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Bam complex associated proteins in *Escherichia coli* are functionally linked to peptidoglycan biosynthesis, membrane fluidity and DNA replication

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This is a **useful** study that generated a rich inventory of genetic interactions with the potential to produce new insight into the molecular function of Bam-associated proteins. The interactions with genes of unknown function are of special interest as they may suggest experiments to find the functions of these genes. The overall data provided to support their conclusions is **solid**, but there is a major concern with known polar effects on certain mutations, which should be addressed by complementation.

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Abstract

Biogenesis of the bacterial outer membrane is key to bacterial survival and antibiotic resistance. Central to this is the β-barrel assembly machine (Bam) complex and its associated chaperones, which are responsible for transport, folding and insertion of outer membrane proteins (OMPs). The *Escherichia coli* Bam complex is composed of two essential subunits, BamA and BamD, and three non-essential accessory lipoproteins, BamB, BamC and BamE. Optimal Bam function is further dependent on the non-essential periplasmic chaperones

DegP, Skp and SurA. Despite intensive study, the specific function of these non-essential Bam-associated proteins remains unknown. Here, we analysed Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* knockout strains by phenotypic screening, conservation analysis and high-throughput genetics. We reveal that Bam complex activity is impacted by changes in outer membrane lipid composition and that enterobacterial common antigen is essential in the absence of the chaperone SurA. We also show components of peptidoglycan are conditionally essential with Bam accessory lipoproteins and that DNA replication control is perturbed in the absence of specific OMP assembly components. Together, our data indicates potential mechanisms for coordination of OMP biogenesis with other cellular growth processes such as LPS and peptidoglycan biogenesis, and DNA replication control.

Introduction

The Gram-negative outer membrane is instrumental for virulence, antimicrobial resistance, and immune evasion. The outer membrane is an asymmetric lipid bilayer consisting of phospholipid on the inner leaflet, lipopolysaccharide (LPS) on the outer leaflet, integral outer membrane proteins (OMPs) and associated lipoproteins. Biogenesis of the outer membrane is completed by several multi-component proteinaceous machines. Central to this is the β-barrel assembly machine (Bam) complex, the essential protein machinery responsible for folding and insertion of OMPs into the outer membrane, which then act as transporters, porins, receptors, virulence factors, adhesins and enzymes [1, 2]. All OMPs are synthesised in the cytoplasm before being trafficked through the inner membrane to the periplasmic space by the Sec machinery. OMPs are prone to aggregation and must be maintained in an unfolded state during their translocation to the Bam complex [3]. Three quality control proteins that chaperone OMPs across the periplasm are SurA, Skp and DegP, the latter of which also serves as a protease to degrade misfolded OMPs in the periplasm. The double mutants Δ*surA*Δ*skp* and Δ*surA*Δ*degP* are not viable, which suggests that Skp, SurA and DegP might be functionally redundant [4]. The primary chaperone pathway to the Bam complex is thought to be SurA, while Skp and DegP are suggested to form a secondary pathway that is amplified in stress conditions [5]. However, single molecule studies with purified OmpC and Skp or SurA demonstrate that the two proteins recognise different conformations of the OMP and that while Skp is capable of dispersing aggregated OmpC, SurA is not [6]. In addition, the absence of SurA increases degradation of OMPs that have stalled on the Bam complex, whereas loss of Skp in this scenario has the opposite effect, enhancing their assembly [7]. This is potentially due to the role of Skp in recognising and removing stalled OMP substrates from the Bam complex before being degraded alongside the misfolded OMP by the protease DegP [8]. Together, these studies challenge the long-standing assumption that these pathways are redundant and suggests that Skp and SurA have specialised roles in maintaining OMPs in a folding competent state during periplasmic transit.

Following transport across the periplasmic space, OMPs are received at the outer membrane by the Bam complex. In *Escherichia coli*, the Bam complex is composed of two essential subunits, the outer membrane β-barrel BamA and the lipoprotein BamD, and three non-essential accessory lipoproteins, BamB, BamC and BamE [9, 10]. BamA is the central component of the complex and consists of a 16-stranded β-barrel and five periplasmically localised POlypeptide-TRansport-Associated (POTRA) domains that function to dock the accessory subunits [11, 12]. The POTRA domains are hypothesised to interact with incoming unfolded OMP substrates and feed them into the assembly machinery [13]. BamD is a lipoprotein composed of five tetratricopeptide repeat (TPR) domains that interact with POTRA 5 of BamA [9, 14, 15]. The remaining components of the complex are the accessory lipoproteins, BamB, BamC and BamE, which interact with BamA and BamD to form a functionally coordinated ring structure under the BamA barrel on the periplasmic face of the outer membrane [16–20].

The Bam complex accessory lipoproteins each contribute to full activity of the complex through a shared redundant function of coordinating efficient BamA/D interaction in OMP engagement and folding. Beyond this shared function, there is some limited information about specific roles of these proteins in the cell [10, 21]. For example, as part of the Rcs stress response signalling pathway, the outer membrane lipoprotein RcsF is threaded through an OMP during folding by the Bam complex. This process specifically requires BamE to directly interact with and coordinate BamA and BamD to enable completion of OMP folding around RcsF [22–24]. Lethal jamming of the Bam complex in the absence of BamE can be prevented by BamB [23, 25]. BamB has also been shown to be important in folding of high-flux substrates [26]. However, further information about the specific roles of BamB, BamC and BamE are lacking [21].

In this study, we provide evidence for specialised functions and show that changes to LPS structure lead to increased membrane fluidity and decreased Bam complex activity. We also identify that the cyclic form of enterobacterial common antigen (ECA), a periplasmic carbohydrate, is essential in the absence of the chaperone protein SurA. We demonstrate that the Bam accessory lipoproteins, including BamC, are essential in the absence of the peptidoglycan stem-peptide component *meso*-DAP, providing a strong phenotype for this poorly understood protein. Lastly, we show that loss of BamB causes the cells to over-initiate DNA replication, indicating potential coordination between OMP biogenesis and DNA replication. Together, our data indicate specialised roles for the Bam-associated proteins and highlights potential mechanisms for coordination of OMP biogenesis with processes such as outer membrane lipid biogenesis, peptidoglycan synthesis and DNA replication control.

Results

Loss of Bam-associated proteins leads to distinct phenotypes

We hypothesised that if the Bam-associated proteins have specialised roles in the cell it would be unlikely that they phenocopy each other. Therefore, we tested fitness of Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* mutants of *E. coli* K-12 BW25113 compared to the parent strain under various stresses. We arrayed the strains in 384-well format on solid agar plates containing each stress or compound. Endpoint pictures were taken to quantify colony fitness based on size by the image analysis software Iris [27]. Fitness was scored using the chemical genomics analysis software ChemGAPP Small by comparing the mean colony size for the mutant in each condition to the mean colony size of that mutant in the LB agar condition, which was normalised to a fitness score of 1 [28]. Fitness scores below 1 represent decreased fitness, as a function of colony size compared to growth on LB agar, and scores above 1 indicate increased fitness in that condition.

The fitness of each mutant across all conditions identified that the Δ*bamB* and Δ*surA* mutants have the strongest fitness defects (**Fig 1**). Correlation between each mutant fitness profile pair was assessed by calculating the Pearson correlation coefficient. This demonstrated that Δ*bamB* and Δ*surA* have a similar phenotypic profile with a correlation coefficient of 0.74, the highest of all pairs of mutants (**Fig S1**). Similar decreased fitness scores were observed for both strains in the presence of bacitracin, carbenicillin, erythromycin, sodium dodecyl-sulfate (SDS), osmotic stress conditions due to varied salt concentrations, and vancomycin (**Fig 1**). This is unsurprising as SurA is the major chaperone pathway for OMPs to reach the Bam complex resulting in low fitness of Δ*bamB* and Δ*surA* when growing on LB at 37°C. However, they do not phenocopy in all conditions. For example, the Δ*bamB* strain grew better in the presence of the MreB inhibitor A22 with a fitness score of 1.59, but the Δ*surA* mutant was less fit in this condition (score of 0.51). They also had opposing fitness scores in the presence of doxycycline (Δ*bamB* – 1.65, Δ*surA* – 0.94) and hydroxyurea (Δ*bamB* – 0.68, Δ*surA* – 1.14) (**Fig 1**). The Δ*bamE* mutant mildly correlated to Δ*bamB* with a coefficient of 0.51 (**Fig S1**). However, both mutants were sensitive to vancomycin (Δ*bamB* – 0.06, Δ*bamE* – 0.66) and bacitracin (Δ*bamB* – 0.02, Δ*bamE* – 0.58) two antibiotics that

exceed the exclusion limit of outer membrane porins and to which sensitivity indicates damage to the permeability barrier. In contrast, Δ*bamC* has no strong phenotypes and correlated most strongly with the WT parent strain (**Fig 1** and **Fig S1**). Similarly to Δ*bamC*, the Δ*skp* mutant also has no strong phenotypes in our screen. The Δ*degP* strain was negatively correlated with the other periplasmic chaperone pathway group mutants Δ*skp* (-0.51) and Δ*surA* (-0.37) and with Δ*bamE* (-0.68) due to opposing fitness scores in a range of stresses (**Fig. 1** and **Fig S1**). Interpretation of this result is complicated by DegP not only functioning as a chaperone, but also as a protease that targets misfolded OMPs in the periplasm [29]. However, due to the role of DegP in degrading stalled OMPs that have been recognised by Skp, they might have been expected to correlate [8]. Together, the phenotypic profiling data indicate that while there are shared phenotypes between each knockout, we observe a broad range of unique, mutant specific phenotypes that suggests specialised roles beyond those observed previously.

TraDIS identifies unique synthetic lethal interactions for Bam-associated proteins

Considering that the Bam-associated proteins have specialised function, we sought to find their genetic interaction networks by using Transposon-Directed Insertion site Sequencing (TraDIS). When TraDIS libraries are generated in single gene deletion mutant backgrounds, the insertion frequencies of genes/regions compared to wild-type cells identifies genes that become more important for survival in the mutant backgrounds. These genes are termed conditionally essential, and help to identify co-dependencies of processes that the candidate gene might be involved in. The method can also identify mutants that are more fit in the given genetic background or condition [30–33]. Therefore, the parent strain (*E. coli* BW25113) and Δ*bamB,* Δ*bamC,* Δ*bamE,* Δ*surA,* Δ*skp,* or Δ*degP* mutants were transformed with a mini Tn5-kanamycin transposon and recovered on LB agar at 37°C. Transposon mutants were then pooled and transposon insertion sites identified by DNA sequencing as described previously [32]. The BioTraDIS pipeline was used for data analysis to classify genes as either essential or non-essential. Essential genes were then compared to the parent BW25113 TraDIS library to identify genes that became essential in the knockout strains (conditionally essential) (Table S1) [34]. For quality control, two independent replicates of each transposon library were sequenced over several sequencing runs. Individual sequencing runs had a high correlation coefficient of ≥0.93 between samples and generated >6 million TraDIS reads and >500,000 unique insertion sites for each strain (**Fig S2** and **S3**). Transposon insertion sites were evenly mapped throughout the genome, with the exception of an increased density around the origin of replication as expected due to gene dosage (**Fig S4**) [35].

These experiments are designed to identify novel genetic interactions, but this unbiased approach will also uncover known synthetic lethal gene pairs that allow benchmarking of our data. We confirmed known genetic interactions between *surA* and *skp* [4], *degP* and *surA* [4], *bamB* and *degP* [26] and *bamE* and *bamB* [25, 36] (**Fig S5**). The Δ*bamB,* Δ*bamC* and Δ*bamE* TraDIS libraries allowed identification of 93, 92 and 29 conditionally essential genes, respectively (**Fig 2A** and Table S1). This is particularly surprising as there are no known roles or dependencies for *bamC* and we did not observe strong phenotypes for Δ*bamC* in our phenotypic screen (**Fig 1**). Unfortunately, 54 of the genes identified as conditionally essential in the Δ*bamC* background are of unknown function, therefore providing no hypotheses for the role of BamC (Table S1). We then compared the conditionally essential gene lists to probe if they are involved in similar processes or contribute to the same function within the cell. In the case that all three Bam accessory proteins solely contribute to Bam function, we would expect to find significant overlap between mutants. However, we found many unique conditionally essential genes for each (62, 57 and 5 for Δ*bamB,* Δ*bamC* and Δ*bamE,* respectively), supporting the hypothesis that each has specialised roles in the cell (**Fig 2A**).

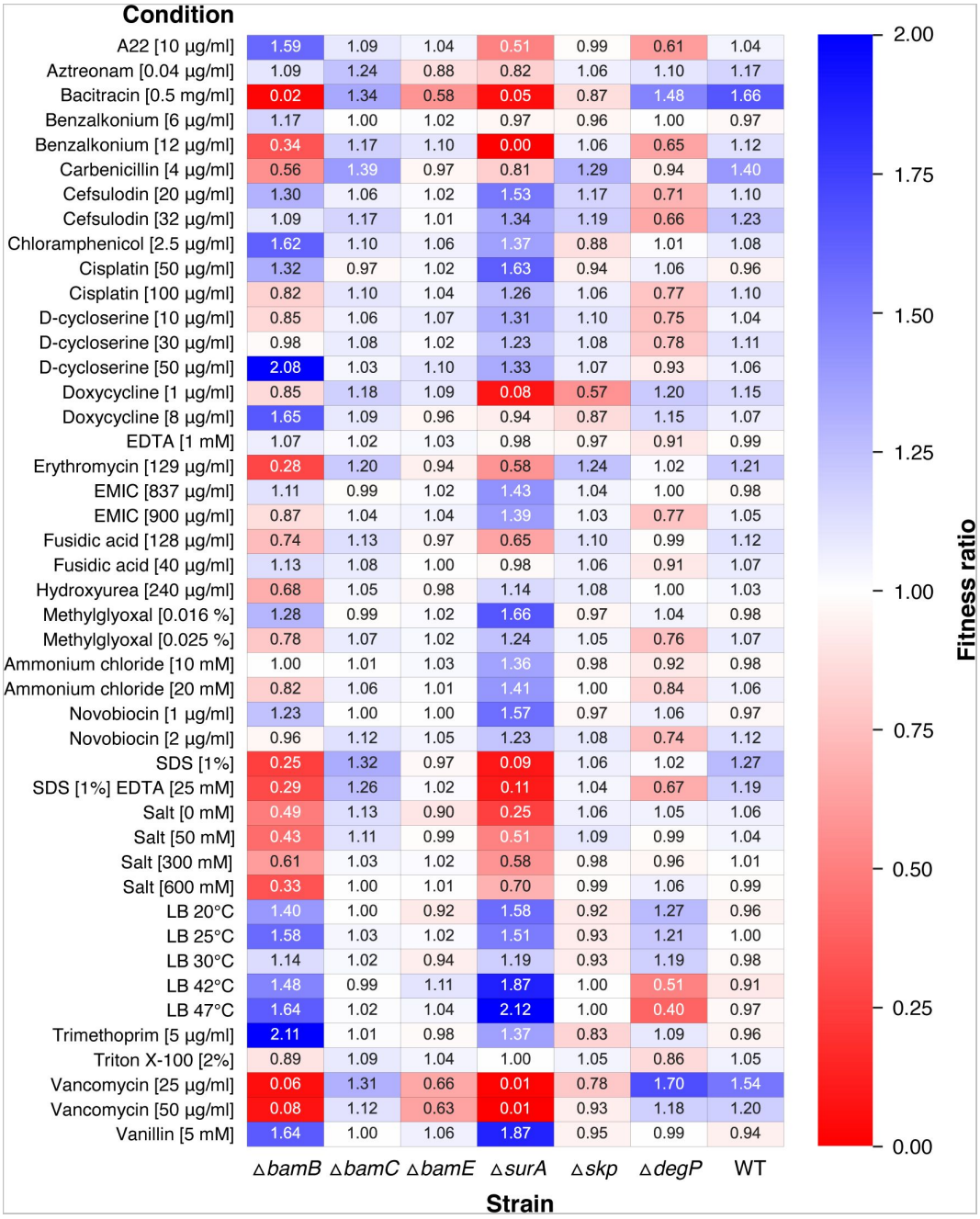

Figure 1

Phenotypic profiling of mutants lacking Bam-associated proteins

Heat map of fitness scores for the *E. coli* BW25113 parent strain and Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp*, or Δ*degP* mutants grown in various stress conditions. Colony size measurements for each condition were normalised by comparing to colony size of that strain when grown on LB medium at 37°C. Growth of each strain on LB is set to 1 and each condition normalised to this. Fitness scores above 1 represent better growth than the LB condition as measured by colony size and scores below 1 indicate smaller colony size than the LB condition. Some conditions are abbreviated due to space restrictions: EMIC - 1-Ethyl-3-methylimidazolium chloride, EDTA - Ethylenediaminetetraacetic acid, SDS – Sodium dodecyl sulfate.

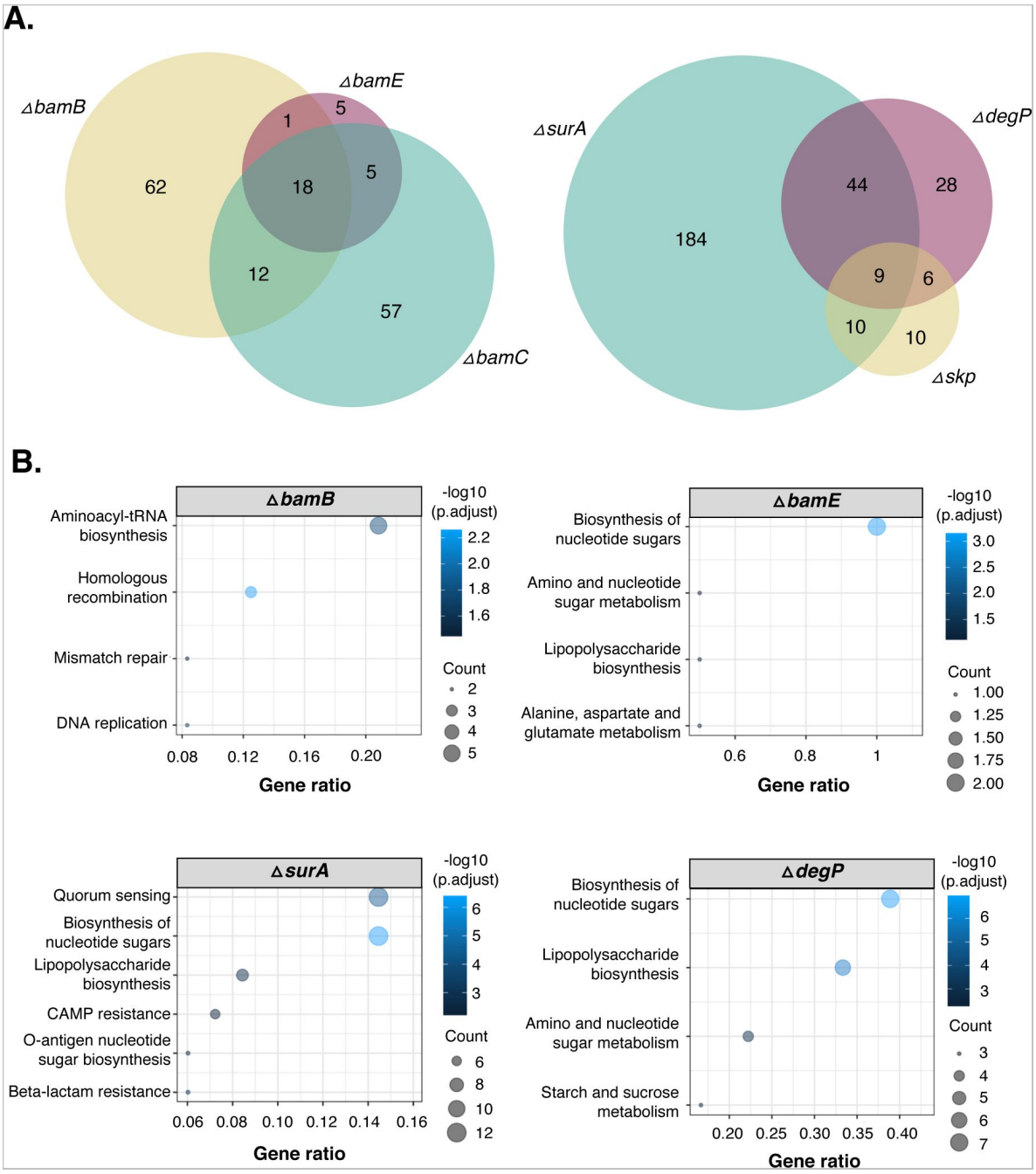

Figure 2

Genetic interaction analysis of Bam-associated genes by TraDIS

TraDIS libraries were constructed in the *E. coli* BW25113 parent strain and Δ*bamB*, Δ*bamC*, Δ*bamE,* Δ*surA*, Δ*skp*, or Δ*degP* mutants to identify genes that become essential in each knockout strain compared to the parent library (conditionally essential). **A.** Venn diagrams showing conditionally essential genes identified within the Δ*bamB*, Δ*bamC* and Δ*bamE* TraDIS libraries or the Δ*surA*, Δ*skp* and Δ*degP* libraries. **B.** Analysis of KEGG categories enriched in conditionally essential gene lists in the Δ*bamB*, Δ*bamE,* Δ*surA* and Δ*degP* TraDIS libraries, represented as bubble plots. No data is shown for the Δ*bamC* and Δ*skp* conditionally essential gene lists as there was no significant enrichment of any KEGG categories.

For the OMP chaperone pathway mutants we identified 247, 87 and 35 conditionally essential genes for the Δ*surA*, Δ*degP* and Δ*skp* mutants, respectively (**Fig 2A**). The Δ*surA* mutant had many strong phenotypes when probed against stresses (**Fig 1**), which could explain the large number of conditionally essential genes compared to the other OMP chaperone mutants (**Fig 2A**). Of the 87 conditionally essential genes in the Δ*degP* dataset, only 15 were also in the Δ*skp* background, despite DegP and Skp functioning together to degrade stalled OMP substrates [8]. This difference is also potentially due to the dual role of DegP as both a chaperone and a protease [29]. These results suggest that while there is functional redundancy between the SurA and DegP/Skp chaperone pathways, the function of the chaperones under specific chemical and genetic conditions is likely specialised (**Fig 1** and **2A**).To determine the functions and associated pathways of the conditionally essential genes identified in the mutant backgrounds, we completed GO and KEGG analyses. Enrichment analysis of KEGG pathways for the Δ*bamC* and Δ*skp* datasets resulted in no significant enrichment of any one category. However, genes involved in "Lipopolysaccharide biosynthesis" and "Biosynthesis of nucleotide sugars" were enriched in the Δ*surA*, Δ*degP* and Δ*bamE* conditionally essential gene lists and "O-antigen nucleotide sugar biosynthesis" was enriched in the Δ*surA* background (**Fig 2B** and Table S2). The most enriched KEGG pathway in the Δ*bamB* dataset was "Aminoacyl-tRNA biosynthesis", however these genes all encode tRNAs and are on average 75 bp in length. Visual inspection of these gene regions identified sparse insertion density within the local genomic area leading to a likely false positive for conditional essentiality. The Δ*bamB* dataset is also significantly enriched for three other KEGG pathways: "Homologous recombination", "Mismatch repair" and "DNA replication" (**Fig 2B** and Table S2). Together, the KEGG pathway enrichment analysis identifies the importance of LPS and ECA biosynthesis in these mutants as well as a potential link between OMP biogenesis and DNA replication. This directed our further investigations.

Genes required for heptose incorporation into LPS are synthetically lethal with *bamB, surA* and *degP*

We identified that fewer mutants were recovered with transposon insertions in genes involved in LPS assembly in the Δ*bamB*, Δ*surA* and Δ*degP* backgrounds than in the parent. In addition, genes involved in "Biosynthesis of nucleotide sugars" were significantly enriched, with genes identified being involved in the synthesis of heptose, a core component of LPS (**Fig 2B** and Table S2). Despite this category being enriched in the Δ*bamE* dataset, we found this was due to one gene in particular, *glmS*, and that the result was actually specific to the Δ*bamB*, Δ*surA* and Δ*degP* TraDIS libraries (**Fig 3A** and **Fig S6**). Synthesis of heptose consists of five main steps before it is incorporated into the LPS inner core (**Fig 3B**). In the Δ*surA* and Δ*degP* TraDIS libraries, all four genes involved in synthesis of heptose were essential: *gmhA*, *gmhB*, *hldE* and *waaD*. In the Δ*bamB* background all were essential except for *gmhB,* which is potentially because a Δ*gmhB* mutant is not completely devoid of heptose [37]. These data suggest that heptose production was functionally more important in Δ*degP* and Δ*surA* than in the Δ*bamB* mutant. The incorporation of heptose into the LPS structure was also functionally important. In Δ*degP* and Δ*surA* mutants, the genes *waaC* and *waaF* were conditionally essential, which are responsible for transfer of the first and second heptose onto the LPS inner core, respectively (**Fig 3A** and **3B**) [38, 39]. In contrast, in the Δ*bamB* mutant, *waaC* was conditionally essential whereas *waaF* was not. This implies that while Δ*degP* and Δ*surA* mutants are not able to tolerate having LPS with only one heptose residue, the Δ*bamB* mutant is.

LPS truncation increases membrane fluidity and decreases Bam complex activity

Increased outer membrane fluidity decreases Bam activity and modifications to LPS structure can lead to changes in membrane fluidity [40]. Thus, we hypothesised that changes in LPS core structure would affect membrane fluidity to differing degrees, which could affect Bam complex

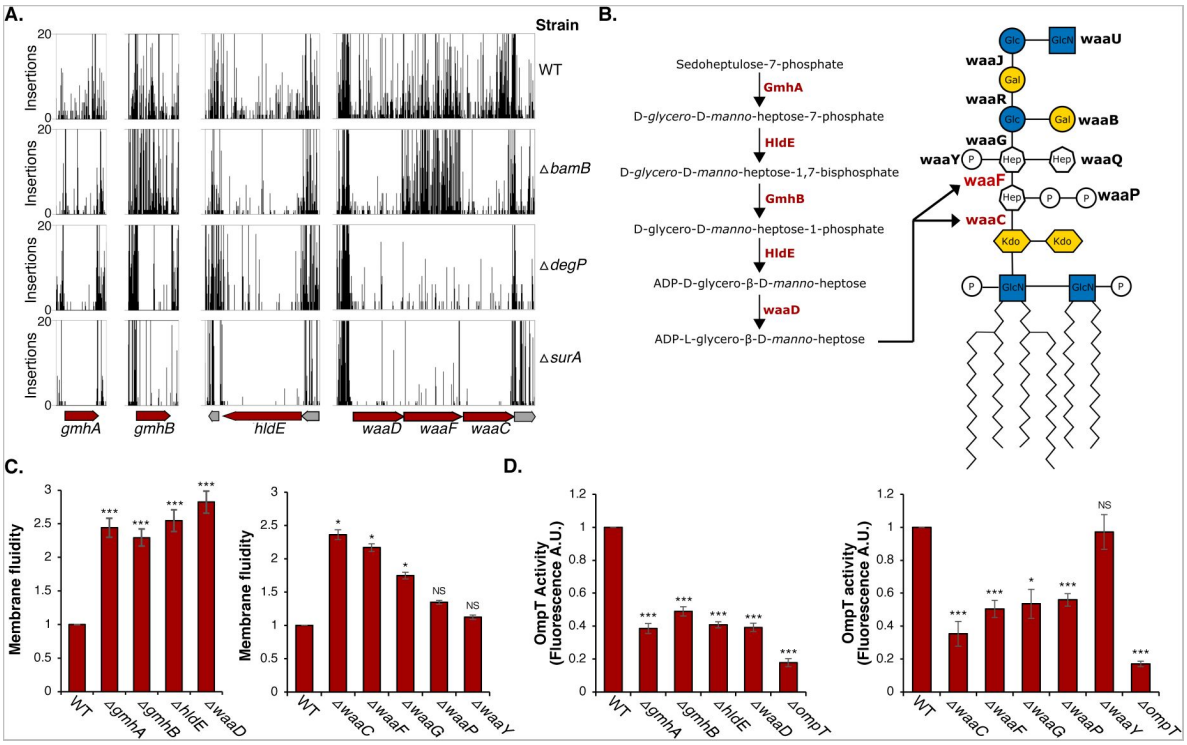

Figure 3

Incorporation of heptose into LPS is essential in Δ*bamB,* Δ*surA* and Δ*degP*

A. Transposon insertions in the genes *gmhA*, *gmhB*, *hldE, waaD, waaC,* and *waaF* in the parent, Δ*bamB,* Δ*surA* and Δ*degP* TraDIS libraries. Transposon cut-off is set to 20. Essential genes are represented as red arrows. **B**. Schematic of the heptose biosynthesis pathway and LPS structure in *E. coli* K-12 BW25113 with gene labels for LPS biosynthesis enzymes indicated next to the linkage they form or component they ligate. Enzymes identified as conditionally-essential are labelled in red text. **C.** Membrane fluidity was measured in Δ*gmhA,* Δ*gmhB,* Δ*hldE,* Δ*waaD,* Δ*waaC*, Δ*waaF*, Δ*waaG*, Δ*waaP* and Δ*waaY* mutants and compared to the parent strain by using pyrenedeconoic acid fluorescence. **D.** To determine the extent of Bam complex activity, successful folding and insertion of OmpT into the outer membrane was measured in the Δ*gmhA,* Δ*gmhB,* Δ*hldE,* Δ*waaD,* Δ*waaC*, Δ*waaF*, Δ*waaG*, Δ*waaP*, Δ*waaY* and Δ*ompT* mutants and compared to the parent strain. For panels C and D, experiments were performed in biological and technical triplicate with standard deviation represented by error bars. Two sample t-tests were used to assess statistical significance of differences from the WT strain with *** indicating p-values of <0.001, * indicating p-values of <0.05, and NS as not significant (p-value ≥ 0.05).

efficiency. To test this hypothesis we assessed membrane fluidity and Bam complex activity in strains lacking the genes encoding the heptose biosynthesis pathway (Δ*gmhA*, Δ*gmhB*, Δ*hldE*, and Δ*waaD*) and LPS core synthesis (Δ*waaC*, Δ*waaF*, Δ*waaP*, Δ*waaG* and Δ*waaY*) (**Fig 3**). There were no differences in transposon insertion index for the gene *waaY*, which encodes the enzyme responsible for phosphorylation of the second heptose in the LPS inner core. Therefore, Δ*waaY* should act as a control in this experiment [41]. In addition, Δ*waaP* and Δ*waaG*, which are responsible for phosphate addition to the first heptose, and incorporation of the first glucose, respectively [42], were included as a small decrease in the insertion indexes for these genes were observed in the Δ*surA* and Δ*degP* TraDIS datasets (**Fig S7**).

Fluidity of the membrane for each mutant was measured using the lipophilic pyrene probe, pyrene decanoic acid. The pyrene monomer can undergo excimer formation and demonstrates a shift in fluorescence, a process which is dependent on the ease of mobility within the membrane [43, 44]. Formation of the excimer was measured and compared to that of the parent strain (**Fig 3C**). The largest increase in membrane fluidity occurred in knockouts of genes required for heptose biosynthesis: Δ*gmhA*, Δ*gmhB*, Δ*hldE*, and Δ*waaD*. Of this group, the Δ*gmhB* mutant had the smallest increase in membrane fluidity and this gene was also not essential in the Δ*bamB* background (**Fig 3A** and **3C**). Of the genes required for LPS core biosynthesis, membrane fluidity was higher in Δ*waaC*, Δ*waaF* and Δ*waaG* than in the parent strain with the biggest change being in Δ*waaC* and the smallest in Δ*waaG*. However, there was no significant increase in fluidity in the Δ*waaP* mutant. Membrane fluidity of the Δ*waaF*, Δ*waaP* and Δ*waaG* mutants was intermediate between that of the heptoseless Δ*waaC* mutant and the parent, with the severity of the effect correlating to the severity of LPS core truncation. No significant change in membrane fluidity was measured in the Δ*waaY* mutant control (**Fig 3C**).

Next, we investigated whether impairment of heptose synthesis affected activity of the Bam complex, which was monitored by an *in vivo* OmpT fluorescence assay. The outer membrane protease OmpT requires the Bam complex for folding and insertion into the membrane and is able to cleave a fluorogenic peptide, which is monitored by increased fluorescence over time [10]. Except for Δ*waaY*, all mutants demonstrated a minimum 40% decrease in OmpT activity compared to the parent strain. The heptoseless Δ*waaC* mutant exhibited the greatest decrease in OmpT activity. The effect of Δ*waaF*, Δ*waaP* and Δ*waaG* mutations on OmpT activity levels was comparable to each other and intermediate between that of Δ*waaC* and the parent strain (**Fig 3D**). In summary, mutations that lead to heptoseless LPS had the biggest increase in membrane fluidity and the lowest levels of OmpT activity with a graded response based on the severity of LPS core truncations. This suggests there is a correlation between LPS core structure, membrane fluidity and Bam complex activity that leads to a variety of Bam complex activity levels depending on the form of LPS in the outer membrane.

Generation of cyclic ECA is essential in the absence of the chaperone SurA

Genes involved in 'Biosynthesis of nucleotide sugars' were significantly enriched as conditionally essential in the Δ*surA* mutant. The genes identified are involved in biosynthesis of ECA (**Fig 2B**), which is a highly conserved carbohydrate-derived molecule present on the external leaflet of the outer membrane and in the periplasm of Enterobacteriaceae [45, 46]. The ECA biosynthesis pathway genes were all conditionally essential with the exception of *wxyE*, which is required for survival of both the parent and the Δ*surA* mutant (**Fig 4A** and **4B**). In addition, fewer mutants were recovered with transposon insertions in the genes *rffH* and *rffG* than in the parent TraDIS library (**Fig 4A**). The gene products RffH and RffG catalyse the same enzymatic reaction and are homologous in sequence to the genes RfbA and RfbB, respectively. However, they form part of different operons and function in separate pathways despite *rffG* being able to complement an RfbB defective strain [47, 48]. This likely explains why *rffH* and *rffG* are not entirely essential in the Δ*surA* background.

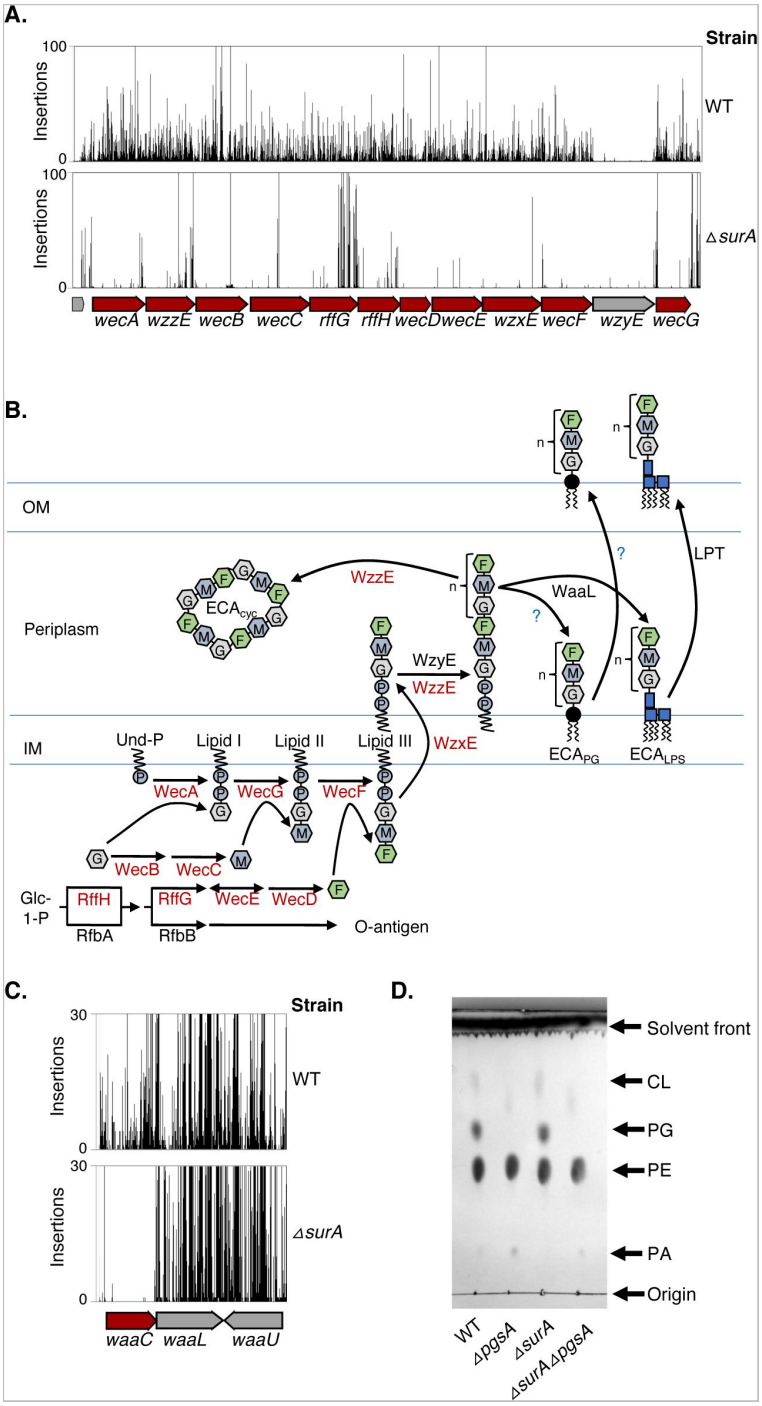

Figure 4

Synthesis of ECA is essential in the absence of the chaperone SurA

A. Transposon insertions in genes of the ECA biosynthesis pathway in the parent or Δ*surA* mutant TraDIS libraries. Transposon cut-off is set to 100. Essential genes are represented as red arrows. **B**. Schematic representation of the ECA biosynthesis pathway with the names of proteins labelled next to the reaction for which they are responsible. Conditionally essential proteins are labelled in red text. **C.** Transposon insertions in the *waaL* gene in either the parent or Δ*surA* mutant TraDIS libraries. Transposon cut-off is set to 100. **D.** Analysis of phospholipid content in the parent or Δ*surA* mutant with further mutations to disrupt synthesis of the major anionic phospholipids. The Δ*pgsA and* Δ*surA*Δ*pgsA* strains are also Δ*lpp*Δ*rcsF*. Phospholipid samples were separated and visualised by TLC using a solvent mixture of methanol/ chloroform/ water with a ratio of 2:2:1.8 before being visualised by phosphomolybdic acid and charring.

ECA exists in three forms that all share the same biosynthetic pathway: ECA that is covalently linked to LPS (ECA_{LPS}), covalently linked to diacylglycerol-phosphate (ECA_{PG}), or a cyclic form (ECA_{CYC}) that is localised to the periplasm as opposed to being surface exposed [45, 49]. We sought to determine which form of ECA is conditionally essential in Δ*surA*. Synthesis of ECA_{LPS} is facilitated by the O-antigen ligase WaaL, which is responsible for attaching the ECA molecule onto the LPS core [50, 51]. However, in the Δ*surA* TraDIS library, the gene *waaL* is non-essential (**Fig 4C**). This suggests that ECA_{LPS} is not essential in the Δ*surA* mutant. The formation of ECA_{PG} is completed by attachment of linear ECA chains to diacylglycerol by a phosphodiester bond through an unknown mechanism [50, 52]. To determine the essentiality of ECA_{PG} we targeted synthesis of the donor molecule for ECA_{PG}, the phospholipid phosphatidylglycerol [53]. The gene *pgsA* encodes phosphatidylglycerophosphate synthase, which catalyses the first committed step in biosynthesis of phosphatidylglycerol. However, loss of *pgsA* is lethal due to mislocalisation of Braun's lipoprotein, Lpp, to the inner membrane and activation of the Rcs stress system. These issues can be resolved by making an Δ*lpp*Δ*rcsF* mutant before constructing the Δ*pgsA* mutation [54–56]. The Δ*pgsA*Δ*surA*Δ*lpp*Δ*rcsF* quadruple mutant was viably constructed and the absence of phosphatidylglycerol was confirmed by phospholipid extraction and thin layer chromatography (**Fig 4D**). This confirmed that the phospholipid donor for synthesis of ECA_{PG} is not essential in the Δ*surA* mutant. Lastly, the gene *wzzE* is not required for the production of ECA_{LPS} or ECA_{PG}, but is required for production of ECA_{CYC} [50, 57, 58]. In the Δ*surA* TraDIS library, the gene *wzzE* contained a conditionally essential region indicating that ECA_{CYC} is likely to be the form of ECA that is conditionally essential in a Δ*surA* mutant (**Fig 4A**) [57].

meso-DAP is essential in the absence of BamB, BamC or BamE

The TraDIS data were searched for conditionally essential genes involved in cell envelope biogenesis pathways other than LPS and ECA biosynthesis. The gene *dapF* was identified as conditionally essential in Δ*bamB* and Δ*bamC* with visual inspection indicating decreased insertions in *dapF* in Δ*bamE* (**Fig 5A**). This was particularly surprising considering the lack of strong phenotypes in the Δ*bamC* background (**Fig 1**). DapF converts LL-diaminopimelate (LL-DAP) to *meso*-diaminopimelate (*meso*-DAP), which is then either decarboxylated to produce L-lysine by the enzyme LysA or is used in biosynthesis of peptidoglycan [59, 60]. The gene *lysA* was non-essential in all strains tested, suggesting the essentiality of *dapF* in these mutants is due to the requirement for *meso*-DAP in peptidoglycan (**Fig 5A** and **5B**). To validate the genetic interaction between Δ*dapF* and components of the Bam complex, the *dapF* gene was knocked out in the BW25113 parent strain, Δ*bamB,* Δ*bamC* or Δ*bamE* mutants. The double knockouts were selected in the presence of externally supplied *meso*-DAP, which alleviated the loss of *dapF*. Cells were then grown in liquid media in the presence of *meso*-DAP before being serially diluted in un-supplemented media and assayed for survival in the presence or absence of *meso*-DAP by efficiency of plating assay. All strains grew equally as well as the parent on LB agar supplemented with 1 mM *meso*-DAP, except for Δ*bamB*Δ*dapF* which showed a minor decrease in CFU and colony size (**Fig 5C**). However, in the absence of *meso*-DAP the single Δ*dapF* mutant demonstrated a decrease in survival and the double mutants were not viable (**Fig 5C**). Lastly, considering that loss of DapF will lead to a weaker peptidoglycan layer due to decreased crosslinks, we sought to negate the synthetic-lethal phenotype by growth in the presence of sucrose as an osmoprotectant [61, 62]. Unexpectedly, while the presence of 10% sucrose enabled survival of the single *dapF* mutant to levels comparable to the parent, this was insufficient to restore growth of the double mutant (**Fig 5C**). This data demonstrates that peptidoglycan structure is of increased importance in the absence of full Bam complex activity and that this may not simply be due to structural support for the envelope.

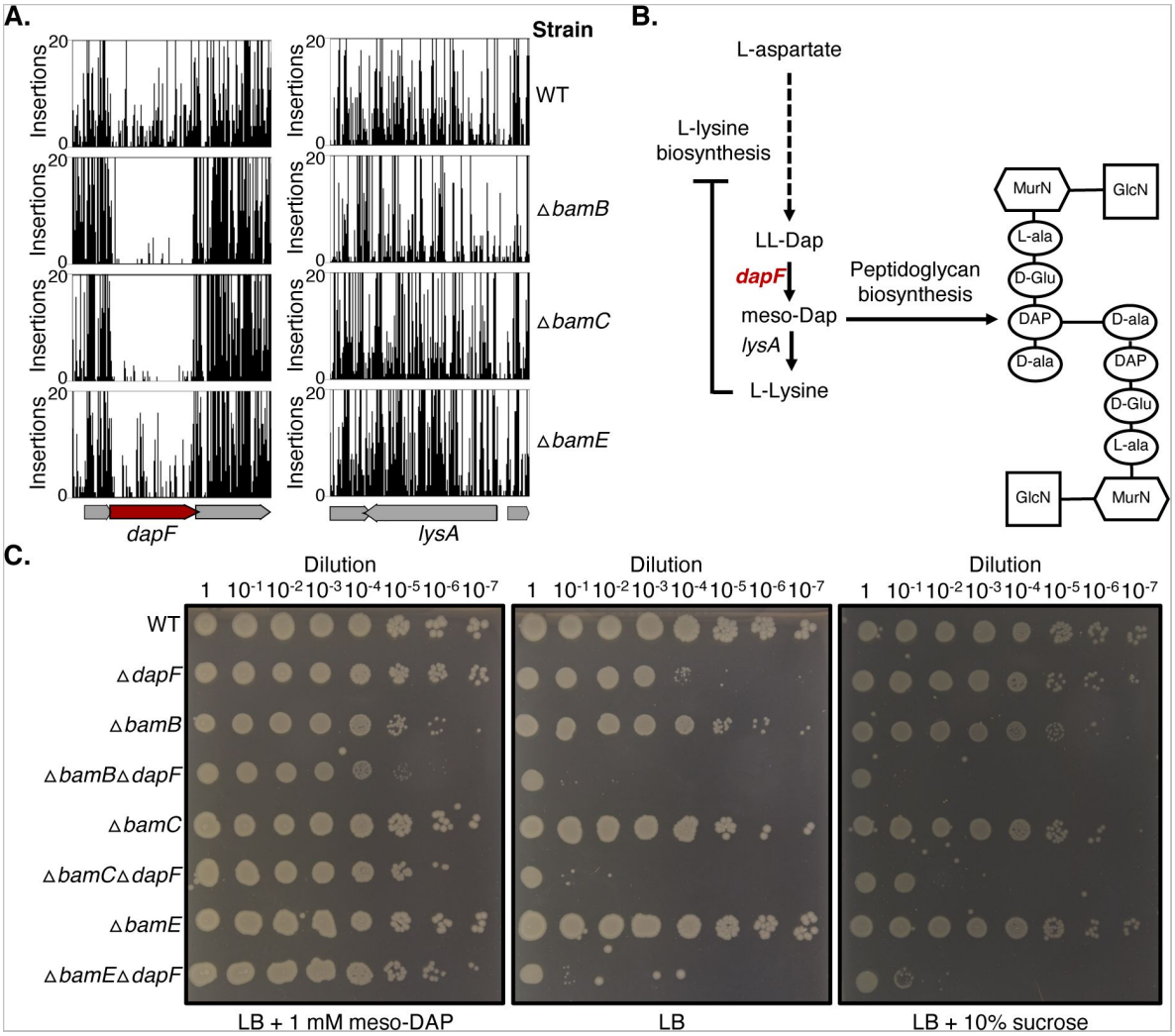

Figure 5

meso-DAP containing peptidoglycan is essential in the absence of Bam accessory lipoproteins

A. Transposon insertions in the genes *dapF* and *lysA* in the parent or Δ*bamB,* Δ*bamC,* Δ*bamE* mutant TraDIS libraries. Transposon cut-off is set to 20. Essential genes are represented as red arrows. **B**. Schematic representation of the L-lysine biosynthesis pathway with the names of proteins labelled next to the reaction for which they are responsible. Conditionally essential proteins are labelled in red text. A schematic structure of *E. coli* peptidoglycan is shown with the site of *meso*-DAP incorporation shown. **C.** Efficiency of plating assay showing survival of the Δ*dapF,* Δ*bamB,* Δ*bamC,* Δ*bamE,* Δ*dapF*Δ*bamB,* Δ*dapF*Δ*bamC, or* Δ*dapF*Δ*bamE* mutants grown on LB with or without 1 mM *meso*-DAP or 10 % sucrose. Cells were grown overnight in LB supplemented with 1 mM *meso*DAP before being normalised to OD_{600} = 1.00 and serially diluted 1:10 before 2 µl was spotted on agar plates and incubated at 37°C overnight.

Regulation of DNA replication initiation is disrupted in the absence of BamB

Our TraDIS experiment identified that in the Δ*bamB* dataset three KEGG pathways were significantly enriched: “Homologous recombination”, “Mismatch repair” and “DNA replication”. Bacterial DNA replication is mainly regulated at the initiation step by DnaA (**Fig 2A** and **6A**). The ATP-bound form of this protein binds to DnaA boxes present at the chromosomal origin of replication (*oriC*), with both high and low affinity, and directs replisome assembly [63]. Briefly, DnaA-ATP forms an oligomeric complex that facilitates unwinding of the DNA strands, which in turn enables assembly of the replisome: the complex responsible for replication of the bacterial chromosome. In *E. coli*, DnaA binding to its cognate DnaA boxes is modulated by regulatory proteins. Negative regulation is achieved by two proteins. The protein SeqA sequesters newly replicated origins, preventing premature reinitiation of replication [64–66]. The Hda protein works together with the β-sliding clamp to stimulate ATP hydrolysis of DnaA-ATP, which in the ADP-bound form is unable to promote initiation of DNA replication [64–68]. Positive regulation is facilitated by the DnaA initiator-associating factor, DiaA, which directly interacts with the N-terminal domain of DnaA to stimulate initiation at *oriC* [69, 70].

Analysis of the TraDIS data identified the genes *holC, holD, matP, seqA* and *hda* as conditionally essential in the Δ*bamB* mutant (Table S1). Within this set of genes, we chose to focus on those with the clearest conditional essentiality from the TraDIS data, *hda* and *seqA*. Insertions within the negative regulator encoding genes, *seqA* and *hda*, led to decreased survival of Δ*bamB*. In contrast, increased fitness of Δ*bamB* was observed for cells containing disruptions of the positive regulator gene *diaA* (**Fig 6B**). We confirmed the findings regarding the negative regulators by using CRISPRi. The *bamB::aph* allele was transferred into the dCas9 encoding strain *E. coli* LC-E18 and the kanamycin resistance marker was removed. The pSGRNA plasmid was used to express guide RNA targeting the *seqA* or *hda* genes in either the parent or Δ*bamB*. Expression of guide RNA targeting the *seqA* and *hda* genes led to significantly decreased survival of Δ*bamB* when compared to the parent strain, therefore confirming the observations made from TraDIS (**Fig 6C**). The positive genetic interaction between *bamB* and *diaA* was confirmed by measuring fitness of the Δ*bamB*Δ*diaA* mutant compared to both single deletion mutants. Fitness of the double mutant was greater than the expected fitness for this strain (**Fig 6D**).

Considering that loss of negative regulators of DNA replication initiation led to decreased fitness of Δ*bamB*, and loss of the positive regulator increases fitness, we hypothesised that the Δ*bamB* mutant may already have defective replication control. Therefore, we compared chromosomal content of the wild-type and Δ*bamB* strain by using an established replication run-out assay [71]. Both strains were grown to early exponential phase under conditions supporting fast cell growth (LB + 0.2% glucose), when cells contain multiple replicating chromosomes. We reasoned that dysfunction of replication control is usually exacerbated when cells grow with overlapping replication rounds. Next, cells were collected and treated with rifampicin to inhibit further rounds of DNA replication initiation, and with cephalexin to prevent cell division. Fixed cells were stained for DNA content with Sytox Green and analysed by flow cytometry. The comparison of chromosomal copy number revealed a higher proportion of cells with 16 chromosome copies for the Δ*bamB* mutant, which implies that initiation of replication occurs earlier during the cell cycle in this strain relative to the parent. Strikingly, a population of Δ*bamB* cells also displayed a number of chromosomes deviating from the normally observed 2^n, as manifested by an additional peak between the 8 and 16 chromosome peaks. This is a hallmark of asynchronous DNA replication events, when not all replication origins in the cell fire simultaneously (**Fig 6E**, S8-10). Analysis of the other Bam-accessory lipoprotein gene mutants and Δ*skp* by replication run-out assay revealed no significant changes from the parent pattern (**Fig S11**). Therefore, our results suggest that *E.*

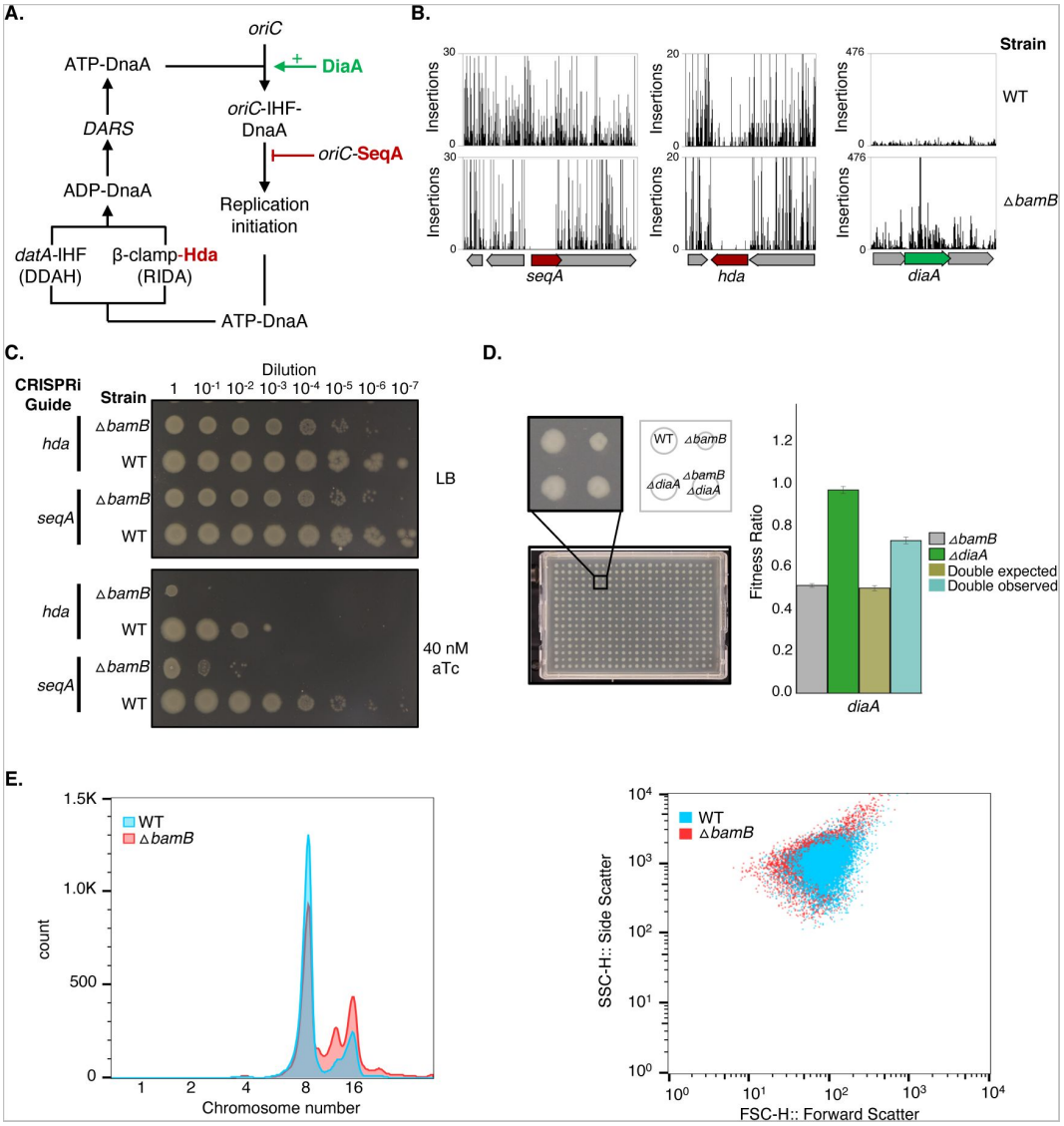

Figure 6

Loss of BamB leads to changes in DNA replication control

A. Schematic representing DNA replication control in *E. coli*. DiaA stimulates initiation at the origin of replication, *oriC*, by DnaA. SeqA prevents premature reinitiation of replication. Hda acts together with the β-sliding clamp to hydrolyse ATP-bound DnaA by regulatory inactivation of DnaA (RIDA). This can also occur through *datA*-dependent DnaA-ATP hydrolysis (DDAH). **B.** Transposon insertions in the genes *hda*, *seqA* and *diaA* in the parent or Δ*bamB* mutant TraDIS libraries. Conditionally-essential genes are represented as red arrows and genes that increase fitness are in green. **C.** Efficiency of plating assay showing survival of the parent dCas9 expressing strain LC-E18 (WT) or Δ*bamB* mutant derivatives carrying the pSGRNA plasmid, which encodes CRISPRi guide RNA targeting either *seqA* or *hda*. Cells were grown on LB with or without 40 nM anhydro-tetracycline (aTc) to induce guide RNA expression. **D**. The *E. coli* BW25113 parent strain, single, or double Δ*bamB* and Δ*diaA* mutants were spotted on LB agar in 384-well format, in triplicate, and grown overnight at 37°C before being imaged. Fitness was calculated based on colony size and fitness ratios generated relative to the WT parent. **E.** Flow cytometry of *E. coli* LC-E18 cells grown in LB followed by replication run-out assay. Forward scatter and side scatter are plotted with WT cell data indicated in blue and Δ*bamB* data points in red. Fluorescence is plotted and represents chromosomal content for each cell with chromosome numbers for each peak marked

coli over-initiates DNA replication in the absence of BamB. This is consistent with the observations that Δ*bamB* cells are more fit if they lose the positive regulator of DNA replication, DiaA, but have decreased fitness in the absence of the negative regulators SeqA and Hda.

OMP trafficking protein conservation varies throughout Gram-negative bacteria

The data presented here demonstrate that in *E. coli*, the Bam-associated proteins likely have specialised functions that are potentially coordinated with LPS structure, the presence and form of ECA, peptidoglycan biogenesis and DNA replication control. Considering these results, we revisited the evolutionary conservation of Bam complex subunits and the periplasmic chaperone pathway proteins [72]. Reference genomes for a diverse range of Gram-negative bacterial representative strains were used to search for sequences coding the Bam complex subunits (as observed in *E. coli*) or the chaperone pathway proteins SurA, Skp and DegP. A combination of Prokka annotation, hmmsearch, SignalP 6.0 [73] and manual curation were used to generate a neighbour joining tree with a heat map of gene counts in each organism (**Fig 7**). While we found broad conservation of all query proteins within the gamma- and beta-proteobacteria, there are some notable exceptions. The BamB and BamC lipoproteins are not conserved within two species of the aphid endosymbiont *Buchnera aphidicola* and the data confirm previous unpublished observations that a strain of *B. aphidicola*, (subspecies *Baizongia pistaciae*) appears to have entirely lost genes encoding the Bam complex [74]. Likely this is due to the strain containing only a few OMPs, therefore no longer requiring a dedicated OMP assembly machinery.

Outside of the gamma- and beta-proteobacteria, requirement for the Bam-associated proteins appears to be diverse. The lipoproteins BamB and BamC are largely absent in the alpha-proteobacteria, and the epsilon-proteobacteria only encode the core components BamA and BamD, demonstrating reductive evolution. This minimalist form of the Bam complex is sufficient for OMP insertion, which is likely due to reduced β-barrel complexity as observed in *Helicobacter pylori* [75] (**Fig 7**). While there is diversity in the presence or absence of these proteins in the organisms analysed, multiple copies of some components were also identified. We found that *Pseudomonas putida* encodes two copies of BamA, BamE and SurA. Outside of the gamma-proteobacteria there are numerous examples of species encoding multiple copies of BamA, particularly within the alpha-proteobacteria, such as the plant pathogen *Agrobacterium tumefaciens* (**Fig 7**). These copies may play specialised roles in folding and inserting specific cargo, or they may alleviate an increased requirement for OMP insertion in these organisms. Taken together, the diverse range of Bam complex subunit compositions within Gram-negative bacteria support the hypothesis that the Bam-associated proteins likely play specialised roles that vary in each organism.

Discussion

The Bam complex has been identified as a significant unrealised target for new antimicrobials, with several new inhibitors of this essential outer membrane biogenesis machine being identified recently [77, 78]. However, despite this focus as an important new avenue for drug discovery, and recent advances in understanding the mechanism by which the Bam complex folds and inserts OMPs into the outer membrane [79, 80], we still do not fully understand the roles of the Bam-associated proteins in *E. coli*. We also do not understand why the complex appears to be modular, with subunits being differentially conserved throughout Gram-negative bacteria as shown through our analysis and those done previously (**Fig 7**) [72]. Constituent parts of the outer membrane such as phospholipid species, type of LPS, O-antigen, ECA, lipoprotein content, and variety/flux of the unfolded OMP proteins can vary across bacterial species [81, 82]. Such variation would likely result in significant differences in the biophysical constraints of the membrane environment in which the Bam complex must operate. In addition, there is likely to be

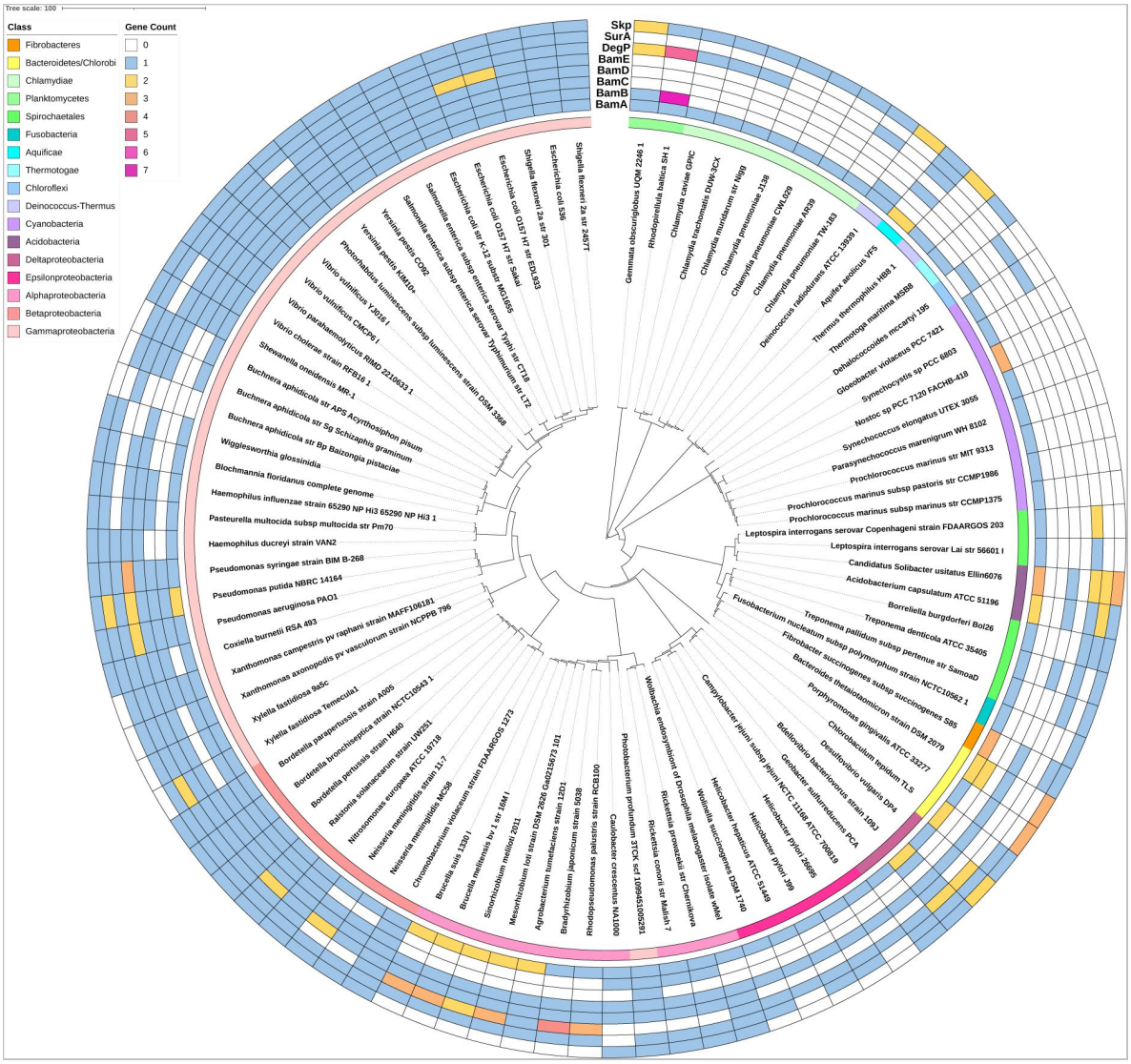

Figure 7

Conservation analysis of Bam-associated proteins in Gram-negative bacteria

Conservation of the BamB, BamC, BamE, SurA, DegP and Skp proteins among key Gram-negative bacterial species represented by a presence/absence map plotted onto a phylogenetic tree generated with iTOL [76]. Gene count in each species is represented by coloured boxes.

differing requirements for the chaperone pathway proteins depending on the pool of client OMPs. Considering that Skp and SurA have been shown to act on different unfolded states of OmpC, with Skp likely being more important during stress conditions, the environmental conditions experienced by each species could also dictate the requirement for each OMP chaperone [6]. Together, this strongly suggests that each accessory lipoprotein is likely to have specialised functions and that the chaperone proteins are unlikely to be truly functionally redundant. This is supported by our phenotypic profiling of knockout strains. Should each of the accessory lipoproteins merely contribute to overall activity of the Bam complex, and if there is true redundancy in the chaperone pathway, then we would expect that the knockout strains should phenocopy each other. However, we found this not to be the case (**Fig 1**). Our TraDIS analysis also supports this conclusion as we saw a large number of unique conditionally essential genes for each mutant (**Fig 2**).

Role of the outer membrane lipid environment for OMP insertion

The Bam complex must function within the constraints of the outer membrane lipid environment, therefore OMP biogenesis is likely to be affected by changes to outer membrane lipids. Indeed, we found conditional essentiality between genes involved in LPS inner core biogenesis and either *bamB*, *surA* or *degP*. Complete truncation of the LPS inner core by disruption of the last enzyme in the heptose biosynthesis pathway has previously been demonstrated to increase membrane fluidity and lead to decreased Bam complex activity [40]. We confirmed this observation and further demonstrate that the changes to membrane fluidity are of graded severity relating to the severity of LPS inner core truncation. This in turn leads to a graded impact on Bam complex activity (**Fig 3**). The Δ*bamB,* Δ*surA* and Δ*degP* knockout strains have the most severe phenotypes within the set of strains tested here (**Fig 1**). Also, loss of BamB, SurA or DegP each leads to OMP assembly defects, decreased numbers of folded OMPs in the outer membrane, and accumulation of unfolded OMPs in the periplasm [9, 26, 83–85]. We hypothesise that the negative effect of increased membrane fluidity on the Bam complex in combination with decreased levels of OMP assembly in the absence of BamB, SurA or DegP leads to OMP assembly levels that are too low to sustain cell viability. This is of particular significance when considering the variation in LPS structure seen between different strains, and the LPS modifications available, which could affect the level of Bam activity and influence the conservation of each Bam-associated protein [81, 82].

A role for ECA_{cyc} in amelioration of periplasmic protein folding stress?

The carbohydrate antigen ECA consists of three repeating sugars, is widely conserved amongst the *Enterobacterales*, and is produced in three forms that are either surface exposed (ECA_{LPS} and ECA_{PG}) or periplasmically localised (ECA_{CYC}). The invariant nature of the antigen suggests it has an important undiscovered function, however the role of ECA in outer membrane biology remains unknown [49]. We identified that ECA biogenesis becomes essential in the absence of the chaperone SurA (**Fig 4A**). Production of ECA requires the lipid carrier undecaprenyl phosphate (Und-P), which is limited in the cell and utilised in numerous metabolic reactions including the production of peptidoglycan, capsule, O-antigen and membrane-derived oligosaccharides. Disruption of ECA biogenesis can cause stress on these other pathways due to Und-P sequestration in ECA dead-end intermediates [49, 57, 86]. However, the first gene in the ECA pathway is conditionally essential in the absence of SurA (**Fig 4A**), therefore the effects are unlikely due to stress on the Und-P pool. Instead, we showed that the periplasmically localised form, ECA_{cyc}, is essential in the absence of SurA (**Fig 4**). It is possible that ECA_{cyc} might help stabilise the outer membrane of a Δ*surA* mutant, as it has been shown to suppress the envelope permeability defect of a Δ*yhdP* mutant. However, the molecular details of this suppression are yet to be discovered [57]. Alternatively, ECA_{cyc} could be performing a chaperone function directly, however this would require *in vitro* assays of protein aggregation with purified products for proof.

Coordination of OMP biogenesis with peptidoglycan biosynthesis

To ensure successful cell growth and division, activity of the Bam complex must be coordinated with biogenesis of the peptidoglycan layer. Our TraDIS analysis identified that in the Δ*bamB*, Δ*bamC* and Δ*bamE* mutants, the gene *dapF* was essential (**Fig 5A**). The DapF enzyme is responsible for synthesis of the peptidoglycan stem-peptide component *meso*-DAP. The single and double mutants could be rescued by exogenous *meso*-DAP, however only the single *dapF* mutant could be rescued by the osmo-protectant sucrose, which is sufficient to allow survival during disruption of peptidoglycan biogenesis (**Fig 5B**) [61]. Loss of *meso*-DAP in the single *dapF* mutant leads to accumulation of LL-DAP, which is incorporated into peptidoglycan and leads to decreased crosslinking [59, 62]. In the Δ*dapF* mutant the peptidoglycan layer would be able to withstand less mechanical load due to decreased crosslinking. This decreased mechanical strength, in combination with the reduced capacity for load bearing by the outer membrane in the Bam complex mutants, means that the cells are unlikely to withstand osmotic stress [87]. However, while this may be true for Δ*bamB* and Δ*bamE* mutants, there are no strong phenotypic effects for Δ*bamC* (**Fig 1**) [88]. Also, the loss of BamC has only a slight effect on the membrane permeability barrier, no detectable changes to the outer membrane proteome, and no effect on OMP folding in an *in vitro* assay [9, 89]. Consequently, the synthetic lethality between *dapF* and *bamC* is unlikely to be due to osmotic stress tolerance, as could be the case for the other mutants.

It has recently been demonstrated that the Bam complex preferentially inserts OMPs at the cell division site and that this is coordinated by interaction with the peptidoglycan layer [90]. While all of the Bam complex proteins were shown to interact with peptidoglycan *in vitro*, only BamA and BamC were found to interact with peptidoglycan in whole cells when analysed by crosslinking and pull-down assays [90]. Considering the interaction of mature peptidoglycan with BamC and the altered peptidoglycan structure in the Δ*dapF* strain [59, 62], we hypothesise that BamC might facilitate coordination between the Bam complex and peptidoglycan biosynthesis, however this requires further investigation.

Link between OMP biogenesis and DNA replication control

Not only must envelope biogenesis processes be coordinated with each other, but synthesis of the envelope must be coordinated with cell growth and cell division. Experimental evidence suggests that both DNA replication control [91, 92] and the rate of cell surface synthesis are coupled to cell volume [93]. This provides the rationale for existence of mechanisms that modulate DNA replication rate in response to cell envelope synthesis perturbation. Here we demonstrated that in the absence of BamB, cells over-initiate DNA replication and that synchrony of replication initiation at multiple origins is compromised (**Fig 6E**). Also, our phenotypic screen revealed that Δ*bamB* has increased fitness at sub-optimal growth temperatures. This would lead to slower growth, a factor that is known to alleviate some DNA replication control defects, such as those arising from loss of SeqA [64]. In addition, we found that the Δ*bamB* mutant is sensitive to hydroxyurea, which specifically inhibits ribonucleotide reductase and leads to depletion of the cellular dNTPs pool, replication fork arrest and genomic instability (**Fig 1**) [94, 95]. Similarly to Δ*bamB*, mutants that over-initiate replication, such as *seqA* mutants, are also sensitive to hydroxyurea [96].

Led by our TraDIS results, we confirmed that *seqA* and *hda* are essential in the Δ*bamB* mutant (**Fig 6**) and that this is likely due to over-initiation of DNA replication, which can be caused by all three of these mutations [64, 66, 97]. SeqA is required to prevent premature re-initiation of replication through sequestration of newly replicated GATC sites within oriC, whereas Hda prevents re-initiation of replication through regulatory inactivation of DnaA (RIDA), in combination with the β-sliding clamp of DNA polymerase [64, 98]. Interestingly, we also identified that two components of the β-sliding clamp loader complex, HolC and HolD, become

essential in the Δ*bamB* mutant (Table S1). These proteins contribute towards activity of the clamp loader. Therefore, the synthetic lethality could be due to decreased RIDA and over-initiation of DNA replication, as observed for the *hda* mutant [68, 99].

This is the first link between the Bam complex and DNA replication control, however links between the envelope and DNA replication have been observed previously. In order to initiate DNA replication, the DnaA protein must be in the ATP-bound form and regeneration of DnaA-ATP from the ADP-bound form is facilitated by interaction with phospholipids. [100, 101]. DNA replication control and envelope biogenesis are also linked by the SeqA protein, which has been associated to the purified outer membrane fraction in a cell-cycle dependent manner, however the mechanism remains unclear [102]. In addition, disrupted origin/terminus ratios in *seqA* mutant strains are suppressed by mutations in the *waaG*, *waaQ* and waa*P* genes, which are responsible for addition of the outer core of LPS, heptose III addition to the inner core and phosphorylation of heptose II of the LPS inner core, respectively [81, 103]. Interestingly, the Δ*bamB* mutant is also sensitive to changes in membrane fluidity brought about by LPS modifications that map to the inner core (**Fig 3**).

We also identified that insertions in the *diaA* gene, which encodes a positive regulator of DNA replication initiation, can suppress Δ*bamB*. The *diaA* gene is part of a conserved four gene cluster that also encodes LpoA - a regulator of peptidoglycan biogenesis, YraN - a protein of unknown function, and DolP - a cell-division site localised outer membrane lipoprotein shown to interact with the Bam complex [104, 105]. The genes in this cluster all appear to be linked to regulating cellular growth processes such as peptidoglycan biogenesis, OMP insertion into the outer membrane and DNA replication. This is of particular interest considering our data demonstrate potential coordination of these processes, which is essential to ensure efficient growth and survival during cell division.

Together, our data demonstrate that the Bam-associated proteins have specialised roles in the cell and highlights potential future targets to understand these roles. We provide further evidence that OMP transport, folding and insertion is affected by, and potentially coordinated with, peptidoglycan and LPS structure. We also show that Bam function is correlated with DNA replication control and highlight the BamB lipoprotein, outer membrane fluidity and LPS structure as a potential route through which this could occur. This provides a strong direction for further study to understand this coordination.

Materials and methods

Bacterial strains and culture conditions

For TraDIS experiments and phenotypic profiling, the parent strain was *E. coli* K-12 BW25113. The *E. coli* Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* mutants were generated by transferring the relevant allele from the Keio library [106] into the clean parent strain by P1 transduction [107]. The kan^Rcassette was then removed by using the pCP20 plasmid [108] to leave a small in-frame 34 amino acid peptide consisting of residues from the FRT scar and the first amino acid and last seven of the target gene. These strains were then used for construction of transposon libraries and phenotypic profiling. The same approach was used for construction of the *E. coli* Δ*wecA*::*aph*, Δ*wecD*::*aph*, Δ*wecF*::*aph*, Δ*ompT*::*aph*, Δ*dapF*::*aph* and Δ*diaA*::*aph* mutants. The *diaA*::*aph* allele was also transferred into the Δ*bamB* derivative of *E. coli* BW25113 to generate the Δ*bamB*Δ*diaA*::*aph* double mutant. The *dapF*::*aph* allele was also transferred from the Keio library into Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* mutants in the presence of 1 mM *meso*-diaminopimelate in order to generate double mutants. The Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* mutant was generated by transfer of alleles from the Keio library and subsequent removal of the kan^R cassette by use of the pCP20 plasmid before incorporation of the next allele by P1 transduction. The *E. coli*

Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* mutants were generated by transferring the relevant allele from the Keio library [106] into the clean parent strain by P1 transduction [107]. The kan^Rcassette was then removed by using the pCP20 plasmid [108] to leave a small in-frame 34 amino acid peptide consisting of residues from the FRT scar and the first amino acid and last seven of the target gene. These strains were then used for construction of transposon libraries and phenotypic profiling. The same approach was used for construction of the *E. coli* Δ*wecA*::*aph*, Δ*wecD*::*aph*, Δ*wecF*::*aph*, Δ*ompT*::*aph*, Δ*dapF*::*aph* and Δ*diaA*::*aph* mutants. The *diaA*::*aph* allele was also transferred into the Δ*bamB* derivative of *E. coli* BW25113 to generate the Δ*bamB*Δ*diaA*::*aph* double mutant. The *dapF*::*aph* allele was also transferred from the Keio library into Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* mutants in the presence of 1 mM *meso*-diaminopimelate in order to generate double mutants. The Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* mutant was generated by transfer of alleles from the Keio library and subsequent removal of the kan^R cassette by use of the pCP20 plasmid before incorporation of the next allele by P1 transduction. The *E. coli* Δ*gmhA*::*aph*, Δ*gmhB*::*aph*, Δ*hldE*::*aph*, Δ*waaD*::*aph*, Δ*waaC*::*aph*, Δ*waaF*::*aph*, Δ*waaP*::*aph*, Δ*waaG*::*aph*, Δ*waaY*::*aph* mutants were created by using λ-Red recombination, as previously described for single-step gene inactivation [109]. All mutants were confirmed by polymerase chain reaction and strains were routinely cultured on LB agar and LB broth. The dCas9 expressing strain *E. coli* LC-E18 and the pSGRNA plasmid was a gift from David Bikard (Addgene plasmid # 115924) and has been described previously [110]. The *E. coli* LC-E18 Δ*bamB* derivative was constructed as described for the BW25113 Δ*bamB* derivative with the kan^R cassette removed in order to allow selection of the pSGRNA plasmid in this background. All strains used in this study are listed in Table S3. Bacterial cultures were grown at 37°C unless otherwise stated. Where stated, the medium was supplemented with kanamycin (50 μg/ml), carbenicillin (100 μg/ml), and *meso*-DAP (1 mM). For micro-dilution survival assays, bacteria were grown in 5 ml LB medium at 37°C with aeration for ~16 hours. Cultures were normalised by optical density to OD_{600} = 1.00, 10-fold serially diluted in LB, and 2 μl of each dilution was inoculated onto LB agar plates.

Phenotypic screening

The *E. coli* K-12 BW25113 parent strain, Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* mutants were screened against diverse stress conditions to phenotypically profile the mutants. Strains were arrayed in 384-well format and inoculated on 2% agar LB plates using a BM3-BC robot (S&P Robotic Inc.). In addition, the mutants and the wild type were subsequently inoculated on LB agar plates containing different stress conditions. The inoculated plates were incubated for 12 to 14 hours at 37°C before being imaged under controlled lighting with an 18-megapixel Canon rebelT3i (Canon) camera on the BM3-BC robot (S&P Robotic Inc.). Images of the plates were analysed using the software IRIS, which measured the size, opacity and circularity of each colony [27]. A total of 4 replica plates were generated for each stress condition. Fitness of the mutants was then scored and analysed using the ChemGAPP Small software [28]. Mean colony size for the mutant in each condition was compared to the mean colony size of that mutant in the LB agar condition, which was normalised to a fitness score of 1 [28]. Fitness scores below 1 represent decreased fitness, as a function of colony size compared to growth on LB agar, and scores above 1 indicate increased fitness in that condition. Each of the plates contained 56 replicates for the parent BW25113 strain (WT), Δ*bamB*, Δ*bamC*, Δ*bamE* and Δ*degP* strains with 52 replicates for Δ*surA* and Δ*skp* for plate space requirements. There were 4 replicate plates for each condition, all of which were treated as individual replicates and compared to their respective wild type parent replicates on each plate before the average was taken. The probability that the two means are equal across replicates was obtained by a one-way ANOVA. Correlation of fitness ratios for each strain was assessed by calculating the Pearson correlation coefficient for averaged fitness scores across replicates using Python before being plotted as a heatmap.

TraDIS library construction

The *E. coli* K-12 BW25113 parent strain, Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* mutants were transformed with the EZ-Tn5™ <KAN-2> Tnp Transposome (Cambio) as previously described [32]. Approximately 1 million mutant colonies were pooled, thoroughly mixed and stored in LB supplemented with 15% glycerol at -80°C. DNA was extracted from at least two samples of each transposon library to generate independent sampling replicates for library generation.

Sequencing of TraDIS libraries

Cells from the pooled mutant library were harvested and genomic DNA was extracted for library preparation and sequencing as previously described [32]. Samples were sequenced using an Illumina MiSeq with a 150 cycle v3 cartridge.

TraDIS data analysis

Raw data were processed using a series of custom scripts as previously described [32]. The data were trimmed using Fastx barcode splitter and trimmer tools (Pearson *et al.*, 1997) and filtered based on inline indexes. The accuracy of the transposon sequences were checked in two steps: the first 22 bases, allowing for three nucleotide base mismatches and the last 10 bases of the transposon, allowing for up to one mismatch. Using Trimmomatic, sequences with less than 20 bases in length were removed (Bolger *et al.*, 2014). TraDIS data were then analysed using BioTraDIS (*https://sanger-pathogens.github.io/Bio-Tradis/*) [34] and aligned to the *E. coli* BW25113 reference genome CP009273.1, available from NCBI (Tatusova *et al.*, 2014). We used SMALT (*https://www.sanger.ac.uk/tool/smalt/*) as an aligner with zero value for mismatch threshold. We also set the parameter smalt_r, determining how to treat multi-mapping reads, to zero. This avoided repetitive elements to count as essential. We used the scripts *tradis_essentiality.R and tradis_comparison.R* as part of the package to produce the list of essentiality and then to compare the control and test libraries, respectively.

Membrane fluidity assay

Membrane fluidity was measured using the membrane fluidity kit (Abcam: ab189819), as previously described, but with minor modifications [104]. Bacterial strains were grown to mid-exponential phase (OD_{600} = ~0.4-0.6) in LB medium. Cells were harvested by centrifugation, washed with phosphate buffered saline (PBS) and incubated with labelling mix (10 µM pyrenedecanoic acid (PDA), 0.08% pluronic F-127, in PBS) in the dark for 20 min at 25°C with rocking. Cells were then washed twice in PBS and re-suspended in PBS prior to measuring fluorescence (excitation = 350 nm, emission = either 400 nm or 470 nm). Membrane fluidity was estimated by measuring the ratio of excimer (470 nm) to monomer (400 nm) fluorescence. The emission spectra were compared to unlabelled cells to confirm membrane incorporation and each experiment contained triplicate technical repeats and was then repeated three times. Membrane fluidity of the mutants of interest were expressed relative to the parent *E. coli* strain. Experiments were performed in technical triplicate and were repeated three times. Two sample t-tests were used to assess statistical significance of differences from the WT strain.

OmpT *in vivo* fluorescence assay

The OmpT assay for monitoring Bam activity was performed as described previously with minor modifications [10, 16, 40]. Bacterial strains were grown to mid-exponential phase (OD_{600} = ~0.4-0.6) in LB medium and were normalised to OD_{600} of 0.2 in growth media. The cell suspension (5 µl) was then added to 95 µl of 25 µM fluorogenic peptide, Abz-ARRAY(NO_2)-NH_2, diluted in PBS. Fluorescence emission was immediately measured (excitation = 325 nm, emission = 430 nm) over

a period of 5 h, with readings every 20 s. OmpT activity was expressed relative to the parent strain. Experiments were performed in technical triplicate and were repeated three times. Two sample t-tests were used to assess statistical significance of differences from the WT strain.

Phospholipid extraction and thin layer chromatography

Phospholipids were extracted using an adapted Bligh-Dyer method [111]. Bacterial cultures were grown overnight (~16 hours) at 37°C with shaking and cells were harvested by centrifugation. The bacterial cell pellets were resuspended and normalised to OD_{600} = 3 and mixed with methanol and chloroform (2:1). The cell suspension was incubated at 50°C for 30 min before additional chloroform and water was added to the samples to produce a final ratio 2:2:1.8 of methanol/chloroform/water. Following centrifugation, the phospholipid-containing phase was extracted and dried under nitrogen before being stored at -20°C. Samples were re-dissolved in chloroform before being separated by thin layer chromatography on silica gel membrane using a solvent system that consisted of chloroform, methanol, acetic acid (65:25:10). The plate was subsequently stained with phosphomolybdic acid (PMA) and warmed until the lipid species were visible.

Efficiency of plating assay

Efficiency of plating assays were completed as previously described [112]. Cells were grown overnight in LB (supplemented with 1 mM *meso*DAP where indicated) before being normalised to OD_{600} = 1.00 and serially diluted 1:10. Following dilution, 2 µl was spotted on agar plates containing supplements where indicated and incubated at 37°C for ~16 hours before being imaged.

Genetic interaction analysis

For genetic interaction analysis, overnight cultures were diluted 1/100 and then grown to OD_{600} = 0.8-1. Cultures were then spread on LB agar plates using sterile glass beads to create source plates, before being incubated for 12 to 14 hours at 37°C. Each strain was arrayed on LB agar plates, from the source plates, to form 384 colonies of 96 replicates for each strain using a BM3-BC robot (S&P Robotic Inc.). Plates were incubated at 37°C for 12 to 14 hours. The plate was then imaged under controlled lighting, with an 18-megapixel Canon rebelT3i (Canon) camera on the BM3-BC robot (S&P Robotic Inc.). Images were then analysed using the software called IRIS to measure the size, opacity and circularity of each colony on the plates [27]. Fitness of the mutants was then analysed and compared using the software ChemGAPP GI [28].

Flow cytometry analysis

Chromosome number measurements were performed as described previously, but with several modifications [71]. Briefly, cells were grown with aeration at 37°C until OD_{600}=0.15 in LB medium supplemented with 0.2% glucose. Samples were collected, treated with 150 µg/ml rifampicin, and 10 µg/ml cephalexin and incubated for 4 h at 37°C with mixing. Incubation with antibiotics results in cells containing an integral number of chromosomes, corresponding to the number of replication origins at the time of drug treatment. Subsequently, cells were harvested, washed with TBS (20 mM Tris-HCl pH 7.5, 130 mM NaCl) and fixed with cold 70% ethanol overnight. Additional samples were collected at OD_{600}=0.15 without antibiotic treatment and fixed as above.

Prior to flow cytometry analysis, cells were resuspended in 50 mM sodium citrate followed by RNA digestion with RNase A for 4 h. Chromosomal DNA was stained with 2 mM Sytox Green (Invitrogen) and DNA content per cell was measured with BD FACS Calibur at 488 nm Argon Ion laser. MG1655 (WT) strain grown in AB medium containing one of the following carbon sources: 0.4% sodium acetate, 0.2% glucose, 0.2% glucose + 0.5% casamino acids or in LB medium with 0.2%

glucose, treated with antibiotics, fixed and stained as above was used as a standard for each flow cytometry measurement, indicating cells containing 1/2 , 2/4, 4/8 or 8/16 chromosomes, respectively. Flow cytometry data was analyzed using FlowJo ver. 10.8.0.

Conservation analysis

Reference genomes were downloaded for each species from NCBI in fasta format and annotated using Prokka [113]. The 16s rRNA sequence for each species was then extracted from Prokka ffn files in fasta format. A Kmer based neighbour joining tree was produced via extracting a one-step sliding window of 8 base kmers from each 16s rRNA sequence. Following this, the number of unique kmers was counted for each species in order to produce a Kmer profile. The Jaccard similarity between each species Kmer profiles was then used to produce a distance matrix. This distance matrix was input into the R module ape::nj to produce the neighbour joining tree. The neighbour joining tree was reformatted as a nexus file and visualised within Figtree (*http://tree.bio.ed.ac.uk/software/figtree/*), where the tree was rooted at the midpoint and branches were transformed to become proportional.

To count the instances of the proteins BamA, BamB, BamC, BamD, BamE, DegP, SurA, and Skp, hmmsearch was used to extract proteins with the relevant domains from the Prokka annotated faa files. The Pfam domains used were PF01103, PF13360, PF06804, PF13525, PF04355, PF09312 and PF03938 for BamA, BamB, BamC, BamD, BamE, SurA, and Skp, respectively. For DegP, both PF00595 and PF13180 were used and the intersect taken. An e-value of 0.001 was used as a threshold for significant hits by hmmsearch. For BamA, hits annotated as the query protein were inputted into hmmscan and hits with at least one POTRA domain and an Omp85 domain were counted as true BamA hits. Hits containing a Tam-POTRA domain were excluded. Where a hit had only an Omp85 gene, the adjacent gene was checked for the POTRA domains. For BamB, any hmmsearch hits annotated as BamB or hypothetical proteins by Prokka were input into SignalP 6.0 [73] and hmmscan. Proteins assigned a LIPO (Sec/SPII) signal peptide within SignalP 6.0, with only one PQQ_2 domain within hmmscan were selected as true BamB hits. For BamC, all hits from hmmsearch were inputted into hmmscan to confirm the presence of the Lipoprotein-18 domain. Hits were also inputted into Phmmer and proteins with significant homology to BamC were counted as true hits. For BamD, hypothetical proteins and BamD hits were inputted into hmmscan and Phmmer and those with homology to BamD and a non-outcompeted YfiO domain counted as true hits. For BamE, hypothetical proteins and BamE hits were inputted into hmmscan and those with a singular SmpA_OmlA domain were counted as true hits. For DegP hypothetical proteins and DegP hits were inputted into hmmscan. If a hit had a PDZ type domain, Peptidase_M50 domain or Trypsin_2 domain then it was visualised on Pfam and manually assigned as DegP based on structure. If a DegP hit had any other type of domain, it was discounted. For SurA, all hits were run through hmmscan and Phmmer. Those with a SurA_N domain, Rotamase domain and a Rotamase_3 domain and homologous to SurA within Phmmer were counted. For Skp, all hits were run through Phmmer and only hits with only an OmpH domain and with homology to Skp counted as true hits. Finally, the neighbour-joining tree was edited in iTOL [76] to produce the heatmap of gene counts.

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Supporting information

Supplemental Table 1

Supplemental Table 2

Supplemental Table 3

Note

This reviewed preprint has been updated to use the correct the funding information.

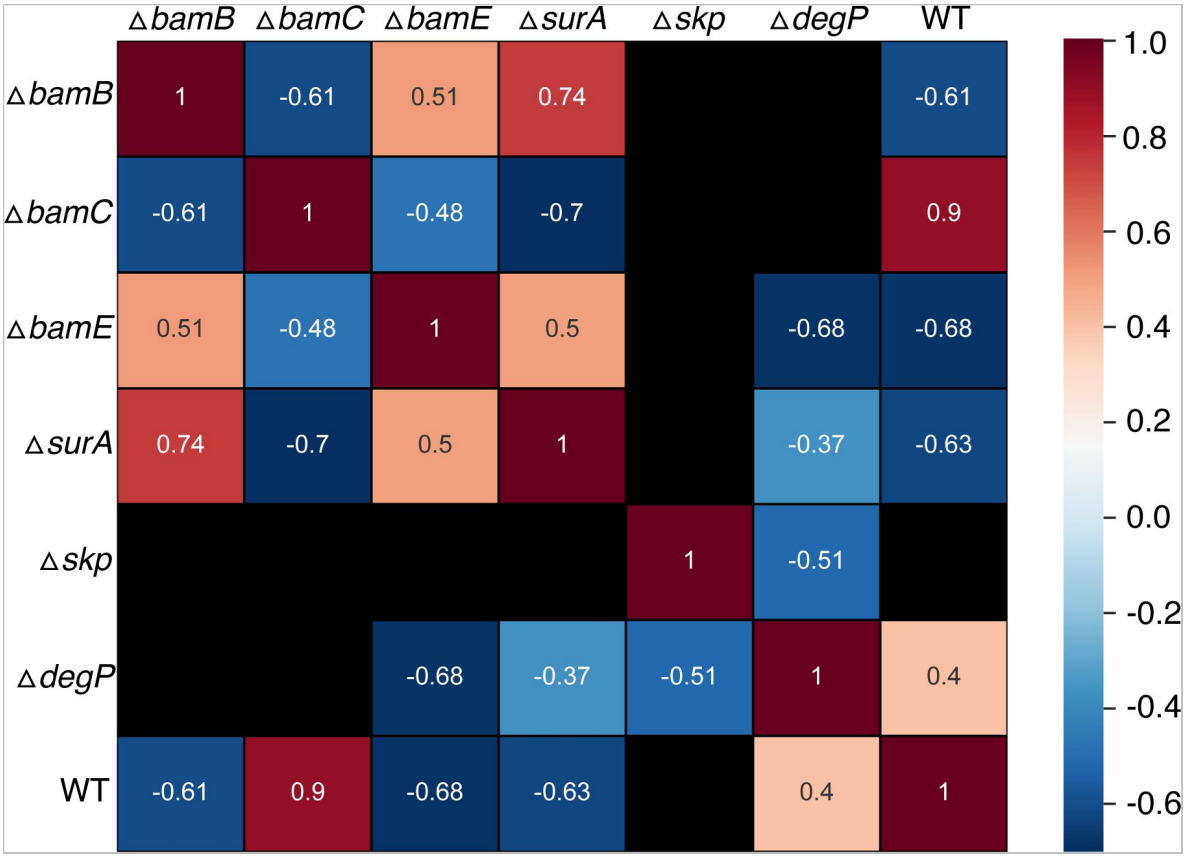


Figure S1

Correlation of phenotypic profiles

Heatmap of Pearson correlation coefficients for each pair of strain phenotypic profiles. Correlation of fitness ratios for each strain was assessed by calculating the Pearson correlation coefficient for averaged fitness scores across replicates using Python before being plotted as a heatmap. Black squares indicate a p-value ≥0.05 meaning the correlation coefficient achieved was not statistically significant.

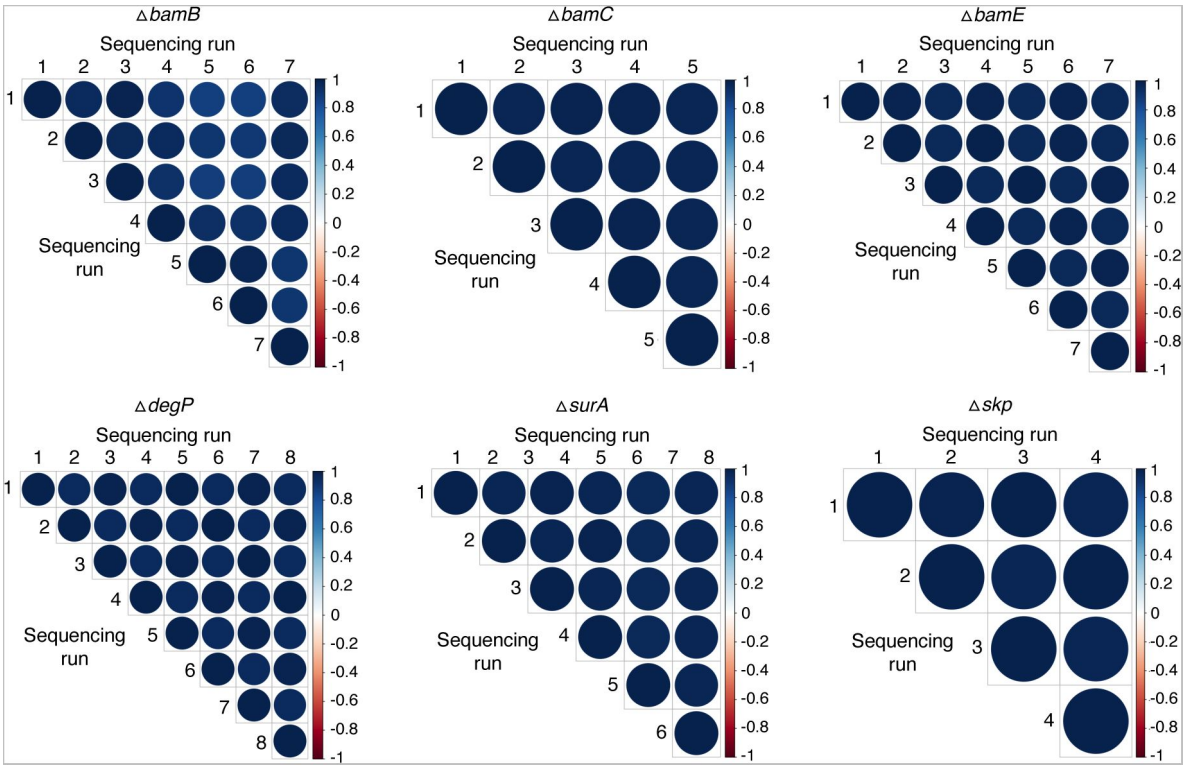


Figure S2

Reproducibility across TraDIS library sequencing runs

Correlogram of insertion indexes between individual sequencing runs for each TraDIS library. The colour and size of the bubbles correspond to the strength of the Pearson correlation coefficient between the indices for the same genes across the runs. The blue colour represents positive correlation.

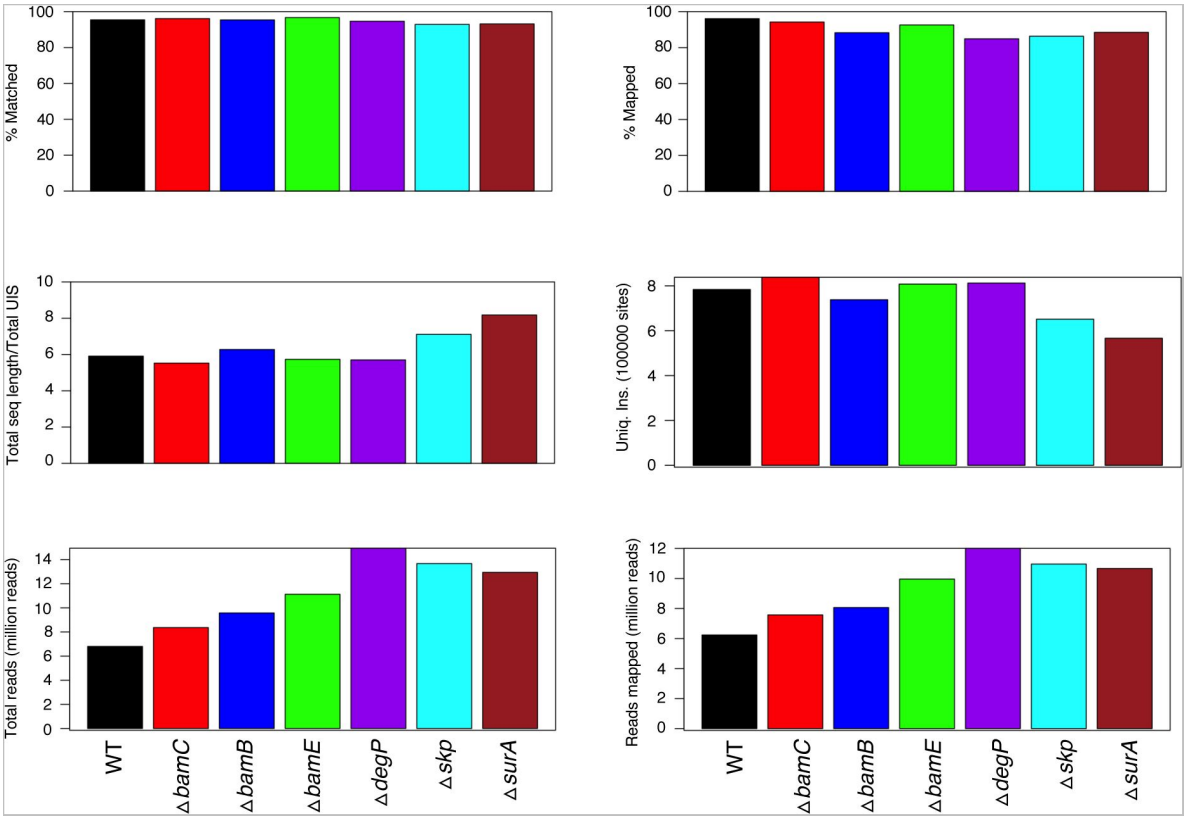


Figure S3

Transposon library construction metrics

TraDIS library construction metrics showing the percentage of sequencing reads that matched the transposon tag and that mapped to the chromosome. Total sequencing length as a function of total unique insertion sites, the total number of unique insertion sites for each sample, total number of sequencing reads, and the number of reads that mapped are also shown.

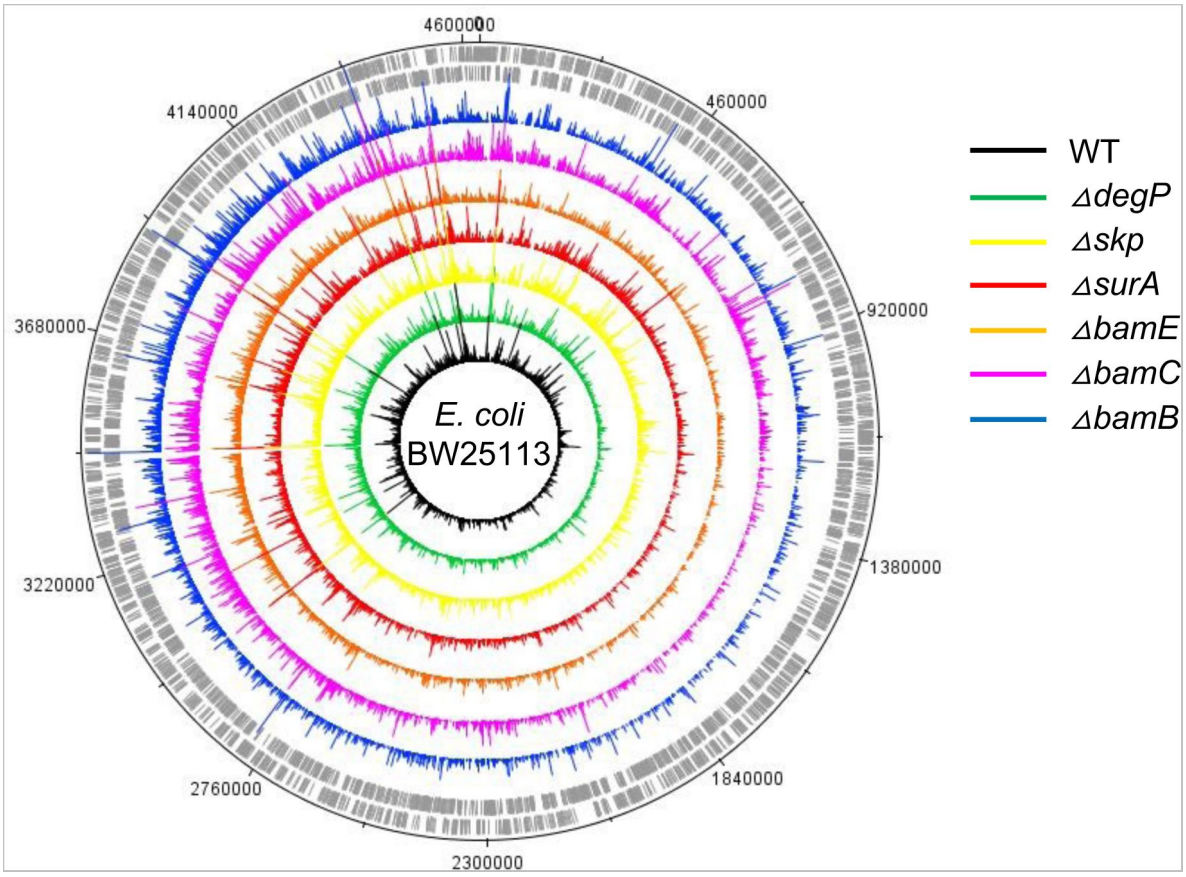


Figure S4

Circular plots of transposon insertion density in TraDIS libraries.

Circular plot of transposon insertion sites in the parent BW25113 (WT), Δ*degP,* Δ*skp,* Δ*surA,* Δ*bamE*, Δ*bamC*, Δ*bamB* TraDIS libraries, listed from innermost tracks outwards, generated using DNAPlotter. The outermost track marks the BW25113 genome in base pairs with the inner grey bar tracks corresponding to sense and antisense CDS.

Figure S5

Examples of known synthetic lethal interactions

Transposon insertions in the genes *degP*, *skp*, *bamB* or *bamE* in the WT parent strain, Δ*bamB*, or Δ*surA* mutant TraDIS libraries. Conditionally-essential genes are represented as red arrows, non-essential genes are represented by grey arrows. Transposon cut-off is set to 50.

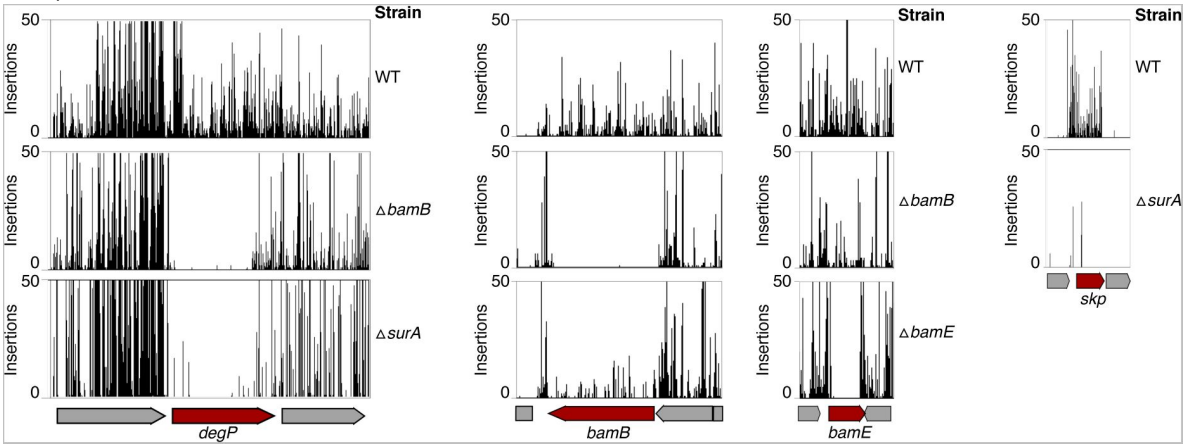
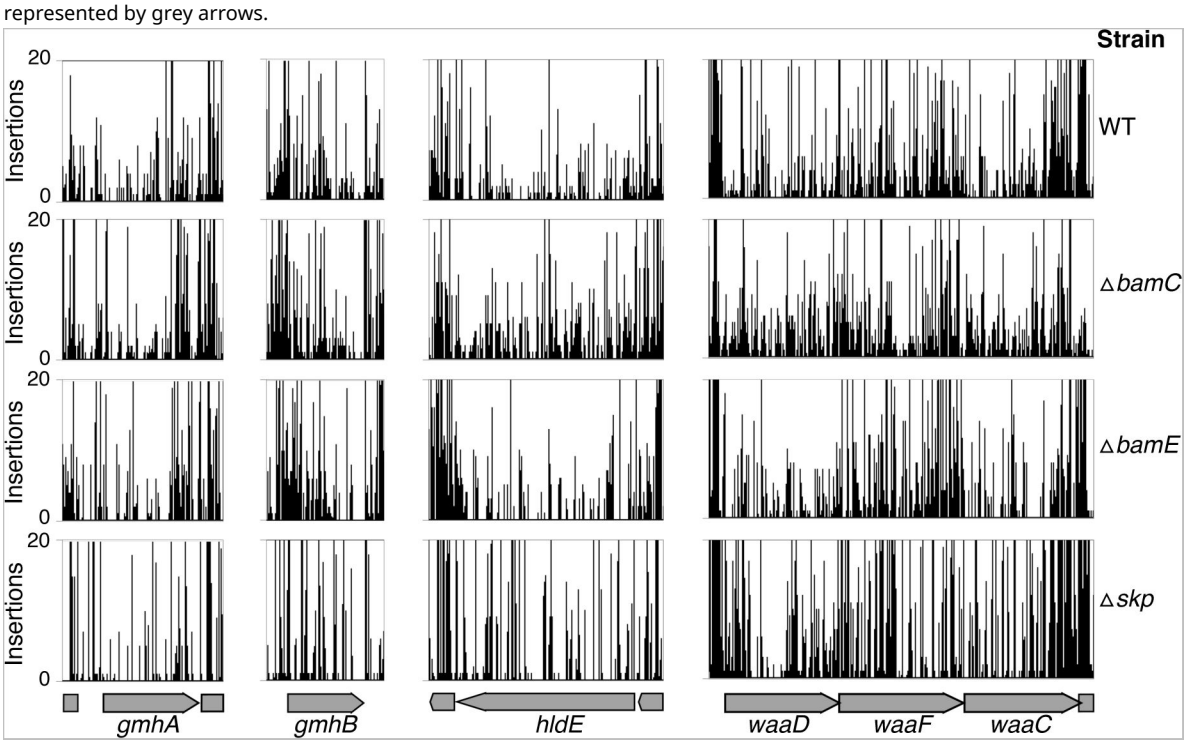


Figure S6

Transposon insertions in genes required for heptose biosynthesis in the Δ*bamC,* Δ*bamE* and Δ*skp* TraDIS libraries

Transposon insertions in the genes *gmhA*, *gmhB*, *hldE, waaD, waaC,* and *waaF* in the parent, Δ*bamC,* Δ*bamE* and Δ*skp* TraDIS libraries. Transposon cut-off is set to 20. Essential genes are represented as red arrows and non-essential genes are represented by grey arrows.



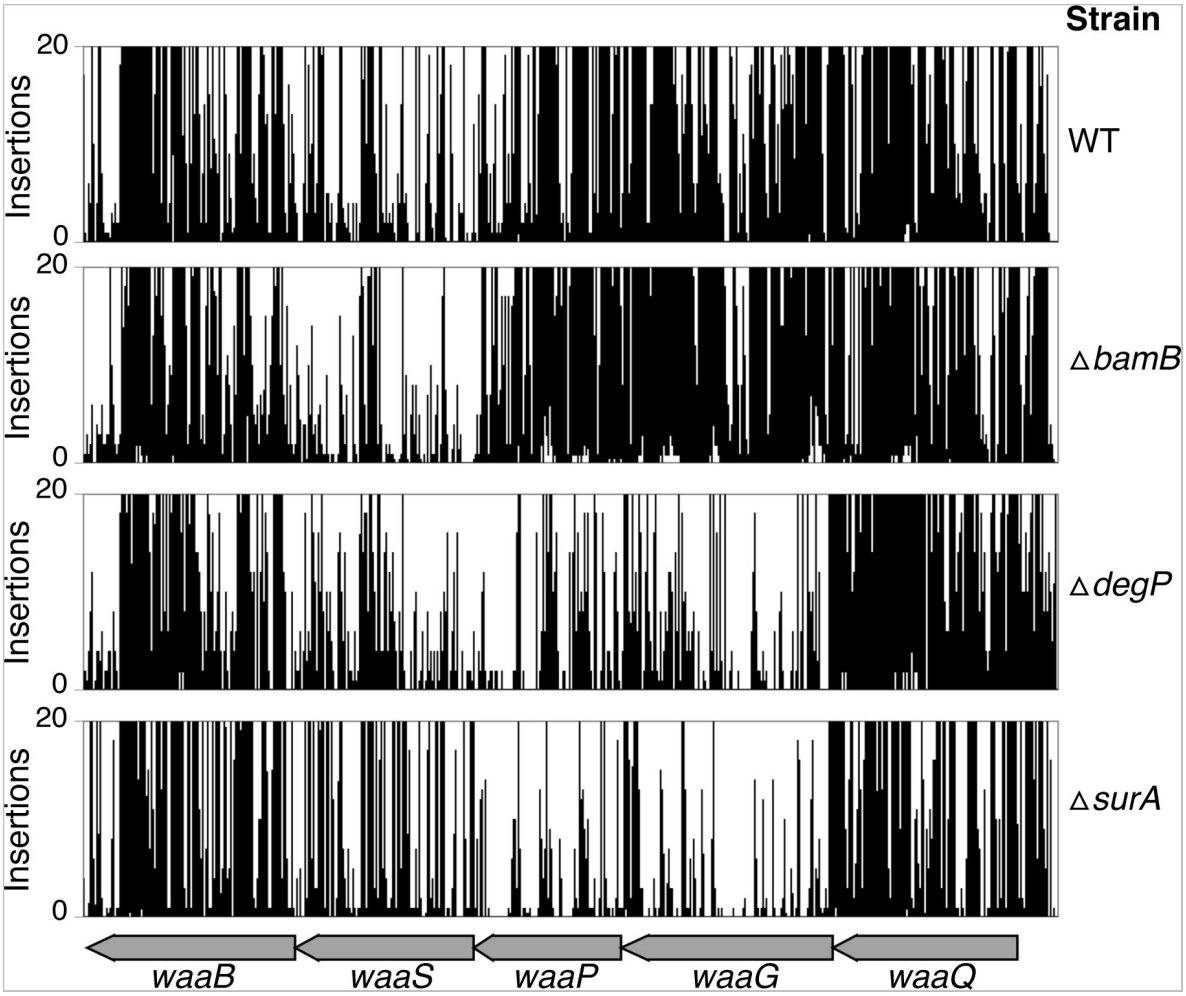


Figure S7

Transposon insertions in the *waaG* and *waaP* genes in the WT, Δ*bamB,* Δ*degP* and Δ*surA* TraDIS libraries

Transposon insertions in the genes *waaB, waaS, waaP, waaG* and *waaQ* in the parent, Δ*bamB,* Δ*degP* and Δ*surA* TraDIS libraries. Transposon cut-off is set to 20. Genes are represented by grey arrows.

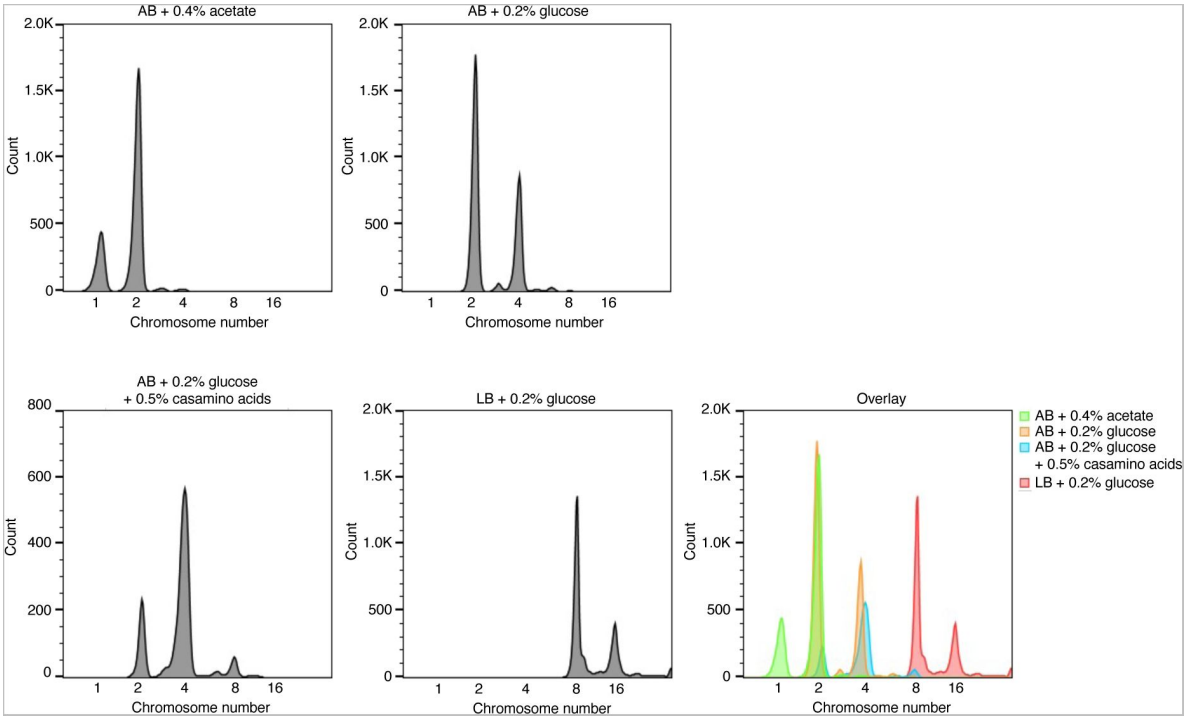


Figure S8

Replication run-out assay standards

Flow cytometry of *E. coli* MG1655 cells grown in media supporting different growth rates followed by replication run-out assay. Cells were grown in AB minimal medium supplemented with either 0.4% acetate, 0.2% glucose, or 0.2% glucose + 0.5% casamino acids, or LB supplemented 0.2% glucose at 37°C with aeration before being treated with rifampicin, cephalexin and stained with Sytox Green. Fluorescence is plotted and represents chromosomal content for each cell with chromosome numbers for each peak marked. An overlay of the separate data panels is presented.

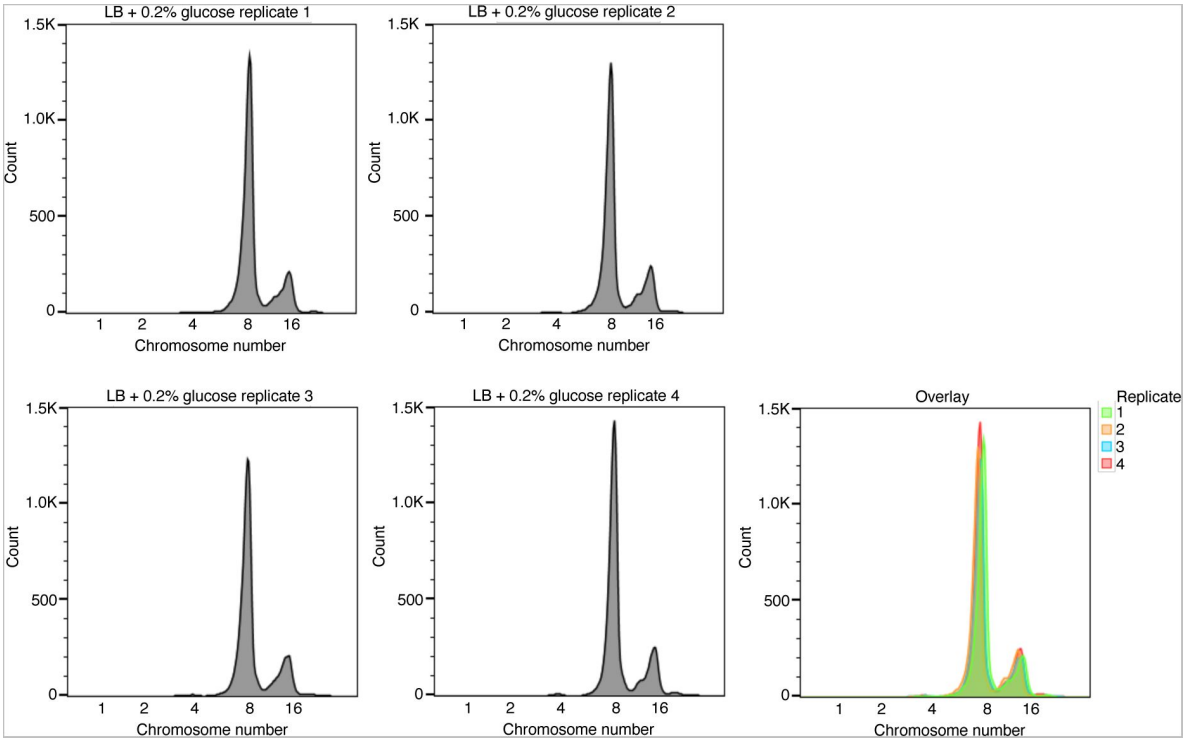


Figure S9

Reproducibility of replication run-out assays for the parent strain

Flow cytometry of *E. coli* LCE-18 cells grown in LB supplemented with 0.2% glucose at 37°C with aeration before being treated with rifampicin, cephalexin and stained with Sytox Green. Fluorescence is plotted and represents chromosomal content for each cell with chromosome numbers for each peak marked. Each experiment was repeated on 4 separate occasions and an overlay of the separate data panels is presented.

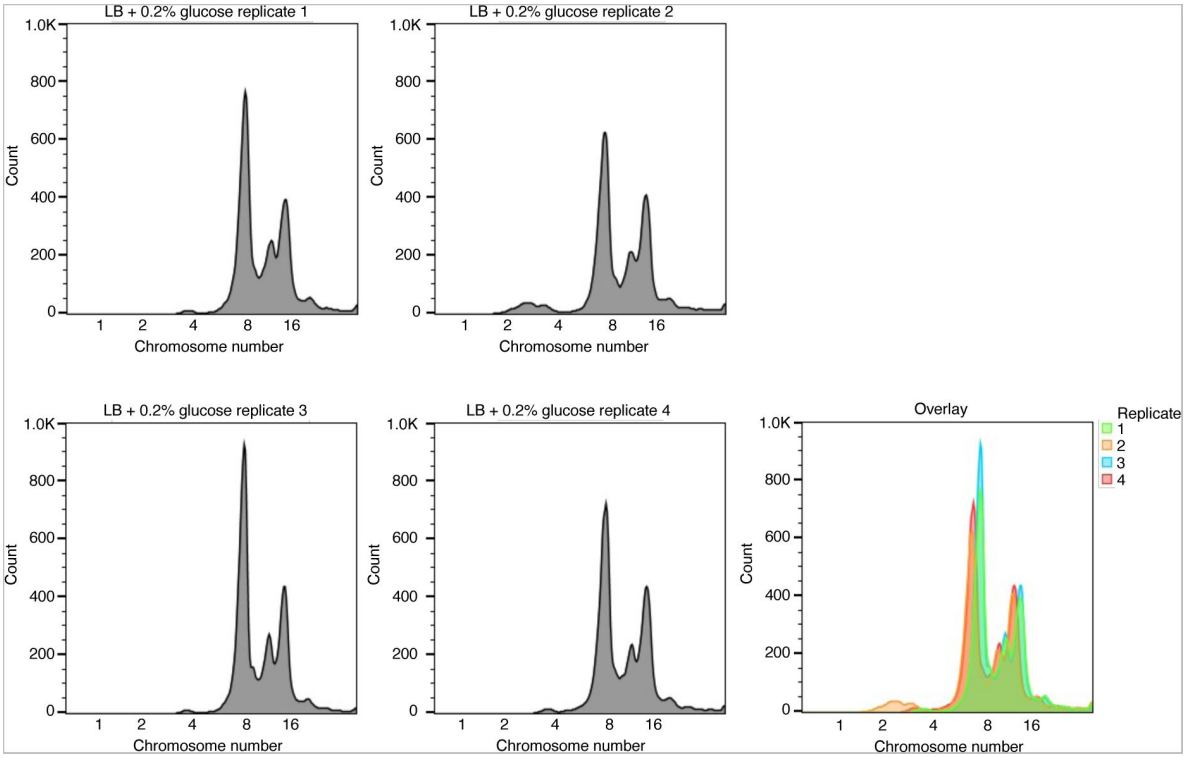


Figure S10

Reproducibility of replication run-out assays for the Δ*bamB* strain

Flow cytometry of *E. coli* LCE-18 Δ*bamB* cells grown in LB supplemented with 0.2% glucose at 37°C with aeration before being treated with rifampicin, cephalexin and stained with Sytox Green. Fluorescence is plotted and represents chromosomal content for each cell with chromosome numbers for each peak marked. Each experiment was repeated on 4 separate occasions and an overlay of the separate data panels is presented.

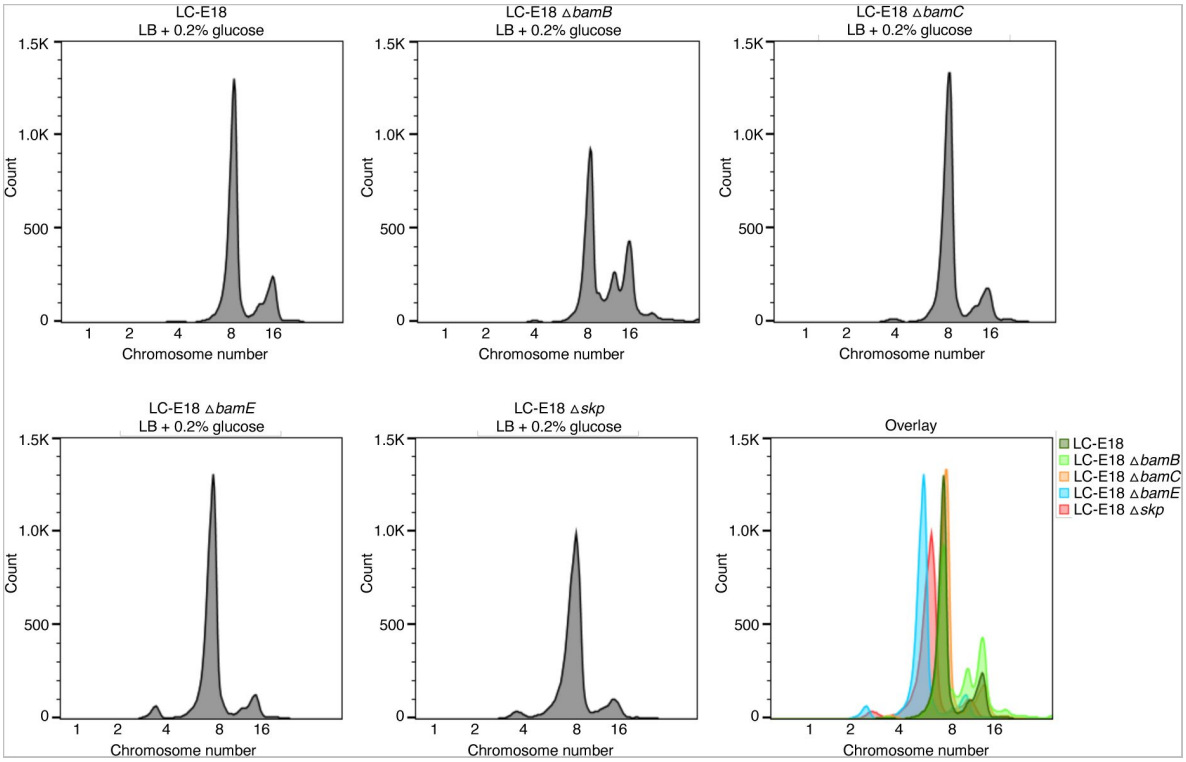


Figure S11

Replication run-out assays for the parent, Δ*bamB,* Δ*bamC,* Δ*bamE* and Δ*skp* strains

Flow cytometry of *E. coli* LCE-18 WT parent, Δ*bamB,* Δ*bamC,* Δ*bamE* or Δ*skp* cells grown in LB supplemented with 0.2% glucose at 37°C with aeration before being treated with rifampicin, cephalexin and stained with Sytox Green. Fluorescence is plotted and represents chromosomal content for each cell with chromosome numbers for each peak marked. An overlay of the separate data panels is presented.

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Reviewer #1 (Public Review):

Summary:

The overall goal of the manuscript is to delineate pathways that are conditionally essential with the Bam complex and associated chaperones. The Bam complex is made of several proteins, including BamA and BamD, which are essential. The protein complex works to insert proteins in the asymmetric outer membrane. Substrates are translated in the cytoplasm prior to transport across the cell envelope to the Bam complex. Transport includes non-essential periplasmic chaperones, SurA, Skp, and DegP. According to the authors, the pathways were assumed to be redundant. The Bam complex also includes non-essential components, BamBCE. These were thought to be accessory components that interact with BamA and BamD to coordinate optimal activity. While some roles have been assigned to BamE and BamB, a detailed understanding of the role of each accessory Bam protein is lacking. In this study, more specific roles for each non-essential Bam component are proposed.

Strengths:

The overall findings are intriguing and could advance our understanding as to how the Gram-negative cell envelope is assembled. These studies could provide new targets for antimicrobial treatment. In general, the manuscript was well-written.

Weaknesses:

While the overall findings are interesting, I had some concerns with the data analysis, presentation, and conclusions. Not all the conclusions are supported by data. The proposed revisions include experimental and editorial work. The manuscript is generally well-written and could provide impactful data to advance the field if the concerns are addressed.

Major concerns:

Overall Comments:

(1) The cutoffs the authors used to define "conditionally essential" mutants are not reported. The results also lack validation for lethality using a titratable system. It would be ideal to validate several genes in each dataset to determine cutoffs (i.e. 5-fold decrease in insertion mutants) for conditional lethality. It was not done (or described) here.

(2) Also, two mutations that both make the cells sick could provide an additive effect (i.e. dapF and BamB), which doesn't necessarily mean the pathways are linked. The authors should revise their wording. They have not shown genetic linkage in some cases.

(3) Mutations throughout the manuscript are not complemented. It would be ideal to add complementation data to show the gene-phenotype relationship is specific.

(4) Also, I would argue the term "conditionally essential genes" should be replaced with "synthetically lethal". Strains were compared in the same conditions but with different genetic backgrounds.

https://doi.org/10.7554/eLife.99955.1.sa2

Reviewer #2 (Public Review):

Summary:
Bryant et al. apply phenotypic profiling and saturating transposon mutagenesis to investigate the role of the non-essential lipoproteins BamB, BamC, and BamE, along with chaperones DegP, Skp, and SurA, in the biogenesis of the bacterial outer membrane. This generated a set of genetic interactions that revealed that changes in LPS and outer membrane fluidity impact Bam activity, and that the cyclic form of enterobacterial common antigen becomes essential in the absence of the chaperone surA. The study also uncovers that peptidoglycan crosslinking and DNA replication control are conditionally essential with the absence of certain Bam components, suggesting a coordination between outer membrane protein (OMP) biogenesis and other cellular processes such as lipid and peptidoglycan synthesis, as well as DNA replication.

Strengths:

(1) This is probably the first comprehensive analysis of genetic interactions involving Bam-associated proteins and should provide rich insight to refine the mechanistic understanding of this complex machine and the process of OM biogenesis.

(2) Good quality data and analysis. Well-presented manuscript.

Weaknesses:

(1) An important control in any genetic interaction study is to do complementation tests to demonstrate that the phenotype observed is indeed due to the missing gene under analysis. Although the Keio library was designed to avoid polar effects, it is impossible to predict other undesirable effects of the deletions (hitting of a non-annotated sRNA or RNA stability effects, for example). Thus, before one can safely conclude that a proposed genetic interaction is real, complementation tests should be carried out. This seems particularly important in the case of a new and surprising interaction, such as that between bamB and DNA replication and repair genes.

(2) Why not include the suppressor interactions in the work? There are probably plenty, and in principle, they should be as informative as the conditional essential (or synthetic lethal) ones. The only one highlighted in the paper is that between bamB and diaA, since it nicely fits with the synthetic lethal effects with initiation inhibitors seqA and hda. Even if the authors cannot make sense of the suppressor interactions, their inclusion in the paper should make the dataset richer and more valuable to the community.

(3) The enrichment analysis in Figure 2B deserves some clarification. What is the meaning of gene ratio? How can single genes of a pathway yield an enrichment signal? Why werenŕ seqA and hda included in the DNA replication class in 2B?

(4) The writing puts too much emphasis on demonstrating that bam lipoproteins and chaperones are specialized instead of fully redundant. However, I have the impression this is

a long-settled conclusion in the field, as the manuscript itself describes at several points when reviewing the literature.

https://doi.org/10.7554/eLife.99955.1.sa1

Reviewer #3 (Public Review):

In this work, Bryant, et al. investigate genetic interactions between non-essential members of the outer membrane protein biogenesis pathway and other genes in the genome using a transposon-directed insertion sequencing (TraDIS) approach in E. coli K-12. The authors identify interactions with other components of the envelope including LPS, peptidoglycan, and enterobacterial common antigen biogenesis, and they tie these interactions to specific members of the outer membrane biogenesis pathway. Although many of these interactions are known and have been previously investigated in the field, the study provides several synthetic phenotypes that could be useful for further investigations.

The strengths of the paper include their unbiased, TraDIS approach, and follow up on the interactions they observe. The interactions with genes of unknown function also are of interest as they may suggest experiments to find the functions of these genes. The largest weakness of this paper is the use of a gene deletion allele for bamB that is known to be polar leading to decreased expression of an essential gene. This largely invalidates all results related to DNA replication. In addition, it is a weakness that the paper does not adequately address its place in the field through discussion of existing results on the interactions they investigate.

https://doi.org/10.7554/eLife.99955.1.sa0

Author response:

We would like to thank the reviewers for their time and for their kind comments about our work. We expect that their comments will help us to improve the manuscript and so will plan the following experiments/revisions to address some of their comments:

> ***Reviewer 1 (Public Review):***
>
> *(1) The cutoffs the authors used to define "conditionally essential" mutants are not reported. The results also lack validation for lethality using a titratable system. It would be ideal to validate several genes in each dataset to determine cutoffs (i.e. 5-fold decrease in insertion mutants) for conditional lethality. It was not done (or described) here.*

We will report the cutoffs used when we generate the revised manuscript. Our experiments identified hundreds of lethal combinations and we have six datasets, validation of several genes from each would require generation of at least 20 depletion strains and subsequent testing of each. Validation using a depletion system would therefore be a significant undertaking and is typically not the standard when using these approaches. However, should time permit then we will attempt a subset of these experiments.

> *(2) Also, two mutations that both make the cells sick could provide an additive effect (i.e. dapF and BamB), which doesn't necessarily mean the pathways are linked. The authors should revise their wording. They have not shown genetic linkage in some cases.*

We will revise the text to address this.

> *(3) Mutations throughout the manuscript are not complemented. It would be ideal to add complementation data to show the gene-phenotype relationship is specific.*

We thank the reviewers for highlighting this and will complete the complementation experiments.

> *(4) Also, I would argue the term "conditionally essential genes" should be replaced with "synthetically lethal". Strains were compared in the same conditions but with different genetic backgrounds.*

We take the reviewers point and will revise the text accordingly.

> ***Reviewer 2 (Public Review):***
>
> *Weaknesses:*
>
> *(1) An important control in any genetic interaction study is to do complementation tests to demonstrate that the phenotype observed is indeed due to the missing gene under analysis. Although the Keio library was designed to avoid polar effects, it is impossible to predict other undesirable effects of the deletions (hitting of a non-annotated sRNA or RNA stability effects, for example). Thus, before one can safely conclude that a proposed genetic interaction is real, complementation tests should be carried out. This seems particularly important in the case of a new and surprising interaction, such as that between bamB and DNA replication and repair genes.*

We thank the reviewers for highlighting this and will complete the complementation experiments.

> *(2) Why not include the suppressor interactions in the work? There are probably plenty, and in principle, they should be as informative as the conditional essential (or synthetic lethal) ones. The only one highlighted in the paper is that between bamB and diaA, since it nicely fits with the synthetic lethal effects with initiation inhibitors seqA and hda. Even if the authors cannot make sense of the suppressor interactions, their inclusion in the paper should make the dataset richer and more valuable to the community.*

These data are available in supplementary table 1. However, we appreciate this is not obvious and so will make a new supplementary table and include a brief description of the data for the revised paper.

> *(3) The enrichment analysis in Figure 2B deserves some clarification. What is the meaning of gene ratio? How can single genes of a pathway yield an enrichment signal? Why weren t seqA and hda included in the DNA replication class in 2B?*

We apologise for the confusion caused and will include a description of the analysis in the methods section.

> *(4) The writing puts too much emphasis on demonstrating that bam lipoproteins and chaperones are specialized instead of fully redundant. However, I have the impression this is a long-settled conclusion in the field, as the manuscript itself describes at several points when reviewing the literature.*

We will revise the text to reduce this emphasis.

> ***Reviewer #3 (Public Review):***
>
> *In this work, Bryant, et al. investigate genetic interactions between non-essential members of the outer membrane protein biogenesis pathway and other genes in the genome using a transposon-directed insertion sequencing (TraDIS) approach in E. coli K-12. The authors identify interactions with other components of the envelope including LPS, peptidoglycan, and enterobacterial common antigen biogenesis, and they tie these interactions to specific members of the outer membrane biogenesis pathway. Although many of these interactions are known and have been previously investigated in the field, the study provides several synthetic phenotypes that could be useful for further investigations.*
>
> *The strengths of the paper include their unbiased, TraDIS approach, and follow up on the interactions they observe. The interactions with genes of unknown function also are of interest as they may suggest experiments to find the functions of these genes. The largest weakness of this paper is the use of a gene deletion allele for bamB that is known to be polar leading to decreased expression of an essential gene. This largely invalidates all results related to DNA replication. In addition, it is a weakness that the paper does not adequately address its place in the field through discussion of existing results on the interactions they investigate.*

We appreciate the reviewers' comments and concerns about the bamB allele, and we will address these concerns by completing complementation experiments for the CRISPRi depletion experiments and the run-out assays. However, despite the statement that it is known to be polar, several previous studies have also used the bamB Keio library strain. Many of these studies transfer the allele to a clean background and use the derivative in which the cassette has been removed as we have done here (Cox et al., 2017, Gunasinghe et al., 2018, Psonis et al., 2019, Storek et al., 2019, Ranava et al. 2021, Steenhuis et al., 2021, Thewasano et al., 2023). Therefore, we feel somewhat justified in our choice of strain.

We are unable to find a reference for the Keio bamB strain causing polar effects and would have appreciated the reviewers' guidance here. However, we believe the concern about polar effects stems from the observations of Ruiz et al., (2005), in which it was observed that a yfgL::ISE1 allele causes polar effects. This was hypothesised to be due to the ORF contained within the IS being transcribed in the opposite orientation to yfgL and the downstream der gene. They subsequently observed that a strain carrying a Tn5KAN-I-SceI insertion in yfgL (yfgL::kan) did not cause polar effects and this was hypothesised to be due to the kan cassette being co-oriented with yfgL. In addition, Charlson et al., 2006 generated a yfgL deletion by replacing the majority of the gene with a kan cassette in a manner similar to that of the Keio library that was subsequently flipped out. This study also found no evidence of polar effects on der. In theory, the strain used here, and in previous studies by other groups, should provide minimal disruption to transcription through generation of a mini-gene from the original bamB sequence to maintain operon expression. This is in contrast to the disruption caused by the yfgL::ISE1 allele.

While we do appreciate the concern, several pieces of evidence lend themselves to counter the statement that our strain choice largely invalidates the results. The der GTPase is essential, hence the concern about polar effects leading to the bamB phenotypes we see. However, depletion of der leads to cold sensitivity, whereas we find that the bamB strain used here actually performs better in colder temperatures. In addition, the der depletion is sensitive to doxycycline, whereas the bamB mutant has increased fitness in this condition (Fig 1) (Bharat and Brown, 2015, Hwang and Inouye, 2008). Hence, should the mutation lead to decreased expression of der then we would expect the bamB strain to phenocopy the der depletion, which it does not. Regardless of this information, we will still address these concerns by completing complementation experiments.

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