3213>

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Reviewer #1 (Public Review):

Summary

This article by Zhai et al, investigates sterol transport in bacteria. Synthesis of sterols is rare in bacteria but occurs in some, such as, *M. capsulatus* where the sterols are found primarily in the outer membrane. In a previous paper the authors discovered an operon consisting of five genes, with two of these genes encoding demethylases involved in sterol demethylation. In this manuscript the authors set out to investigate the functions of the other three genes in the operon. Interestingly, through a bioinformatic analysis they show that they are an inner membrane transporter of the RND family, a periplasmic binding protein and an outer membrane associated protein, all potentially involved with lipid transport, so providing a means of transporting the lipids to the outer membrane. These proteins are then extensively investigated through lipid pulldowns, binding analysis on all three, and X-ray crystallography and docking of the latter two.

Strengths

The lipid pulldowns and associated MST binding analysis are convincing, clearly showing that sterols are able to bind to these proteins. The structures of BstB and BstC are high resolution with excellent maps that allow docking studies to be carried out. These structures are distinct from sterol binding proteins in eukaryotes.

Weaknesses

While the docking and molecular dynamics studies are consistent with the binding of sterols to BstB and BstC, this is not backed up particularly well. Their discussion, however, is measured and clearly provides a strong case for further investigation.

- <https://doi.org/10.7554/eLife.90696.2.sa2>

Reviewer #2 (Public Review):

Summary:

In eukaryotes, sterols are crucial for signaling and regulating membrane fluidity, however, the mechanism governing cholesterol production and transport across the cell membrane in bacteria remains enigmatic. The manuscript by Zhai et al. sheds light on this topic by uncovering three potential cholesterol transport proteins. Through comprehensive bioinformatics analysis, the authors identified three genes *bstA*, *bstB*, and *bstC* encoding proteins which share homology with transporters, periplasmic binding proteins, and periplasmic components superfamily, respectively. Furthermore, the authors confirmed the specific interaction between these three proteins and C-4 methylated sterols and determined the structures of BstB and BstC. Combining these structural insights with molecular dynamics simulation, they postulated several plausible substrate binding sites within each protein.

Strengths:

The authors have identified 3 proteins that seem likely to be involved in sterol transport between the inner and outer membrane. The structures are of high quality, and the sterol binding experiments support a role for these proteins in sterol transport.

Weaknesses:

While the author's model is very plausible, direct evidence for a role of BstABC in transport, or that the 3 proteins function together in a single pathway, is limited.

- <https://doi.org/10.7554/eLife.90696.2.sa1>

Reviewer #3 (Public Review):

Summary:

The work in this manuscript builds on prior efforts by this team to understand how sterols are biosynthesized and utilized in bacteria. The study reports a new function for three genes

encoded near sterol biosynthesis enzymes, suggesting the resulting proteins function as a sterol transport system. Biochemical and structural characterization of the two soluble components of the pathway establishes that both proteins can bind sterols, with a preference for 4-methylated derivatives. High-resolution x-ray structures of the apoproteins reveal hydrophobic cavities of the appropriate size to accommodate these substrates. Docking and molecular dynamics simulations confirm this observation and provide specific insights into residues involved in substrate binding.

Strengths:

The manuscript is comprehensive and well-written. The annotation of a new function in a set of proteins related to bacterial sterol usage is exciting and likely to enable further study of this phenomenon - which is currently not well understood. The work also has implications for improving our understanding of lipid usage in general among bacterial organisms.

- <https://doi.org/10.7554/eLife.90696.2.sa0>

Author Response

The following is the authors' response to the original reviews.

Public Reviews:

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Summary

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The lipid pulldowns and associated MST binding analysis are convincing, clearly showing that sterols are able to bind to these proteins. The structures of BstB and BstC are high resolution with excellent maps that allow docking studies to be carried out. These structures are distinct from sterol-binding proteins in eukaryotes.

We thank the reviewer for their favorable impression of this work.

Weaknesses

While the docking and molecular dynamics studies are consistent with the binding of sterols to BstB and BstC, this is not backed up particularly well. The MST results of mutants in the binding pocket of BstB have relatively little effect, and while I agree with the authors this may be because of the extensive hydrophobic interactions that the ligand makes with the protein, it is difficult to make any firm conclusions about binding.

We agree with the reviewer that at this point, there is no experimental evidence to define the sterol binding site in BstB. While in the manuscript we allude to the extensive hydrophobic interactions as being especially stabilizing and difficult to eliminate with one or two mutations, we are now also aware that hydrogen-bonding interactions with the polar head of the sterols are quite important (see data on BstC, where disruption of that interaction significantly reduces the equilibrium affinity for sterols). Our MD simulations show that at least 3 protein amino acids can participate in H-bonding with the sterols. Moreover, recent work from our lab show that even ligand site waters can extend an H-bonding network around the polar head of the lipid (Zhai et al., ChemBioChem 2023, 24, e202300156), thereby enabling H-bonding with amino acids that are further away from the ligand site. It is therefore difficult to predict which mutations will sufficiently destabilize the binding. While this question is one we will tackle in future studies focused on obtaining high-resolution substrate-bound structures of BstB or homologs, the findings reported here are still relevant and timely, and we posit will spur the discovery of functional homologs, including some in organisms that are more tractable.

The authors also discuss the possibility of a secondary binding site in BstB based on a slight cavity in domain B next to a flexible loop. This is not backed up in any way and seems unlikely.

The reviewer is correct in that the evidence for this second binding site weak. While the crystallographic structure shows a highly hydrophobic region and the binding studies suggests cooperativity exists in the binding of the 4methylsterol substrate, the docking studies do not strongly support binding at that site. As such, we have clarified in the manuscript that a second hydrophobic cavity is observed, but that its role in ligand interaction remains unexplored.

Reviewer #2 (Public Review):

Summary:

*In eukaryotes, sterols are crucial for signaling and regulating membrane fluidity, however, the mechanism governing cholesterol production and transport across the cell membrane in bacteria remains enigmatic. The manuscript by Zhai et al. sheds light on this topic by uncovering three potential cholesterol transport proteins. Through comprehensive bioinformatics analysis, the authors identified three genes *bstA*, *bstB*, and *bstC* encoding proteins which share homology with transporters, periplasmic binding proteins, and periplasmic components superfamily, respectively. Furthermore, the authors confirmed the specific interaction between these three proteins and C-4 methylated sterols and determined the structures of BstB and BstC. Combining these structural insights with molecular dynamics simulation, they postulated several plausible substrate binding sites within each protein.*

Strengths:

The authors have identified 3 proteins that seem likely to be involved in sterol transport between the inner and outer membrane. The structures are of high quality, and the sterol binding experiments support a role for these proteins in sterol transport.

We thank the reviewer for this positive view of our work.

Weaknesses:

While the author's model is very plausible, direct evidence for a role of BstABC in transport, or that the 3 proteins function together in a single pathway, is limited.

The reviewer is correct that we were unable to demonstrate that the three proteins work together to transport 4methylsterols. This is not for lack of trying. We first attempted gene deletion studies, and as mentioned in the manuscript (with more details now provided in the experimental section), this appeared to be lethal. We then attempted in vitro exchange experiments, in which the proteins would be used to transfer sterols from sterol-loaded “heavy” liposomes to a sterol-free “light” liposomes – such exchange assays are frequently performed with eukaryotic sterol transporters (see Chung et al., Science 2015, <https://doi.org/10.1126/science.aab1370>). These assays were not successful because 1) sterols incorporated poorly into liposomes made with E. coli polar lipids and yielded leaky liposomes; 2) use of liposomes prepared with the TLE of M. capsulatus proved more stable, but no appreciable exchange was observed; we reasoned that this might be due to the absence of an energy source for BstA, the RND component for which we have expressed and purified only the soluble periplasmic domain. Given the technical difficulty of these in vitro transport experiments, we will continue to pursue in vivo demonstration of function as new homologs are identified.

Reviewer #3 (Public Review):

Summary:

The work in this manuscript builds on prior efforts by this team to understand how sterols are biosynthesized and utilized in bacteria. The study reports a new function for three genes encoded near sterol biosynthesis enzymes, suggesting the resulting proteins function as a sterol transport system. Biochemical and structural characterization of the two soluble components of the pathway establishes that both proteins can bind sterols, with a preference for 4methylated derivatives. High-resolution x-ray structures of the apoproteins reveal hydrophobic cavities of the appropriate size to accommodate these substrates. Docking and molecular dynamics simulations confirm this observation and provide specific insights into residues involved in substrate binding.

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The manuscript is comprehensive and well-written. The annotation of a new function in a set of proteins related to bacterial sterol usage is exciting and likely to enable further study of this phenomenon - which is currently not well understood. The work also has implications for improving our understanding of lipid usage in general among bacterial organisms.

We thank the reviewer for this synopsis of our work.

Weaknesses:

The authors might consider moving some of the bioinformatics figures to the main text, given how much space is devoted to this topic in the results section.

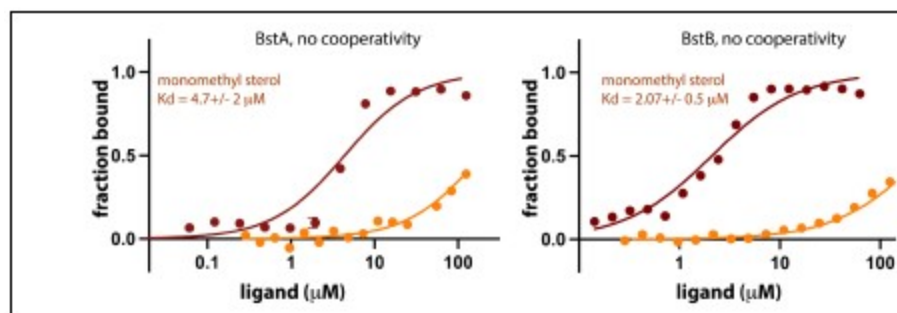
We have taken this advice and moved Figure S1 to the main manuscript.

Reviewer #1 (Recommendations For The Authors):

- 1. In the analysis of the MST data, the authors quote Hill coefficients. How reliable are these numbers? For BstB, for instance, it seems unlikely that more than one molecule would bind. Can the analysis be done without needing to include Hill coefficients?*

We used fits that did and did not invoke cooperativity – see below. We are certain that both BstA and BstB are better fit with cooperativity invoked.

Author response image 1.



1. In looking at the maps associated with the structures, which were included in the review package, I see that two citric acid molecules fit beautifully into the density where currently PEG has been modelled. This needs to be fixed and some comments may be appropriate in the manuscript.

We thank the reviewer for calling our attention to this. Citric acid has now been added to the model, and we reason that these are present in the structure because citric acid was used in the crystallization condition. The revised model is now present in the PDB.

1. It is not necessary to show the two molecules in the asymmetric unit in Figure 4 given that it is not a dimer. This doesn't add anything to the manuscript.

We now show a single molecule of BstC in Figure 4 (now Figure 5).

1. I wouldn't consider the loops shown in Figure S4 as disordered. They have slightly higher B-values but are not completely mobile.

We did not refer to these loops as disordered. In the text, we say they “exhibit poor electron densities, suggesting conformational sampling of more than one state (Fig. S4A).”

Reviewer #2 (Recommendations For The Authors):

pg 7, "hinting at an astounding distinction": I might suggest a word other than astounding that conveys how statistically unlikely, unusual, etc. this result is.

Thank you – we have removed “astounding”.

pg 7, paragraph 2: Here the authors show that in the SSN analysis, BstB proteins cluster separately and suggest this implies a distinction in function. However, they also show that PhnD homologs do not cluster separately (distributed across multiple clusters), yet presumably have similar functions. I am not familiar with SSN, but it seems to me that the second statement about PhnD implies that the first statement about BstB might not be valid, i.e., if PhnD doesn't cluster based on function, on what basis can we conclude that BstB does? On what basis does clustering occur in the SSN analysis? Might it be driven by things other than function? This comment also concerns the final paragraph of this section.

The reviewer is correct in that PhnD homologs occupy separate clusters of the SSN. Many of these homologs were crystallized with phosphate-like compounds, but it is possible that they

have non-overlapping substrate scopes and are therefore functionally distinct. As for the basis of clustering, the SSN is fully sequence-based. What has been observed is that proteins with highly similar sequences can have similar functions – but this is not always true.

*pg 8, paragraph 1: The authors suggest that BstABC may be essential. This is probably not a critical claim and it might be simplest to just remove it, but if it is mentioned, the authors should probably explain what was attempted that failed, so a reader can assess the strength of the evidence supporting essentiality. For example, I don't see anything in the methods about genetic manipulations of *M. capsulatus*, so currently, this falls within the realm of "Data not shown".*

We have provided additional information about the experimental techniques used to do this. This statement was included so that it is understood that the reason for the experimental failure is unlikely to be technical in nature, as we have successfully deleted some sterol related genes while others remain intractable.

Fig. 2A: It is unclear to me what is being plotted here, perhaps more experimental detail is required in the form of labels and/or legend. Is this a quantification of each sterol in each fraction separated by GC? There are essentially no methods provided for the GC-MS experiments. A reference is provided, but I think providing detailed methods for these specific experiments will provide a higher degree of scientific rigor. I am not sure what is standard for GCMS, but perhaps showing spectra in the supplement that establish the identity of the bound molecules as species I and II would be appropriate?

Additional experimental details have been provided and the figure legend changed to be more clear. Moreover, we now clearly state that the chromatograms shown were used to identify lipids due to retention times for spectra that were previously published in Wei et al., 2016.

pg 10-11, comparison with PhnD structure: Perhaps it is worth mentioning a 3rd possible explanation for the relative opening/closing of the cleft is simply crystal packing? I don't think it necessarily has to imply anything about a difference in function. Also, the focus seems to be on this pairwise comparison, but perhaps more insights could be gleaned from an analysis that included a wider range of homologs, especially if any are thought to bind hydrophobic substrates.

This could be true, and we have included a statement to that effect. We are unaware of homologs shown to bind to large, hydrophobic molecules.

I think that BstB is shown upside-down in sup movies relative to other figures. If it isn't changed, perhaps adding some labels would help orient the reader.

We have rotated the movies to be more consistent with the figures.

Fig. S7: No units are indicated for Kds (uM?).

Thank you – this has been fixed.

pg 11, paragraph 2. "adjacent to three residues: Glu118, Tyr120 and Asn192": The residue number used in the text doesn't seem to match the numbering in the PDB file. I think these residues correspond to Glu98, Tyr100, and Asn172 in the PDB file.

We regret this error. The correct numbering for both structures is now present in the deposited PDB files (7T1M for BstB and 7T1S for BstC).

pg 12, final paragraph: The authors present binding data for BstB variants with mutations in the putative sterol binding pocket identified in the structural and MD analyses. However, these mutants had no effect on binding. The authors rationalize this in terms of the size of the interface and hydrophobic nature (which indeed, may be correct and is very plausible), and it is worth noting that many of their mutations are to Ala and would largely preserve the hydrophobic nature of the cleft. However, these mutants raise questions about where sterols actually bind. No experimental evidence is presented that substrates bind in the cleft, it is only hypothesized based on structural homology, MD simulations, etc. These mutations formally provide evidence against the hypothesis being tested; I think that has to be discussed a bit more directly, alongside the caveats the authors already discuss about hydrophobicity, etc.

This is a valid point by the reviewer, and it is one we have attempted to address with our statement in the manuscript and in our response to reviewer 1. We have modified the relevant text to more clearly state that there is as of yet no experimental evidence for the binding of sterols to the cavity identified via molecular docking.

pg 13: Presumably this is not the full-length lipoprotein, but has been truncated/mutated in some way? Some statement of roughly what was purified/crystallized should be stated.

The SI methods on protein purification states that the genes of BstB and BstC without their respective signal peptides were obtained.

pg 13, last paragraph "TN1 exhibits hybrid hydrophobicity, with the sides horizontal to cavities being hydrophobic while the vertical sides are more hydrophilic". I don't really follow the horizontal vs vertical sides. Perhaps this could be described in a different way.

Noted and changed to “TN1 is closer to the N-terminal face of the structure, while CA1 and CA2 are proximal to the C-terminal face and form two open hydrophobic pockets; TN1 exhibits a mixture of hydrophobic and hydrophilic amino acids (Fig. 4B and Fig. S9B, Table S4).”

pg 15-16, "Comparison to eukaryotic sterol transporters": Perhaps this would be better suited for the discussion section? Could also be streamlined; it is mostly discussing and comparing eukaryotic sterol binding domains to each other, not to BstABC.

Given that BstB and BstC are the first identified proteins (and putative transporters) for bacterial sterol engagement, we thought a careful description of the existing sterol transporters (which are all eukaryotic) was warranted.

Reviewer #3 (Recommendations For The Authors):

I have just two minor suggestions for the authors if they wish to comment on or address them.

- 1. Do the three proteins (BstA/B/C) form any sort of complex? Perhaps this property was not assessed - but it seemed possible that the B and C components might constitute a shuttle for the membrane-bound transporter?*

This is an important observation – the unliganded version of these proteins show no appreciable affinity for each other. However, BstB (which would be expected to engage both with BstA and BstC) belongs to a family of proteins known to undergo significant

conformational change upon substrate binding. It is possible that with substrate present, complexes are formed – we have yet to investigate this.

1. In Figure S1, panel C - it appears that the label for the BstC cluster may have migrated away from the intended location. In this figure, it might also be useful to indicate in the caption the meaning of the red coloring of the nodes?

The label is now fixed – thank you for drawing our attention to this.