

***Caulobacter* lipid A is conditionally dispensable in the absence of *fur* and the presence of a novel anionic sphingolipid**

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Summary

Lipid A, the membrane-anchored portion of lipopolysaccharide (LPS), is an essential component of the outer membrane (OM) of nearly all Gram-negative bacteria. Here, we identify regulatory and structural factors that together render lipid A nonessential in *Caulobacter crescentus*. Mutations in the ferric uptake regulator *fur* allow *Caulobacter* to survive in the absence of either LpxC, which catalyzes an early step of lipid A synthesis, or CtpA, a tyrosine phosphatase homolog which we find is needed for wild-type lipid A structure and abundance. Alterations in Fur-regulated processes, rather than iron status *per se*, underlie the ability to survive when lipid A synthesis is blocked. Fitness of lipid A-deficient *Caulobacter* requires a previously uncharacterized anionic sphingolipid, ceramide phosphoglycerate (CPG), which also mediates sensitivity to the antibiotic colistin. Our results demonstrate that, in an altered regulatory landscape, anionic sphingolipids can support the integrity of a lipid A-deficient OM.

Introduction

Gram-negative bacteria are enclosed in a three-layer envelope, composed of the inner or cytoplasmic membrane (IM), a thin layer of peptidoglycan (PG), and an outer membrane (OM). The OM is an asymmetric bilayer, with phospholipids populating the inner leaflet and lipopolysaccharide (LPS) predominating in the outer leaflet. The canonical LPS structure, first described in *Escherichia coli*, consists of three segments: 1) lipid A, a hexa-acylated, phosphorylated glucosamine disaccharide anchored in the membrane; 2) a core oligosaccharide usually shared by members of the same species; and 3) a repeating polysaccharide (O-antigen) which can vary highly among strains of the same species (Whitfield and Trent, 2014). LPS confers robust barrier function upon the OM, making it inherently less permeable than the IM to small hydrophobic compounds (Nikaido, 2003).

Although the O-antigen and core polysaccharide are nearly always dispensable, it is widely accepted that the lipid A portion of LPS is essential for the viability of Gram-negative bacteria. Some exceptions to this rule are species that possess a dual membrane system but naturally lack lipid A, such as *Sphingomonas* spp. and the spirochetes *Borrelia burgdorferi* and *Treponema pallidum* (Kawahara et al., 1991; Kawasaki et al., 1994; Radolf and Kumar, 2018). Efforts to eliminate lipid A from *E. coli* strains have demonstrated that the intermediate molecule lipid IV_A is sufficient for viability, but only if the strain also has compensatory mutations that promote the export of this species across the IM (Mamat et al., 2008; Meredith et al., 2006). To

date, lipid A-deficient mutants have been recovered in *Neisseria meningitidis*, *Moraxella catarrhalis*, and *Acinetobacter baumannii* (Moffatt et al., 2010; Peng et al., 2005; Steeghs et al., 1998). It remains unclear why at least a minimal lipid A structure is essential in some Gram-negative bacteria but not others.

Lipid A is synthesized by the highly conserved Raetz pathway (Whitfield and Trent, 2014), yet significant variation exists in lipid A structures. In many species, mechanisms exist to modify the 1- and 4'-phosphates of lipid A to decrease its negative charge and reduce susceptibility to cationic antimicrobial peptides (Moffatt et al., 2019). In a few species, replacement of the 1- and/or 4'-phosphates of lipid A with sugars is constitutive (De Castro et al., 2008; Plötz et al., 2000). The predominant lipid A species in the alphaproteobacterium *Caulobacter crescentus* (Smit et al., 2008) varies from that of *E. coli* (Qureshi et al., 1988) in that the central glucosamine disaccharide is replaced by two 2,3-diamino-2,3-dideoxy-D-glucopyranose (GlcN3N) residues, and the 1- and 4'-phosphates are replaced by galactopyranuronic acid (GalpA) residues.

The tyrosine phosphatase homolog *ctpA* (for *Caulobacter* tyrosine phosphatase *A*) is essential for viability and is implicated in cell envelope maintenance, but its molecular function remains unknown (Shapland et al., 2011). Depletion of *ctpA* causes extensive OM blebbing, failure to resolve PG at the division site, and cell death. Here, we show that *ctpA* is required for the wild-type structure and abundance of lipid A. A screen for suppressors of *ctpA* essentiality recovered strains with null mutations in the O-antigen biosynthetic pathway or in the ferric uptake regulator *fur*. Surprisingly, mutations in *fur* also permitted the deletion of *lpxC*, which encodes an otherwise essential enzyme catalyzing the first committed step in lipid A synthesis. $\Delta ctpA$ and $\Delta lpxC$ strains containing suppressor mutations have significantly reduced or undetectable levels of lipid A, respectively.

To identify mechanisms that promote survival in the absence of lipid A, we used random barcode-transposon site sequencing (RB-Tnseq) to identify genes important for fitness when lipid A synthesis is chemically inhibited. Interestingly, we obtained several hits in genes required for sphingolipid synthesis in *Caulobacter* (Stankeviciute et al., 2019, 2021). Since *Sphingomonads* naturally lack LPS and bear anionic sphingolipids on the cell surface (Kawasaki et al., 1994), we hypothesized that anionic sphingolipids could support viability in the *Caulobacter* $\Delta lpxC$ strain. Indeed, we identified a previously unknown sphingolipid species, ceramide phosphoglycerate, which is produced in wild-type cells and is a critical fitness factor in the absence of lipid A. Further, we found that ceramide phosphoglycerate, rather than LPS, underlies *Caulobacter*'s sensitivity to the cationic antimicrobial peptide colistin.

Results

Suppressor mutations affecting fur or O-antigen synthesis permit the loss of ctpA

We used a CtpA depletion strain developed in a prior study to identify mutations that would support *Caulobacter* viability in the absence of CtpA (Shapland et al., 2011). Regulated depletion of CtpA in KR3906 is achieved by expressing *ctpA::3xFLAG::ssrA* from a xylose-inducible promoter (Meisenzahl et al., 1997) on a high-copy plasmid in a $\Delta ctpA$ strain also lacking the proteolytic adaptor *sspB* (Levchenko et al., 2000). The native CtpA protein could not be depleted without an *ssrA* tag to target it for proteolysis. However, addition of this tag made CtpA proteolysis so rapid that xylose-dependent expression of CtpA-3xFLAG-ssrA did not support viability. Further deleting *sspB*, which encodes a proteolytic adaptor for *ssrA*-tagged substrates, reduced the basal rate of CtpA-3xFLAG-ssrA degradation enough to permit complementation in xylose-supplemented PYE medium (PYEX).

When depleting CtpA by transferring cells from PYEX to dextrose-supplemented PYE medium (PYED), KR3906 exhibits division defects, significant OM blebbing, and death (**Fig. 1**). We UV-mutagenized KR3906, plated on solid PYED medium to deplete CtpA, and grew the recovered colonies in PYED liquid medium to allow loss of the plasmid bearing *ctpA*. Isolates cured of the plasmid were identified via chloramphenicol sensitivity and the inability to PCR-amplify *ctpA* (**Fig. 1A**). Genome resequencing of 17 confirmed suppressors yielded 15 strains with mutations in nine genes predicted to participate in O-antigen biosynthesis, one strain with a single mutation in *fur*, encoding the ferric uptake regulator, and one strain harboring a mutation in *fur* along with a mutation in an O-antigen biosynthetic gene (**Table S1**). Although individual strains sometimes contained point mutations in other genes, every suppressor strain harbored a mutation predicted to affect either O-antigen synthesis or Fur. Due to the frequent occurrence of frameshift or nonsense mutations, we assumed that each mutation disrupted the function of the affected gene.

We chose for further analysis candidate suppressor genes whose functions were well-established in *Caulobacter* or other bacteria. *CCNA_00497* encodes a predicted glycosyltransferase necessary for wild-type levels of smooth LPS (S-LPS) containing the O-antigen in *Caulobacter* (Hershey et al., 2019). *CCNA_01553* encodes a glycosyltransferase which initiates O-antigen synthesis on undecaprenyl-phosphate (Toh et al., 2008). *CCNA_03733* encodes a homolog of *manC* involved in synthesizing the activated sugar GDP-D-mannose (Samuel and Reeves, 2003), which is present in the core oligosaccharide and O-antigen of *Caulobacter* S-LPS (Jones et al., 2015). *CCNA_00055* encodes the iron-

responsive transcriptional regulator Fur (da Silva Neto et al., 2009) and is predicted to be functionally distinct among the genes harboring suppressor mutations. We deleted each gene in the wild-type background (NA1000) or in combination with $\Delta sspB$, but we were unable to subsequently delete *ctpA* in these strains by double homologous recombination. Therefore, we individually deleted these genes in the CtpA depletion strain KR3906 while propagating the strains on PYEX to supply CtpA.

To determine how each deletion affects cells during acute CtpA depletion, we shifted each mutant onto liquid or solid PYED medium and observed cell morphology and viability (**Fig. 1**). Compared to CtpA depletion in KR3906, depletion of CtpA in the Δfur mutant caused much less OM blebbing, but still yielded elongated cells indicative of a division defect (**Fig. 1C**). Surprisingly, neither OM blebbing nor cell chaining/elongation was markedly improved when CtpA was depleted from the strains lacking *CCNA_00497*, *CCNA_01553*, or *CCNA_03733*. Despite the persistence of one or more morphological defects, deletion of *fur*, *CCNA_01553*, or *CCNA_03733* significantly improved cell viability during CtpA depletion on solid PYED (**Fig. 1B**). The deletion of *CCNA_00497* improved survival on PYED to a lesser extent, despite the fact that two independent strains with point mutations in *CCNA_00497* were isolated in the suppressor screen. Notably, each strain with a mutation in *CCNA_00497* also harbored 1-2 other mutations (**Table S1**), which may have augmented the fitness of the original isolates.

We acquired stable, suppressed $\Delta ctpA$ mutants by passaging each modified CtpA depletion strain (above) in PYED medium, plating on solid PYED medium, and screening viable colonies for loss of the *ctpA*-bearing plasmid. The OM of each stable $\Delta ctpA$ strain was smooth with minimal blebbing, but chains of cells were still prevalent in the $\Delta ctpA \Delta sspB$, $\Delta CCNA_01553$ and $\Delta ctpA \Delta sspB \Delta fur$ mutants (**Fig. 1D**). The reconstituted suppressor strains were morphologically similar to the original isolates containing point mutations in the same genes (**Fig. S1A**). Suppressed $\Delta ctpA$ mutants grew more slowly than the wild-type strain NA1000 and the corresponding *ctpA*⁺ strains, but all achieved similar stationary phase densities in PYE medium (**Fig. S1B**). As expected, restoring the expression of *fur*, *CCNA_00497*, or *CCNA_03733* using a xylose-inducible promoter reduced the viability of each corresponding stable $\Delta ctpA$ strain (**Fig. S1C**). Thus, null mutations affecting *fur* or O-antigen biosynthesis allow *Caulobacter* to survive in the absence of *ctpA*.

It was puzzling that we could not obtain the strains $\Delta ctpA \Delta CCNA_00497$, $\Delta ctpA \Delta CCNA_1553$, $\Delta ctpA \Delta CCNA_03733$, or $\Delta ctpA \Delta fur$ (with or without $\Delta sspB$) by double homologous recombination, yet they were accessible by depleting CtpA and curing the *ctpA* covering plasmid. A key difference between these two procedures is that double homologous

recombination relies on *sacB* counterselection, where the desired mutants must survive on medium containing 3% sucrose. Unlike the parent $\Delta fur \Delta sspB$ strain, the $\Delta ctpA \Delta fur \Delta sspB$ strain displayed a growth defect on PYE medium containing sucrose (**Fig. S1D**), indicating that $\Delta ctpA$ confers susceptibility to sucrose, which likely accounts for the discrepancy between the two genetic methods.

To confirm the functions of *CCNA_00497*, $\Delta CCNA_01553$, and *CCNA_03733* in O-antigen synthesis, we deleted individual genes in a strain lacking *sspB*. Whole-cell lysates treated with proteinase K were probed with antibodies recognizing S-LPS (**Fig. S2A**) or stained with Pro-Q Emerald 300 to detect carbohydrates (**Fig. S2B**). As previously observed, strains lacking *CCNA_01068* (*wbqA*) or *CCNA_01553* lacked S-LPS, while $\Delta CCNA_000497$ contained reduced amounts of S-LPS, which migrates as a single high-molecular weight species in *Caulobacter* (Walker et al., 1994; Awram and Smit, 2001; Hershey et al., 2019). Deletion of *CCNA_03733* (*manC*) eliminated S-LPS and reduced the size of a species that we propose to be lipid A + core polysaccharide (**Fig. S2B-C, ****). The core oligosaccharide of *Caulobacter* LPS contains a single penultimate mannose residue (Jones et al., 2015); thus, the reduced size of the indicated band for $\Delta CCNA_03733$ (**Fig. S2B and S2C, ***) may arise from an incomplete core oligosaccharide. S-LPS was restored to each mutant by xylose-driven complementation of the respective genes (**Fig. S2C**). In contrast to strains with mutations in *CCNA_00497*, *CCNA_01553*, or *CCNA_03733*, the $\Delta fur \Delta sspB$ mutant contained wild-type levels of S-LPS (**Fig. S2A-B**), indicating that *fur* mutations do not suppress the lethality of $\Delta ctpA$ by eliminating the O-antigen.

$\Delta ctpA$ and $\Delta lpxC$ strains containing suppressor mutations contain little or no lipid A

ctpA is transcribed divergently from an operon containing *msbA*, *lpxJ*, *kdtA*, and *lpxK* (Zhou et al., 2015), which in other bacteria participate in the synthesis and export of lipid A + core polysaccharide (Whitfield and Trent, 2014). Like *ctpA*, these genes are essential for *Caulobacter* viability (Christen et al., 2011). Since CtpA depletion results in OM defects, and suppressor mutations were identified in O-antigen biosynthetic genes, we hypothesized that *ctpA* is required for some aspect of LPS synthesis or export.

We performed hot aqueous-phenol extraction of LPS from suppressor mutants lacking *ctpA*, along with their *ctpA*⁺ counterparts, and analyzed them by PAGE and Pro-Q Emerald 300 staining. As expected, full-length S-LPS was recovered from NA1000, $\Delta sspB$, and $\Delta fur \Delta sspB$ (**Fig. 2B, *****), but was absent from $\Delta sspB$ strains lacking *CCNA_00497*, *CCNA_01553*, or *CCNA_03733*. Interestingly, all $\Delta ctpA$ strains were deficient in low-molecular weight species

that could represent lipid A +/- core oligosaccharide (**Fig. 2B**). We therefore used the Limulus Amebocyte Lysate (LAL) assay to measure lipid A abundance in live *Caulobacter* strains. All $\Delta ctpA$ mutants contained ~1,000-fold less lipid A than strains encoding this gene (**Fig. 2A**).

Since $\Delta ctpA$ suppressor mutants could survive with drastically reduced amounts of lipid A, we asked if mutations in *fur* or O-antigen synthesis could render lipid A completely dispensable. LpxC catalyzes the first committed step in lipid A synthesis, removal of the 2-acetyl group from acylated UDP-GlcNAc (Whitfield and Trent, 2014). The *lpxC* homolog CCNA_02064 is essential for viability in wild-type *Caulobacter* (Christen et al., 2011). We constructed an LpxC depletion strain (KR4007) analogous to the CtpA depletion strain. We subsequently deleted *fur*, CCNA_00497, CCNA_01553, or CCNA_03733 in this strain and examined the effects of acute LpxC depletion during growth in PYED. When these O-antigen synthesis genes and *fur* were intact, LpxC depletion yielded chains of cells with extensive membrane blebs. Cells lacking a gene for O-antigen synthesis still showed OM blebs and chaining when LpxC was depleted (**Fig. 3A**). Cells lacking *fur* had far fewer OM blebs upon LpxC depletion but were still frequently elongated or chained (**Fig. 3A**). These morphologies are generally similar to those seen during CtpA depletion, but unlike CtpA, only Δfur allowed significant growth of the LpxC depletion strain on solid PYED medium (**Fig. 3B**).

When we attempted to isolate stable $\Delta lpxC$ mutants by depleting LpxC and curing the covering plasmid, only the strain harboring a Δfur mutation permitted complete loss of *lpxC* (KR4103). Importantly, we recovered two stable $\Delta lpxC$ isolates (KR4224 and 4225) from an LpxC depletion strain harboring only $\Delta CCNA_00497$ and not $\Delta sspB$ (KR4223), but genome resequencing revealed that they had acquired additional point mutations in *fur* (**Table S2**). Together, these results indicate that *fur* mutations are necessary to render $\Delta lpxC$ inessential, and that deletion of *sspB* is not required for the viability of $\Delta lpxC$ cells. As in $\Delta ctpA \Delta fur \Delta sspB$, the stable $\Delta lpxC \Delta fur \Delta sspB$ mutant still formed chains (**Fig. 1D**), and xylose-driven *fur* expression induced lethality in this strain (**Fig. 3C**).

Background levels of lipid A were detected in $\Delta lpxC \Delta fur \Delta sspB$ cells in the LAL assay (**Fig. 2A**), strongly suggesting that lipid A is absent. To corroborate this result, we extracted LPS species by three distinct methods, separated them by PAGE, and stained with Pro-Q Emerald 300. Hot aqueous-phenol extracts of $\Delta lpxC \Delta fur \Delta sspB$ cells were deficient in S-LPS and putative lipid A +/- core species (**Fig. 2B**). However, unknown carbohydrate-containing species were extracted by this method. Extraction of free lipid A (El Hamidi et al., 2005) revealed that a species of ~1800 Da, consistent with the mass of *Caulobacter* lipid A (Smit et al., 2008), is present in NA1000 but absent from $\Delta lpxC \Delta fur \Delta sspB$ (**Fig. 2C, left**). Again, however,

unidentified carbohydrate species were present in these extracts. Lastly, the method of Darveau and Hancock (Darveau and Hancock, 1983) yielded a single rough LPS species which was present in NA1000 and absent from $\Delta lpxC \Delta fur \Delta sspB$ (**Fig. 2C, right**); this method resulted in no unidentified contaminants. Although some *Caulobacter* extracts contain unidentified lipids, these assays together strongly imply that lipid A is absent from the $\Delta lpxC \Delta fur \Delta sspB$ mutant. Xylose-driven expression of *lpxC* or *ctpA* restored the production of lipid A-containing species to $\Delta lpxC \Delta fur \Delta sspB$ or $\Delta ctpA \Delta CCNA_03733 \Delta sspB$, respectively (**Fig. 2D**).

Lipid A extracts from $\Delta ctpA \Delta fur \Delta sspB$, $\Delta lpxC \Delta fur \Delta sspB$, and control strains were further analyzed by matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-MS/MS). Wild-type NA1000, $\Delta sspB$, and $\Delta fur \Delta sspB$ extracts contained predominantly the full-length lipid A (m/z 1874, (Smit et al., 2008) and lesser amounts of an ion at m/z 1858 that differs from 1874 by 16 m/z , consistent with the absence of one hydroxyl group (**Fig. S3A-C**). MALDI-MS analyses of lipid A extracts from $\Delta ctpA \Delta fur \Delta sspB$ cells revealed no ions consistent with full-length *Caulobacter* lipid A, but identified ions at m/z 1682 and m/z 1486 (**Fig. S3D**) that appeared to be missing the GalpA residues at the 1 and 4' positions. Tandem MS analysis of these ions revealed losses of mass consistent with the loss of phosphates, as would be expected for the dissociation of canonical, phosphate-bearing lipid A structures. Although additional characterization is needed, our results suggest that *Caulobacter* mutants lacking CtpA produce a lipid A species which retains phosphate at the 1 and 4' positions, and which lacks one or more of the secondary fatty acids. While these lipid A species were detectable by mass spectrometry, gel electrophoresis and LAL assays indicate that they are much less abundant than the lipid A in wild-type strains.

Lipid A extracts from the $\Delta lpxC \Delta fur \Delta sspB$ mutant yielded no ions consistent with wild-type lipid A and instead contained an unknown lipid (**Fig. S3E**, m/z 1412). Numerous attempts to interpret the structure of this ion using the same type of tandem MS data as used in Fig. S3A-D failed to generate a structural hypothesis resembling lipid A derivatives or other known lipids. Again, it is important to note that while this unknown ion was detected by mass spectrometry, gel electrophoresis and LAL assays together indicate that lipid A is absent from $\Delta lpxC \Delta fur \Delta sspB$ cells.

Lipid A-deficient Caulobacter mutants produce a three-layer cell envelope

We analyzed NA1000, $\Delta ctpA \Delta fur \Delta sspB$ and $\Delta lpxC \Delta fur \Delta sspB$ strains via electron cryotomography to assess the effects of mutations on cell envelope structure (**Movies S1-S4**). As expected, the S-layer is absent from both mutants due to the loss of its O-antigen

attachment site (Walker et al., 1994). Despite drastic reductions in lipid A levels, the $\Delta ctpA \Delta fur$ $\Delta sspB$ and $\Delta lpxC \Delta fur \Delta sspB$ mutants still generate a three-layer cell envelope, including an OM (**Fig. 2E**). Although much less severe than during acute CtpA depletion (**Movie S4**), membrane blebs were often observed at the cell poles or division sites in $\Delta ctpA \Delta fur \Delta sspB$ and $\Delta lpxC \Delta fur \Delta sspB$ cells (**Fig. 2E**). A large fraction of *ctpA* and *lpxC* mutant cells exhibited defects in stalk structure or internal membrane folds at the pole or midcell (N = 100; $\Delta ctpA \Delta fur$ s4

The ability to produce an outer membrane in the absence of LPS, along with excess membrane folds visible in the cytoplasm, suggest that Δfur could suppress $\Delta ctpA$ and $\Delta lpxC$ by increasing the synthesis of other lipids. We examined published transcriptomic data (Leaden et al., 2018; da Silva Neto et al., 2013) for a group of 22 genes predicted to participate in fatty acid or phospholipid synthesis. For 19 of these genes, expression was not altered in a Δfur mutant or in wild-type *Caulobacter* treated with 2,2-dipyridyl to limit iron (**Table S3**). Of the three remaining genes, two were downregulated and one was upregulated only by iron limitation. Thus, Fur seems not to exert a direct or indirect transcriptional effect on genes related to lipid synthesis. However, post-transcriptional effects in Δfur strains could cause an increase in the production of lipids other than lipid A.

Fur-regulated processes, rather than available iron levels, control the conditional essentiality of lipid A

LPS defects are usually associated with increased chemical sensitivity (Nikaido, 2003). Mutations in *fur* or O-antigen synthesis genes did not appreciably increase chemical sensitivity compared to NA1000, while strains lacking *ctpA* or *lpxC* had greater sensitivity to a subset of antibiotics and to all tested detergents (**Fig. 4A**). In sharp contrast, the $\Delta lpxC \Delta sspB$ and $\Delta ctpA \Delta sspB$ strains with suppressor mutations were much less susceptible to CHIR-090, an inhibitor of LpxC (McClerren et al., 2005) (**Fig. 4B**). We infer that suppressed $\Delta lpxC$ and $\Delta ctpA$ mutants are relatively insensitive to CHIR-090 because they already produce little lipid A or lack the target enzyme.

In agreement with its ability to suppress the lethality of $\Delta lpxC$ and $\Delta ctpA$ mutations, the Δfur allele by itself greatly reduced the sensitivity of *Caulobacter* to CHIR-090 (**Fig. 4B**). Fur is a widespread bacterial regulator of iron homeostasis that senses available Fe^{2+} , likely by reversibly binding a [2Fe-2S] cluster (Andrews et al., 2013; Fontenot et al., 2020). When bound to iron, Fur represses the transcription of genes for iron uptake and activates (directly or indirectly) the transcription of genes for iron-utilizing enzymes. Since iron is required for Fur-

directed transcriptional regulation, we asked whether iron limitation could mimic the phenotypes of a Δfur mutant. Culturing NA1000 with the iron chelator 2,2'-dipyridyl reduced its susceptibility to CHIR-090 to match that of the Δfur mutant (**Fig. 4B**). Neither depleting LpxC in fur^+ cells nor inducing fur in $\Delta lpxC \Delta fur \Delta sspB$ cells caused a reduction in viability in the presence of 2,2'-dipyridyl (**Fig. 4C, D**). The NA1000, $\Delta sspB$, and $\Delta fur \Delta sspB$ strains cultured in 2,2'-dipyridyl retained LPS and lipid A +/- core species (**Fig. 4E**). Therefore, low iron availability does not induce the loss of lipid A, but is sufficient to maintain *Caulobacter* viability when lipid A is eliminated by chemical or genetic means.

Because they are impaired in iron sensing, fur mutants of other bacteria accumulate more available iron than the corresponding wild-type strains (Liu et al., 2020; Wofford et al., 2019). We measured available iron levels using a streptonigrin (SNG) sensitivity assay (Justino et al., 2007; Nachin et al., 2001), because SNG killing is linked to the intracellular formation of oxygen radicals in the presence of iron (Hassett et al., 1987; Yeowell and White, 1982). Growth of the Δfur and $\Delta fur \Delta sspB$ strains was almost completely inhibited by 0.25 $\mu\text{g/ml}$ SNG, while NA1000 was only mildly inhibited (**Fig. 4F**), consistent with higher levels of available iron in Δfur mutants. These findings indicate that both excess available iron (in fur mutants) and iron depletion (by 2,2-dipyridyl) can support viability when lipid A synthesis is blocked chemically or genetically. Since fur deletion and iron chelation have the same effect on Fur-regulated gene expression, but are predicted to have opposite long-term effects on Fur-independent iron signaling (**Fig. 4G**), this implies that processes regulated by Fur in concert with iron are specifically responsible for the survival of lipid A-deficient *Caulobacter* (Leaden et al., 2018; da Silva Neto et al., 2013).

RB-Tnseq identifies sphingolipid synthesis genes needed for fitness when lipid A synthesis is chemically inhibited

To uncover additional factors that promote the survival of lipid A-deficient *Caulobacter*, we challenged an RB-Tnseq library constructed in NA1000 (Price et al., 2018) with CHIR-090. Individual barcode frequencies were measured by high-throughput sequencing before each trial and after growth in either PYE or PYE + 2 $\mu\text{g/ml}$ CHIR-090. To identify genes that are particularly important when LpxC is inhibited, we averaged and compared the gene fitness scores (Wetmore et al., 2015) from three trials in each condition (**Fig. 5A**). We anticipated that mutations in fur would increase fitness in CHIR-090, but the NA1000 RB-Tnseq library contained no insertions in fur . Surprisingly, nearly all genes known to be regulated by Fur

(Leaden et al., 2018; da Silva Neto et al., 2013) had similar fitness scores in unstressed and CHIR-090-exposed cultures (**Fig. 5A**).

Focusing on genes whose average fitness scores were ≥ 1 point lower in CHIR-090-treated cultures than in control cultures (**Table S4**), we identified five genes involved in sphingolipid synthesis: serine palmitoyltransferase (*spt*, *CCNA_01220*), acyl-carrier protein (*acp*, *CCNA_01221*), ceramide reductase (*cerR*, *CCNA_01222*), ACP-synthetase (*acps*, *CCNA_01223*), and bacterial ceramide synthase (*bcerS*, *CCNA_01212*) (Olea-Ozuna et al., 2021; Stankeviciute et al., 2021). Fitness scores were also significantly lower for transposon insertions in a neighboring operon of three uncharacterized genes predicted to modify lipids (*CCNA_01217-01219*, Marks et al., 2010) (**Fig. 5A**). None of the genes listed above is known to be regulated by Fur or iron (Leaden et al., 2018; da Silva Neto et al., 2013). Using qPCR, we demonstrated that transcription of *CCNA_01217-01219* is unchanged in $\Delta fur \Delta sspB$ cells and in the $\Delta lpxC \Delta fur \Delta sspB$ mutant (**Fig. 5B**). As controls, *lpxC* transcripts were not detected in $\Delta lpxC \Delta fur \Delta sspB$ cells, and *bfd* (*CCNA_03372* bacterioferritin-associated ferredoxin) transcripts were significantly increased in both mutants, as previously observed (**Fig. 5B**, (Leaden et al., 2018).

To examine the roles of genes in the uncharacterized operon, we constructed unmarked deletions in the NA1000 and $\Delta fur \Delta sspB$ backgrounds and complemented them with the corresponding genes expressed from the inducible *vanA* promoter (Thanbichler et al., 2007). Loss of *spt*, *CCNA_01217*, *CCNA_01218*, or *CCNA_01219* greatly increased the susceptibility to CHIR-090, either in NA1000 or in $\Delta fur \Delta sspB$ cells (**Fig. 5C**), and expression of the complementing gene from the *vanA* locus restored the wild-type level of susceptibility, validating the RB-Tnseq results.

Mutations in *CCNA_01217-01219* or *spt* could increase CHIR-090 sensitivity via distinct mechanisms: by damaging the cell's permeability barrier and giving easier access to CHIR-090, by making it more difficult for cells to grow after lipid A synthesis is inhibited, or both. To eliminate changes in drug access as a factor in the experiment, we measured the effects of *CCNA_01217*, *CCNA_01218*, and *spt* upon cell viability when LpxC was depleted. We deleted individual genes in the strain $\Delta lpxC \Delta fur \Delta sspB + P_{xyl-lpxC}::3xFLAG-ssrA$ (KR4091) and complemented them with *vanA*-driven copies as described above. The parent strain lacks *fur* and grows in PYED medium (with or without vanillate) when LpxC is depleted. In contrast, KR4091 lacking *CCNA_01217*, *CCNA_01218*, or *spt* grew poorly in PYED medium, and growth in PYED was fully or partially restored by expressing the complementing gene from the vanillate promoter (**Fig. 5D**). Since this assay does not rely on an exogenous inhibitor of LpxC, we

conclude that *CCNA_01217-8* and *spt* are critical for the fitness of lipid A-deficient *Caulobacter*, not simply for the exclusion of CHIR-090.

KR4091 lacking *CCNA_01218* grew poorly in PYEX medium without vanillate, where *lpxC* is transcribed (**Fig. 5D**), and we were unable to isolate a stable derivative of KR4091 harboring $\Delta CCNA_01219$ and *vanA::01219*. These findings could indicate that $\Delta CCNA_01218$ and $\Delta CCNA_01219$ have a negative genetic interaction with Δfur and/or $\Delta sspB$. However, we detected no significant additive growth phenotypes when $\Delta CCNA_01218$ or $\Delta CCNA_01219$ was combined with $\Delta fur \Delta sspB$ in strains where LpxC was expressed from the native locus (**Fig. 5E**). We therefore favor the hypothesis that $\Delta CCNA_01218$ or $\Delta CCNA_01219$ is particularly detrimental in the highly modified KR4091 background, where *fur* and *sspB* are absent, and an epitope-tagged version of LpxC is expressed from a nonnative promoter.

CCNA_01217-01219 convert neutral ceramide to an anionic sphingolipid, ceramide phosphoglycerate

The importance of Spt for viability in the absence of lipid A indicated a role for sphingolipids in this phenotype. Since *Sphingomonads* produce anionic glycosphingolipids (GSLs) on the outer membrane (Kawasaki et al., 1994), we initially hypothesized that *Caulobacter* responds to *lpxC* deletion by upregulating GSL production. The *Caulobacter* sphingolipid glycosyltransferases Sgt1 and Sgt2 are expressed specifically in phosphate-limiting conditions (Stankeviciute et al., 2019), and their transcripts were not upregulated in $\Delta fur \Delta sspB$ or $\Delta lpxC \Delta fur \Delta sspB$ cells grown in PYE (**Fig. 5B**). Further, transposon insertions in *sgt1* or *sgt2* did not reduce the fitness of CHIR-090-treated cells in RB-Tnseq experiments. Thus, while Sgt1 and Sgt2 are not critical in these experiments, they may be important for the fitness of lipid A-deficient *Caulobacter* under other conditions.

A careful analysis of the *Caulobacter* lipidome revealed a previously unidentified sphingolipid species, ceramide phosphoglycerate (**Fig. 6A**). In fact, we identified two forms of this lipid containing either one or two phosphoglycerate moieties (**Fig. 6A**) that we have designated CPG and CPG2. LC/MS/MS analysis confirmed the proposed structures of these lipids (**Fig. 6B**). To determine whether *CCNA_01217-01219* are involved in CPG/CPG2 synthesis, we analyzed lipid extracts from mutant and complemented mutant strains. Deletion of *CCNA_01218* led to the loss of CPG, CPG2 and ceramide phosphate, but preserved neutral ceramide (**Fig. 6C**). *CCNA_01218* is annotated as a sphingosine kinase-related protein and has a conserved LCB5 domain (Nagiec et al., 1998). Therefore, we propose that *CCNA_01218* adds the initial phosphate on the ceramide (**Fig. 6D**). The $\Delta CCNA_01219$ mutant lost CPG and

CPG2 but retained ceramide-phosphate (**Fig. 6C**). This is consistent with CCNA_01219 adding a glycerate molecule to ceramide phosphate to form CPG (**Fig. 6D**). CCNA_01219 has no conserved domains, and a BLAST analysis identified homologs only in Caulobacterales and Sphingomonadales. Deletion of CCNA_01217 resulted in the loss of CPG2 but had no effect on CPG, ceramide phosphate, or neutral ceramide (**Fig. 6C**). CCNA_01217 has a conserved phosphatidylglycerophosphate synthase (PgsA) domain which is normally involved in phosphatidylglycerol (PG) synthesis. PG is the dominant phospholipid in *Caulobacter* (Stankeviciute et al., 2019), but the essential PgsA ortholog CCNA_03002 is likely responsible for PG synthesis (Christen et al., 2011; Marks et al., 2010). Thus, we conclude that CCNA_01217 adds a second phosphoglycerate to CPG to form CPG2 (**Fig. 6D**). Each deletion could be complemented by expressing the respective gene from a vanillate-inducible promoter (**Fig. 6C**).

We note that the amount of CPG/CPG2 detected appears to be a relatively small percentage of the total lipidome (**Fig. S4**), raising the question of how these lipids can enable survival in the absence of lipid A. The CPG2 molecule is very polar, as evidenced by its very long LC retention time, and we expect that this lipid is not efficiently extracted by standard methods. Though we tried several modifications to increase the extraction yield, we made only marginal improvements. Additionally, our genetic data show that CpgA adds the second phosphoglycerate molecule to generate CPG2, but we cannot rule out the possibility of higher-order polymers containing additional phosphoglycerates, which would be even more polar and difficult to extract.

Ceramide phosphoglycerate mediates susceptibility to colistin

Cationic antimicrobial peptides (CAMPs) have been demonstrated to kill Gram-negative bacteria by first interacting with negatively charged groups on surface-exposed LPS . Phosphates at the 1 and 4' positions of lipid A are particularly important for this interaction, and several bacteria possess mechanisms to modify them, reducing their negative surface charge and sensitivity to CAMPs (Moffatt et al., 2019; Velkov et al., 2010). Despite lacking phosphate groups on its lipid A, *Caulobacter* is highly sensitive to colistin (**Fig. 7A**), and the antimicrobial effect is retained in the lipid A-deficient strain $\Delta lpxC \Delta fur \Delta sspB$ (**Fig. 7A**). Since the CPG/CPG2 lipids are anionic, we considered whether they may be the colistin target in *Caulobacter*. Indeed, the growth of mutants lacking *cpgA*, *cpgB*, or *cpgC* was unaffected by colistin (**Fig. 7A**). Since the deletion of *cpgA*, catalyzing the conversion of CPG to CPG2, can alone greatly reduce

colistin sensitivity, and since the elimination of lipid A had no effect, we infer that a primary target of colistin on the *Caulobacter* surface is CPG2. Furthermore, these findings are consistent with our hypothesis that CPG lipids are a significant component of the outer leaflet of the OM whose detection is limited by inefficient extraction.

Discussion

CtpA is required for wild-type lipid A structure and abundance

We performed a suppressor screen to discover the essential function of CtpA, which contains active site residues characteristic of tyrosine phosphatases. Inactivation of *fur* or genes involved in O-antigen synthesis permitted the deletion of *ctpA* and yielded cells with drastically reduced amounts of lipid A. MS/MS analysis of the remaining lipid A extracted from $\Delta ctpA \Delta fur \Delta sspB$ were consistent with species that retain phosphoryl groups at the 1 and 4' positions of the central disaccharide, suggesting that CtpA is responsible for dephosphorylating at least one of these positions, in preparation for the addition of GalpA residues.

Some alphaproteobacteria produce lipid A species with a tri- or tetrasaccharide backbone (De Castro et al., 2008). In *Rhizobia*, the phosphatases LpxE and LpxF dephosphorylate the 1 and 4' positions, respectively, of lipid A at the periplasmic surface of the IM (Karbarz et al., 2003; Wang et al., 2006). Sugars are then added to the 1 and 4' positions by the glycosyltransferases RgtF and RgtD, respectively, before the transport of mature LPS molecules to the OM (Brown et al., 2012, 2013). NA1000 harbors a gene (*CCNA_03113*) with similarity to *lpxE*, but none with similarity to *lpxF*, raising the possibility that CtpA substitutes for LpxF. Additional work is needed to test this hypothesis and to understand how mutations in *ctpA* affect the structure and abundance of lipid A.

Caulobacter requires a novel anionic sphingolipid to survive without lipid A

Here we demonstrate that the enzyme LpxC and lipid A itself are dispensable for viability in *Caulobacter crescentus*, conditional upon the absence of Fur and the presence of a previously uncharacterized anionic sphingolipid, ceramide phosphoglycerate (**Fig. 7B**). LPS molecules form a robust permeability barrier based on 1) tight packing of the six saturated acyl chains of lipid A, and 2) a lateral network formed by the bridging of phosphate groups on lipid A or the core polysaccharide by divalent cations such as Mg^{2+} and Ca^{2+} (Nikaido, 2003). *Caulobacter* lipid A and core polysaccharide lack the phosphates that would participate in a lateral network with divalent cations (Smit et al., 2008). We propose that negative charges on

CPG/CPG2 can provide this function in the *Caulobacter* OM, accounting for the observation that *cpgABC* and other sphingolipid synthesis genes are important for fitness even in non-stress conditions (**Fig. 5A** and Christen et al., 2011). Evidence that CPG/CPG2 contribute negative charge to the *Caulobacter* OM comes from studies of CAMP sensitivity. We previously observed that Spt is necessary for susceptibility to polymyxin B, but Sgt1 and Sgt2, which convert neutral ceramide to the anionic glycosphingolipid GSL-2, are not required (Stankeviciute et al., 2019). This result was puzzling, because neutral ceramide was not expected to be a target for CAMP activity. Here we provide an explanation by showing that neutral ceramide is converted by CpgABC to a different anionic species, CPG2, and that this lipid, rather than LPS, is critical for colistin susceptibility.

Inhibition of Fur-mediated gene expression is necessary to survive in the absence of lipid A

In contrast to Sphingomonads, the presence of cell surface sphingolipids is not sufficient for *Caulobacter* to survive in the absence of lipid A. Instead, the iron-responsive transcription factor Fur must also be deactivated. Both iron limitation (via growth in 2,2-dipyridyl) and excess available iron (due to a disruption in iron homeostasis in Δfur) supported the viability of lipid A-deficient *Caulobacter*. These results imply that genes or processes regulated by Fur in complex with iron, rather than those regulated by iron independently of Fur, are the critical factors.

Fur controls iron homeostasis in *Caulobacter* by directly or indirectly regulating ~120 genes in combination with iron (Leaden et al., 2018; da Silva Neto et al., 2009, 2013). A significant fraction of the Fur regulon, comprising 45 genes, is predicted to encode membrane proteins functioning in transport reactions or energy metabolism. *Caulobacter* Fur represses the transcription of genes predicted to mediate iron uptake, and it activates the expression of genes encoding iron-containing enzymes, including respiratory complexes harboring Fe/S clusters or heme groups. Fur is linked to oxygen signaling in *Caulobacter* by activating the transcription of *fixK*, which mediates the response to hypoxia (Crosson et al., 2005). In addition, the Δfur mutant has a constitutively elevated level of intracellular oxidation and displays impaired growth under oxidative stress, implicating Fur in the prevention of oxidative stress (da Silva Neto et al., 2009; Leaden et al., 2018).

Our *ctpA* suppressor screen retrieved mutations in *fur*, but not in genes whose transcription is activated by Fur. Thus, there is unlikely to be a singular Fur-activated gene whose expression is lethal when lipid A is depleted. Consistently, RB-Tnseq revealed that no transposon insertions in Fur-activated genes led to significantly increased fitness during challenge with CHIR-090. Since mutations would be more likely to cause loss than gain of

function, we might not retrieve suppressors that work by increasing gene expression or activity. However, if there were a singular Fur-repressed gene whose upregulation was required to render lipid A dispensable, then transposon insertions in this gene would be expected to reduce the fitness of CHIR-090-treated cells. Again, no individual gene fits this profile, but one caveat is that essential genes are excluded from RB-Tnseq analysis.

Based on our genetic data, mutations in *fur* could support the viability of lipid A-deficient *Caulobacter* via 1) downregulation of multiple Fur-activated genes, 2) upregulation of multiple Fur-repressed genes, and/or 3) activation of compensatory cellular stress responses. Since Fur regulates the expression of many OM and IM proteins (da Silva Neto et al., 2009; Leaden et al., 2018), deletion of *fur* could alter envelope composition in a manner that renders lipid A nonessential. Alternatively, the transcriptional changes and oxidative stress which follow from *fur* deletion could activate a network of stress responses which together make it possible to survive in the absence of lipid A.

The search for principles governing lipid A essentiality

Hypotheses to explain the essential nature of lipid A include its chemical barrier function, the detrimental activation of stress responses when it is depleted, its role in OM protein biogenesis or function, and its mechanical role in resisting turgor pressure (Rojas et al., 2018; Zhang et al., 2013). *Caulobacter* is only the fourth LPS-bearing Gram-negative bacterium demonstrated to survive in the absence of lipid A, following *N. meningitidis*, *M. catarrhalis*, and *A. baumannii*. So far, however, no single theme has emerged to explain why this select and phylogenetically diverse group of Gram-negative species is capable of surviving without lipid A. In *A. baumannii*, proteins which synthesize PG in lateral cell walls (the elongasome) are critical for the fitness of lipid A-deficient strains, suggesting that alterations in PG structure are needed to compensate for the OM's loss of mechanical strength (Simpson et al., 2021). However, since elongasome components are essential for viability in wild-type *Caulobacter* (Christen et al., 2011), RB-Tnseq could not reveal their fitness effects in CHIR-090-treated cultures. Lipid A-deficient strains of *A. baumannii* consistently display increases in the expression of lipoproteins and the Lol pathway for lipoprotein transport to the OM (Boll et al., 2016; Henry et al., 2015). Two lipoprotein synthesis genes, *lgt* (CCNA_00525) and *Int* (CCNA_00050), had markedly reduced fitness scores in CHIR-090-treated *Caulobacter* cultures compared to unstressed cultures, so OM lipoproteins may help to compensate for the absence of lipid A in diverse species.

A. baumannii Δ lpxC mutants have growth and morphological defects that are corrected when the growth rate is limited by environmental factors such as low temperature or nutrient limitation (Nagy et al., 2019), suggesting that one barrier to the elimination of lipid A is the rate of synthesis of alternative molecules to constitute the OM. Although Δ fur slows the growth of *Caulobacter* (Fig. S1, Table S5, and da Silva Neto et al., 2009), we found that slow growth in PYE medium at a reduced temperature was not sufficient to support the viability of *fur*⁺ *Caulobacter* depleted of LpxC (Fig. S5A), or of Δ lpxC Δ fur Δ sspB cells with *fur* expression restored (Fig. S5B). Furthermore, if slow growth were sufficient to render lipid A nonessential, then our screen for *ctpA* suppressors should have retrieved a wider variety of mutations that slow *Caulobacter* growth, rather than repeated mutations in *fur* and genes for O-antigen synthesis. Although reduced growth rate may play a role, we propose that the Δ fur mutation provides specific, unknown benefits which support viability in the absence of lipid A.

Our work suggests that possession of genes to produce anionic sphingolipids may provide certain Gram-negative bacteria with an unusual capacity to survive without lipid A. Anionic sphingolipids can also underlie clinically important phenotypes in wild-type membranes that retain LPS, such as the susceptibility to CAMPs, which are used as a last line of defense against multidrug-resistant bacterial infections. Thus, functions traditionally attributed to lipid A may be performed wholly or in part by alternative lipids, underscoring the need to study lipid A functions in diverse species and to identify and functionally characterize novel lipids.

Limitations of the study

Although the absence of Fur is clearly important for survival without lipid A, we do not yet understand how Δ fur renders lipid A inessential in *Caulobacter*. Deletion of *fur* may upregulate the synthesis of lipids other than lipid A posttranscriptionally, which was not assessed in this study. Although CtpA is needed for the correct structure and abundance of *Caulobacter* lipid A, this work did not uncover its precise biochemical function. Finally, due to technical difficulties with extracting polar lipids, we do not yet know how much CPG or CPG2 is present in the outer membrane, or if CPG polymers exist with more than two glycerates per molecule.

Acknowledgments

The authors thank Charlie Huang, Morgan Price, Laura Herron and Henry Seaborne for experimental assistance. Cryoelectron microscopy data acquisition was performed using instrumentation of Lawrence Berkeley National Laboratory and the Bay Area CryoEM resource, maintained by Jonathan Remis and Daniel Toso. Linux computer maintenance was provided by

Paul Tobias. D.R.G. thanks the International Centre for Cancer Vaccine Science project of the International Research Agendas program of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund MAB/2017/03 at the University of Gdansk. Funding for this work was provided by National Science Foundation awards 1553004 and 2031948 to E.A.K. and 1615287 to K.R.R., and National Institutes of Health awards GM111066-01 and 1R01AI123820-01 to D.R.G. Cryoelectron tomography work was supported by the Laboratory Directed Research and Development Program of Lawrence Berkeley National Laboratory under U.S. Department of Energy Contract No. DE-AC02-05CH11231.

Author contributions

J.J.Z., E.A.K., and K.R.R. conceived the project. Mass spectrometry experiments were performed and analyzed by S.H.Y., D.R.G., and Z.G. Electron cryotomography experiments were performed and analyzed by R.G. and K.M.D. RB-Tnseq experiments, sequencing, and data analysis were performed by J.J.Z and A.M.D., and K.R.R. All other experiments were performed and analyzed by J.J.Z., G.S, E.A.K., and K.R.R. The manuscript was written by J.J.Z., E.A.K, and K.R.R. with input from all authors.

Declaration of interests

The authors declare no competing interests.

Figure Legends

Figure 1. Suppressor mutations affecting *fur* or O-antigen biosynthesis permit the loss of *ctpA*. (A) Strategy for isolation of Δ *ctpA* suppressor mutants. (B) Viability of suppressor strains during CtpA depletion on PYED. (C) Differential interference contrast (DIC) images of the indicated strains grown in CtpA-expressing (PYEX) or non-expressing (PYED) conditions. D) DIC images of the indicated strains grown to exponential phase in PYE. Scale bar, 3 μ m.

Figure 2. Δ *ctpA* and Δ *pxC* strains with suppressor mutations contain little or no lipid A. (A) Endotoxin units (EU) per mL of whole cells of the strains indicated in panel B (mean $\pm$ S.D.). Dots represent individual data points, and mean values are displayed above bars. (B) Hot aqueous-phenol LPS extracts of the indicated strains. *** = S-LPS, ** = putative full-length lipid A-core, * = putative lipid A. (C) Lipid A (left) or rough LPS (right) extracted from the indicated strains. * = lipid A. ** = rough LPS. (D) Proteinase K-treated lysates of the indicated strains.

Cells were maintained in PYED or shifted into PYEX for 6 hours before harvesting. Samples were normalized by OD₆₆₀. NA1000 was grown in PYE. Leaky expression of LpxC can generate S-LPS (***) and lipid A + core (**) in PYED. Full-length S-LPS is not restored to *ctpA* and the lipid A-core species is reduced in size (*) because *CCNA_03733* is needed for mannose incorporation. (E) Electron cryotomography images of the indicated strains noting the inner membrane (IM), peptidoglycan (PG), outer membrane (OM), and S-layer. All strains were grown to exponential phase in PYE medium, except that CtpA was depleted from KR3906 during 12 hours of growth in PYED prior to analysis. Scale bars, 100 nm.

Figure 3. Deletion of *fur* supports the viability of Δ *lpxC* cells. (A) DIC images of the LpxC depletion strain alone or harboring the indicated mutations, grown in PYEX or PYED for 10 hours. Scale bar, 3 μ m. (B) Viability of the LpxC depletion strain, alone or harboring the indicated mutations, plated on PYEX or PYED. (C) Viability of Δ *lpxC* Δ *fur* Δ *sspB* cells harboring a P_{xyl}-*fur* or a P_{xyl}-*cerulean* expression vector. Plates included kanamycin to retain expression vectors.

Figure 4. Fur-regulated processes control the conditional essentiality of lipid A. (A) Sensitivity to the indicated chemicals measured by disc diffusion assay. Dots indicate individual data points. Suppressor mutations present in strains represented by blue or green bars are, from left to right, Δ *CCNA_00497*, Δ *CCNA_01553*, Δ *CCNA_03733*, and Δ *fur*. (B) CHIR-090 sensitivity measured by disc diffusion assay. Partial clearing indicates the diameter of a ring of intermediate growth. Dots indicate individual measurements of cleared and partially cleared zones (mean $\pm$ S.D.). ****, $p < 0.0001$; n.s., $p > 0.05$; one-way ANOVA followed by Dunnett's posttest comparison to NA1000. Strains exhibiting rings of partial growth were excluded from the analysis. (C) Viability of the LpxC depletion strain in inducing (PYEX) or depleting (PYED) conditions, in the presence or absence of 100 μ M 2,2'-dipyridyl. (D) Viability of the Δ *lpxC* Δ *fur* Δ *sspB* strain harboring a P_{xyl}-*fur* plasmid, grown in noninducing (PYED) or inducing (PYEX) conditions, in the presence or absence of 100 μ M 2,2'-dipyridyl. Plates included kanamycin to retain the expression vector. Brightness was reduced and contrast increased to improve the clarity of colonies grown on 2,2'-dipyridyl. (E) Proteinase K-treated lysates of the indicated strains grown overnight in the presence or absence of 100 μ M 2,2'-dipyridyl. Samples were normalized by OD₆₆₀. (F) Growth inhibition by SNG in liquid PYE cultures of the indicated strains. Dots represent individual OD₆₆₀ ratios (mean $\pm$ S.D.). ****, $p < 0.0001$; n.s., $p > 0.05$; one-way ANOVA followed by Šídák's multiple comparisons test, where each strain was compared to NA1000 grown in the same

condition. (G) Changes in Fur-regulated gene expression correlate with the ability to survive in the absence of lipid A. Genes regulated by Fur in concert with iron (sets A and B) are modulated similarly by deletion of *fur* or by iron limitation, while genes regulated by iron alone (sets C and D) are modulated in opposite directions in these two conditions.

Figure 5. RB-Tnseq identifies sphingolipid synthesis genes needed for fitness when LpxC is inhibited. (A) Average gene fitness scores for three challenges of the NA1000 RB-Tnseq library with PYE or PYE+2 μ g/ml CHIR-090. Fitness scores are color-coded based on regulation of the corresponding genes. Fitness scores of selected genes are indicated by colors matching the open reading frame diagram below. (B) Wild-type, $\Delta fur \Delta sspB$, and $\Delta fur \Delta lpxC \Delta sspB$ cells were grown to mid-log phase in PYE, and expression of the indicated genes was measured by qRT-PCR (n = 3 biological replicates, mean $\pm$ S.D). Gene expression is normalized to the wild-type sample for each gene tested. n.s., $p > 0.05$; *, $p < 0.05$; one-way ANOVA followed by Šídák's multiple comparisons test, where each strain was compared to wild-type. (C) CHIR-090 sensitivity of the indicated strains measured by disc diffusion assay. Where indicated, 0.5 mM vanillate was included in the medium. Dots represent individual measurements (mean $\pm$ S.D.). n.s., $p > 0.05$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ****, $p < 0.0001$; one-way ANOVA followed by Šídák's multiple comparisons test, where each condition was compared to NA1000 without vanillate or, for strains harboring $\Delta fur \Delta sspB$, to $\Delta fur \Delta sspB$ without vanillate. The shaded bar indicates a ring of partial growth, rather than complete clearing, and this sample was excluded from the analysis. (D) Overnight growth of strains expressing (xylose) or depleting (dextrose) LpxC, and expressing (vanillate) or not expressing the indicated genes. Dots represent individual measurements (mean $\pm$ S.D.). ****, $p < 0.0001$; n.s., $p > 0.05$; one-way ANOVA followed by Šídák's multiple comparisons test, where each condition was compared to growth of the same strain in PYED. (E) Growth curves of the indicated strains in PYE medium (mean $\pm$ S.D.).

Figure 6. CCNA_01217-01219 convert neutral ceramide to an anionic sphingolipid, ceramide phosphoglycerate. (A) Extracted ion-chromatograms identified the indicated sphingolipid species in NA1000 lipid extracts. (B) Structural determination of anionic sphingolipids was performed by MS/MS analysis. (C) The presence of the indicated sphingolipids was assessed in each deletion mutant and its respective complemented strain. x, no lipid of that type was detected in the indicated strain. The presence of neutral ceramide served as an extraction control for each strain. (D) Proposed mechanism for CPG2 synthesis.

Figure 7. Ceramide phosphoglycerate mediates susceptibility to colistin. (A) Overnight growth of the indicated strains in the presence or absence of 10 µg/ml colistin. Dots represent individual OD₆₆₀ measurements (mean ± S.D.) ****, $p < 0.0001$; n.s., $p > 0.05$; one-way ANOVA followed by Šídák's multiple comparisons test, where each strain treated with colistin was compared to the same strain without colistin. (B) Model of the *Caulobacter* cell envelope containing LPS and CPG2. Consequences for OM composition and colistin sensitivity when either lipid A (Δ pxC Δ fur) or sphingolipids (Δ spt) are eliminated. The presence of CPG2 and the absence of *fur* are together required for the viability of lipid A-deficient *Caulobacter*.

STAR Methods

RESOURCE AVAILABILITY

Lead contact

- Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kathleen R. Ryan (krr@berkeley.edu).

Materials availability

- Plasmids and bacterial strains generated in this study are available upon request from the lead contact.

Data and code availability

- Sequence data that support the findings of this study are openly available in the Sequence Read Archive at <https://www.ncbi.nlm.nih.gov/sra>, under BioProject ID PRJNA526705, with specific NCBI BioSample accession numbers listed in the key resources table and **Table S6**. RB-Tnseq data are publicly available at <https://fit.genomics.lbl.gov/>, with set and index numbers listed the key resources table and under RB-Tnseq analysis. Microscopy and gel electrophoresis images and mass spectrometry data reported here will be shared by the lead contact upon request. This paper analyzes existing, publicly available data, whose sources are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All *Caulobacter crescentus* strains were derived from NA1000 (Evinger and Agabian, 1977) and are listed in **Table S6**. *Caulobacter* was grown in peptone-yeast extract medium (PYE, Ely, 1991) at 30°C. PYE was supplemented with 0.3% xylose (PYEX) or 0.2% dextrose (PYED) where indicated. When changing between inducing and non-inducing conditions, cells were washed twice with PYE medium lacking supplemental sugars or vanillate before being released into or plated on medium with a different supplement. Counter-selection using *sacB* was performed using 3% sucrose. 100 µM 2,2'-dipyridyl was added to culture media to achieve low-iron conditions. Vanillic acid was added to PYE media at final concentrations of 0.5 mM (plate assays) or 0.1 mM (liquid assays) to drive gene expression from the *vanA* promoter. Antibiotics added to PYE were used at the following concentrations (µg/mL) for liquid (L) or solid (S) medium: kanamycin, 5 (L), 25 (S); chloramphenicol, 1 (L/S); nalidixic acid, 20 (S); gentamycin, 25 (L), 5 (S); oxytetracycline, 1 (L), 2 (S); spectinomycin, 25 (L), 100 (S); hygromycin, 100 (L/S); streptonigrin 0.025 or 0.25 (L). *E. coli* strains were grown in lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 37°C, supplemented with antibiotics at the following concentrations (µg/mL) for liquid (L) or solid (S) medium: kanamycin, 30 (L), 50 (S); chloramphenicol, 20 (L), 30 (S); gentamicin, 15 (L), 20 (S); tetracycline, 12 (L/S); spectinomycin, 50 (L/S); hygromycin, 100 (L/S). Diaminopimelic acid (0.3 mM) was added to solid or media to support the growth of *E. coli* strain WM3064 (Dehio and Meyer, 1997).

METHOD DETAILS

Plasmid construction. Plasmid descriptions are listed in **Table S7**. Primer sequences used for plasmid construction are listed in **Table S8**. Gibson assembly was performed using vectors linearized with restriction enzymes, DNA fragments amplified with Q5 High-Fidelity DNA Polymerase, and NEBuilder HiFi DNA Assembly Master Mix.

pZIK133. The LpxC depletion vector was constructed by placing the *lpxC* coding region, C-terminally fused to a 3xFLAG tag (amino acid sequence: DYKDHDGDYKDHDIDYKDDDDK) followed by the *Caulobacter* *ssrA* tag (amino acid sequence: AANDNFAEEFAVAA), under control of the *xylX* promoter. The *xylX* promoter was amplified using the pJS14-P_{xylX} and P_{xylX}-lpxC R primers. The P_{xylX}-lpxC F and lpxC-3xFLAG R primers were used to amplify *lpxC*. The C-terminal fusion was amplified from pAB6 using the lpxC-3xFLAG F and *ssrA*-pJS14

primers. The final plasmid was assembled via Gibson cloning into a BamHI/EcoRI-digested pJS14 backbone.

pZIK134. For the *lpxC* knockout construct, flanking homology regions were amplified using the primers *lpxC* UpF and *lpxC* UpR for the 5'- region, and *lpxC* DownF and *lpxC* DownR for the 3'- region. The 5'- arm included a 5'- *SpeI* site and a 3'- *EcoRI* site, and the 3'- arm included a 5'- *EcoRI* site and a 3'- *SphI* site. These fragments were digested with the indicated enzymes and ligated into *SpeI*/*SphI*-digested pNPTS138. This intermediate plasmid was linearized with *EcoRI*, and the *EcoRI*-digested *tetAR* cassette from pKOC3 was inserted to make the final construct.

pZIK73 and *pZIK78*. For knockouts of *CCNA_01553* or *CCNA_00497*, flanking homology regions were amplified using the following primer pairs: *pZIK73* 5'- region (01553 UpF; 01553 UpR), *pZIK73* 3'- region (01553 DownF; 01553 DownR), *pZIK78* 5'- region (00497::hyg UpF; 00497::hyg UpR), *pZIK78* 3'- region (00497::hyg DownF; 00497::hyg DownR). For each construct, the 5'- arm included a 5'- *SpeI* site and a 3'- *SmaI* site, and the 3'- arm included a 5'- *SmaI* site and a 3'- *EcoRI* site. These fragments were digested with the indicated enzymes and ligated into *SpeI*/*EcoRI*-digested pNPTS138. The intermediate plasmids were linearized with *SmaI*, and the *SmaI*-digested *hyg* cassette from pHP45Ω-*hyg* was inserted to make the final constructs.

pZIK80, *pZIK81*, *pZIK82*, and *pZIK161*. For the knockouts of *CCNA_03733*, *CCNA_01068*, *CCNA_01055*, or *CCNA_00055*, flanking homology regions were amplified using the following primer pairs: *pZIK80* 5'- region (03733::hyg UpF; 03733::hyg UpR), *pZIK80* 3'- region (03733::hyg DownF; 03733::hyg DownR), *pZIK81* 5'- region (01068::hyg UpF; 01068::hyg UpR), *pZIK81* 3'- region (01068::hyg DownF; 01068::hyg DownR), *pZIK82* 5'- region (01055::hyg UpF; 01055::hyg UpR), *pZIK82* 3'- region (01055::hyg DownF; 01055::hyg DownR), *pZIK161* 5'- region (*fur* UpF; *fur* UpR), *pZIK161* 3'- region (*fur* DownF; *fur* DownR). Each 5'- arm included a 5'- *SpeI* site and a 3'- *BamHI* site, and each 3'- arm included a 5'- *BamHI* site and a 3'- *EcoRI* site. These fragments were digested with the indicated enzymes and ligated into *SpeI*/*EcoRI*-digested pNPTS138. The intermediate plasmids were linearized with *BamHI*, and the *BamHI*-digested *hyg* cassette from pHP45Ω-*hyg* was inserted to make the final constructs.

781 *pZIK172-174*. *CCNA_00497*, *CCNA_01553*, or *CCNA_03733* were placed under control of the
782 *xyIX* promoter on pXCERN-2, which integrates at the *xyIX* promoter. The corresponding genes
783 were initially cloned into pVCERN-2 before being moved into pXCERN-2. Genes were amplified
784 with the following primer pairs: *CCNA_00497* (pVCERN-2 00497 F; pVCERN-2 00497 R),
785 *CCNA_01553* (pVCERN-2 01553 F; pVCERN-2 01553 R), *CCNA_03733* (pVCERN-2 03733 F;
786 pVCERN-2 03733 R). Primer sets replace the start codon with an NdeI site and add a SacI site
787 after the stop codon. The corresponding gene fragment and pVCERN-2 were digested with
788 NdeI and SacI and ligated together. An NdeI/MluI fragment was subsequently excised from
789 each vector and moved to pXCERN-2 cut with the same enzymes.

790

791 *pZIK175*. *CCNA_00055 (fur)* was placed under control of the *xyIX* promoter on pXCERN-2,
792 which integrates at the *xyIX* promoter. *CCNA_00055* was initially cloned into pVCERN-2 before
793 being moved into pXCERN-2. *CCNA_00055* was amplified using the P_{van-fur} and fur-pVCERN
794 primers, and this fragment was inserted into NdeI/SacI-digested pVCERN-2 via Gibson
795 assembly. The NdeI/MluI fragment was subsequently excised and ligated into NdeI/MluI-
796 digested pXCERN-2.

797

798 *pGS74 and pGS76*. For markerless deletions of *CCNA_01217* or *CCNA_01219*, 5'- and 3'-
799 flanking homology regions, respectively, were amplified using the primer pairs EK1047/1048
800 and EK1049/1050 (*CCNA_01217*) and EK1055/1056 and EK1057/1058 (*CCNA_01219*).
801 pNPTS138 was amplified with primers EK897/898, and vectors were constructed by Gibson
802 assembly.

803

804 *pKR429*. For markerless deletion of *CCNA_01218*, 5'- and 3'- flanking homology regions,
805 respectively, were amplified using the primer pairs 01218 up_fwd/01218 up_rev and 01218
806 down_fwd/01218 down_rev. pNPTS138 was digested with EcoRI and HindIII, and the vector was
807 constructed by Gibson assembly.

808

809 *pEK406*. For complementing the deletion of *CCNA_01217* in LC-MS/MS experiments,
810 *CCNA_01217-FLAG* was amplified using primers EK1357/1358. The PCR product was ligated
811 into the NdeI/NheI sites of pVCHYC-5.

812

813 *pKR432-4*. For appending a C-terminal FLAG tag to the *CCNA_01218-20* open reading frames.
814 The indicated genes were amplified from NA1000 genomic DNA using primer pairs Nde-

815 01218/01218-Mlu, Nde-01219/01219-Mlu, Nde-01220/01220-Mlu. Fragments were digested
816 using NdeI/MluI and ligated into pFLGC-1 digested with the same enzymes.

817

818 *pKR435*. For expressing *CCNA_01218-FLAG* from the chromosomal *vanA* promoter.

819 *CCNA_01218-FLAG* was amplified from pKR432 using primers 01218-FLAG F/01218-FLAG R
820 and inserted in NdeI-digested pVGFP-2 by Gibson assembly.

821

822 *pKR436*. For expressing *CCNA_01219-FLAG* from the chromosomal *vanA* promoter.

823 *CCNA_01219-FLAG* was amplified from pKR433 using primers 01219-FLAG F/01219-FLAG R
824 and inserted in NdeI-digested pVGFP-2 by Gibson assembly.

825

826 *pKR437*. For expressing *CCNA_01220-FLAG* from the chromosomal *vanA* promoter.

827 *CCNA_01220-FLAG* was amplified from pKR434 using primers 01220-FLAG F/01220-FLAG R
828 and inserted in NdeI-digested pVGFP-2 by Gibson assembly.

829

830 *pKR438*. For expressing *CCNA_01217-FLAG* from the chromosomal *vanA* promoter.

831 *CCNA_01217-FLAG* was amplified from pEK406 using primers 01217-FLAG F/01217-FLAG R
832 and inserted in NdeI-digested pVGFP-4 by Gibson assembly.

833

834 **Strain construction.** Unless otherwise stated, plasmids were mobilized from *E. coli* into *C.*
835 *crescentus* by conjugation. *E. coli* donors were counterselected by the addition of nalidixic acid,
836 or when WM3064 was used as the donor, by omitting diaminopimelic acid from selection plates.
837 Gene deletion or disruption was achieved by two-step homologous recombination using *sacB*
838 counterselection (Ely, 1991).

839

840 *LpxC depletion strain.* The LpxC depletion strain KR4007 was constructed in a parallel manner
841 to the CtpA depletion strain KR3906. pZIK133 was introduced to KR1499 (Δ *sspB*) by
842 conjugation and selection on PYE/chloramphenicol. pZIK134 was conjugated into this
843 intermediate strain, and colonies were selected on PYEX/chloramphenicol/oxytetracycline. After
844 *sacB* counterselection on PYEX/sucrose/oxytetracycline, colonies were screened for
845 chloramphenicol^R kanamycin^S on PYEX.

846

847 *Stable Δ ctpA or Δ lpxC strains.* To generate stable Δ *ctpA* or Δ *lpxC* strains without covering
848 plasmids, candidate suppressor genes identified by screening were disrupted in KR3906 or

KR4007, respectively, using two-step homologous recombination while cultivating the cells on PYEX. Intermediate strains (sucrose^R hygromycin^R kanamycin^S) were grown in liquid PYED without chloramphenicol to permit loss of the *ctpA* or *lpxC* covering plasmid, plated on PYED, and tested for chloramphenicol^S. Absence of *ctpA* was confirmed using primers *ctpA* KO F and *ctpA* KO R, and absence of *lpxC* was confirmed using primers *lpxC* KO F and *lpxC* KO R. The genomes of Δ *ctpA* Δ *fur* Δ *sspB* (KR4102) and Δ *lpxC* Δ *fur* Δ *sspB* (KR4103) were resequenced and contained no additional mutations. Stable Δ *ctpA* or Δ *lpxC* strains were further modified by electroporation with purified plasmids (Gilchrist and Smit, 1991) to restore xylose-driven suppressor gene expression.

Deletions of sphingolipid synthesis genes. Deletions in *CCNA_01217*, *CCNA_01218*, *CCNA_01219*, and *CCNA_01220* in NA1000 or KR4077 were made by conjugation of the appropriate pNPTS138-based plasmid, followed by selection on PYE/kanamycin/nalidixic acid. After overnight growth in PYE, cells were plated on PYEX/sucrose, and sucrose^R colonies were screened for kanamycin^S. Colony PCR with the following primers was used to detect the deletion of the indicated chromosomal genes: *CCNA_01217*, EK S238/S239; *CCNA_01218*, EK S240/S241; *CCNA_01219*, EK S242/S243; *CCNA_01220*, EK S216/S217. Loci were sequenced with the indicated primers to ensure the accuracy of in-frame deletions. Unmarked deletions of *CCNA_01217*, *CCNA_01218*, *CCNA_01219*, and *CCNA_01220* were made in KR4091 by conjugation of KR4091 with WM3064 harboring the appropriate pNPTS138-based plasmids, followed by selection on PYEX/kanamycin medium omitting diaminopimelic acid. After growth overnight in PYEX, cells were plated on PYEX/sucrose, and sucrose^R colonies were screened for kanamycin^S. Colony PCR with the following primers was used to detect the deletion of the indicated chromosomal genes: *CCNA_01217*, EK S238/S239; *CCNA_01218*, EK S240/S241; *CCNA_01219*, EK S242/S243; *CCNA_01220*, EK S216/S217. Loci were sequenced with the indicated primers to ensure the accuracy of in-frame deletions. Strains were screened for oxytetracycline^R and hygromycin^R to ensure that they maintained deletions of *lpxC* and *fur*, respectively.

*Complementation of *spt* and *cpg* genes.* To complement deletions of *CCNA_01217-01220*, the following plasmids were introduced by conjugation to place the complementing gene under control of the chromosomal *vanA* promoter: Δ *CCNA_01217*, pEK406 (for LC-MS studies) or pKR438 (for growth and chemical sensitivity assays); Δ *CCNA_01218*, pKR435; Δ *CCNA_01219*, pKR436; or Δ *CCNA_01220*, pKR437. When introducing plasmids into strains capable of *lpxC*

depletion (based on KR4091), plasmids were delivered from WM3064 to avoid the use of multiple antibiotics for selection/counterselection. Correct integration of plasmids at the *vanA* locus was confirmed by colony PCR using primers RecUni-1 and RecVan-2 (Thanbichler et al., 2007).

Suppressor selection. KR3906 was grown to full density in PYEX. 300 µL of culture was transferred onto an open, sterile Petri dish and mutagenized in a UV Stratalinker 1800 (Stratagene) with 30,000 µJ of energy. Mutagenized cells were plated on PYED. Recovered colonies were grown in liquid PYED overnight to allow loss of the covering plasmid, and samples were streaked onto PYED. Isolated colonies were screened for chloramphenicol sensitivity. Chlor^s isolates were grown in PYE and saved at -80°C in 10% dimethylsulfoxide. Loss of *ctpA* was confirmed via PCR using the primers *ctpA* KO F and *ctpA* KO R, which anneal to the interior of the open reading frame.

Genome resequencing. Strains were grown to full density in PYE, and genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). Genomic DNA was submitted to the UC Berkeley Functional Genomics Laboratory, where libraries were prepared using a PCR-free protocol with multiplexing (<http://qb3.berkeley.edu/gsl/>). Samples were sequenced at the UC Berkeley Vincent J. Coates Genomics Sequencing Laboratory using a 300PE or 150PE MiSeq v3 run. Genomic sequencing data were analyzed for variants using the Galaxy platform at usegalaxy.org (Afgan et al., 2016). Adapter sequences were removed using Cutadapt (Martin, 2011(Martin, 2011)), and sequences were aligned to the NA1000 genome (Marks et al., 2010) using Bowtie2 (Langmead and Salzberg, 2012). FreeBayes (Garrison and Marth, 2012) was used to analyze the BAM files for variants. Variants with quality scores below 300 were discarded as noise.

qRT-PCR assays. RNA from mid-log phase cells was extracted with the RNeasy kit (Qiagen). Purified RNA was treated with DNase (Thermo Scientific) to remove any contaminating DNA, and the RNA was re-purified using the RNeasy kit to remove the DNase. RNA concentrations were measured on a Nanodrop and normalized to 10 ng/µl. The RNA was reverse-transcribed with the High Capacity cDNA Reverse-Transcription Kit (Thermo Scientific). qRT-PCR was performed with technical duplicate and biological triplicate samples on a QuantStudio 6 instrument (Thermo Scientific) using the PowerUP SYBR Green master mix (Thermo Scientific)

and 300 nM primers (**Table S8**). Relative expression was determined by the relative $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) and normalized to *rpoD* expression.

Growth and viability assays. For plate assays, strains were grown to $OD_{660} = 0.2-0.5$ in permissive media, washed twice in PYE medium with no additions, and diluted to $OD_{660} = 0.1$. 10 μ l drops of ten-fold serial dilutions were pipetted onto permissive and nonpermissive media. Plates were incubated for 3 days at 30°C, and images are representative of at least three independent trials. For end-point growth assays in liquid media, strains were grown in permissive media to $OD_{660} = 0.2-0.5$. After washing in PYE medium without additions, cells were resuspended at $OD_{660} = 0.01$ in permissive and nonpermissive media. OD_{660} values were measured after 24 h growth at 30°C. Growth curves where OD_{660} and colony forming units (cfu)/ml were measured at 3h intervals were performed using 3-4 ml cultures shaken at 250 rpm in a 30°C incubator. Growth curves where OD_{660} was measured every 15 min were performed in a BioTek Epoch2 microplate reader held at 30°C with constant shaking at 567 cpm between reads.

Disc diffusion assays. Cultures were grown to mid-exponential phase (OD_{660} 0.2-0.5), and an amount of cells equivalent to 250 μ l of culture at $OD_{660} = 0.2$ was added to 4 ml of PYE swarm agar (0.3% w/v agar) pre-warmed to 42°C. Swarm agar containing bacteria was spread onto solid PYE and allowed to set. Antibiotics or detergents (10 μ l each) were added to sterile Whatman filter disks and allowed to dry in a fume hood before discs were placed onto swarm agar surfaces. Plates were incubated upright at 30°C for 24 hours. The diameters of the zones of clearing or haze were measured, and the diameter of the disk (6 mm) was subtracted from all measurements to yield the reported values. The total amount of antibiotic or detergent added to each disk is as follows: kanamycin (100 μ g), rifampicin (100 μ g), vancomycin (1 mg), CHIR-090 (100 μ g, APExBIO), bacitracin (50 μ g), TWEEN 20 (10 μ L of 10% solution), Triton X-100 (10 μ L of 10% solution), sodium dodecyl sulfate (10 μ L of 10% solution). Tests using CHIR-090 used one quarter of the standard amount of cells to reduce growth haze. For strains overexpressing genes integrated at the *vanA* locus, uninduced cells were grown in PYE/kanamycin or PYE/gentamicin, and aliquots were plated in PYE swarm agar on PYE medium. Induced cells were grown in PYE/kanamycin or PYE/gentamicin containing 0.5 mM vanillate before plating in/on PYE medium containing 0.5 mM vanillate. 100 μ M 2,2'-dipyridyl was included in media for testing chemical sensitivity in iron-restricted conditions.

Streptonigrin sensitivity. Isolated colonies of the indicated strains were grown in PYE medium to $OD_{660} = 0.2-0.5$ and diluted to $OD_{660} = 0.01$. The diluted culture was aliquoted into separate tubes, which received 0.025 $\mu\text{g/ml}$, 0.25 $\mu\text{g/ml}$, or no streptonigrin (SNG). After 24 h of growth at 30°C, OD_{660} values were measured, and optical density ratios (0.25 $\mu\text{g/ml}$ SNG/no addition and 0.025 $\mu\text{g/ml}$ SNG/no addition) were calculated as a measure of growth inhibition.

Limulus amebocyte lysate assay. The ToxinSensor Chromogenic LAL Endotoxin Assay kit (GenScript) was used to determine endotoxin units/mL of culture. Cells were grown to mid-exponential phase (OD_{660} 0.2-0.5), washed twice with non-pyrogenic LAL reagent water, and normalized in this water to $OD_{660} = 0.1$. Cell suspensions were serially diluted in non-pyrogenic water and analyzed according to manufacturer's instructions.

Gel electrophoresis of lipid A species. For visualizing LPS species from whole-cell lysates, cells were harvested after overnight growth in the indicated medium. All cultures were normalized by OD_{660} , pelleted, and resuspended to 100 μL in 1x tricine loading buffer (100 mM Tris-HCl pH 6.8, 1% sodium dodecyl sulfate (SDS), 20% glycerol, 0.02% Coomassie G-250, 1% 2-mercaptoethanol). Proteinase K (125 ng/ μL final concentration) was added, and samples were incubated overnight at 55°C. Lysates were boiled 5 min, and equal volumes (10% of each sample) were analyzed by gel electrophoresis.

Hot aqueous-phenol LPS extractions were adapted from Westpahl and Jann (Davis and Goldberg, 2012; Westphal and Jann, 1965). 1 mL of culture at $OD_{660} = 0.75$ was pelleted and resuspended in 200 μL 1x tricine loading buffer. Suspensions were boiled for 15 min and cooled to room temperature. 5 μL of 20 mg/mL Proteinase K was added to each sample before incubation at 55°C for three hours. Suspensions were mixed with 200 μL ice-cold Tris-saturated phenol, vortexed, and incubated at 65°C for 15 minutes before being cooled to room temperature. 1 mL diethyl ether was added to each sample before vortexing and spinning for 10 minutes in a table-top centrifuge at 16,000 x g. The bottom blue layer was removed to a fresh tube, and the extraction was repeated on the blue layer starting from the phenol step. 200 μL 2x tricine loading buffer was added to each sample before gel electrophoresis.

Rough LPS was extracted by the method of Darveau and Hancock (Darveau and Hancock, 1983), modified as described (Hershey et al., 2019), beginning with 50 ml PYE cultures grown to $OD_{660} = 0.85$. Cultures were centrifuged, and cell pellets were resuspended in 2 ml 10 mM Tris-HCl (pH 8.0) containing 2 mM MgCl_2 . Samples were sonicated (Qsonica Q500) on ice for 5 min at 20% amplitude, in cycles of 10 sec on/20 sec off so that fewer than 5% of

cells remained intact. DNase I and RNase A were added to final concentrations of 100 µg/ml and 25 µg/ml, respectively, and lysates were incubated at 37°C for 1 hour. Additional DNase I and RNase A were added to reach final concentrations of 200 µg/ml and 50 µg/ml, respectively, and lysates were incubated for 1 hour at 37°C. SDS and EDTA were added to achieve final concentrations of 2% and 100 mM, respectively, and lysates were incubated for 2 h at 37°C before centrifugation (30 min at 50,000 x g, 30 min, 4°C). Proteinase K (50 µg/ml) was added to each supernatant, followed by incubation for 2 h at 60°C. LPS was precipitated by the addition of 2 volumes of ice-cold 0.375 M MgCl₂/95% ethanol and collected by centrifugation (12,000 x g, 15 min, 4°C). Precipitates were resuspended in 3.3 ml 10 mM Tris-HCl (pH8.0)/2% SDS/100 mM EDTA and incubated with shaking overnight at 37°C. Rough LPS was reprecipitated using 2 volumes ice-cold 0.375 M MgCl₂/95% ethanol and collected by centrifugation (12,000 x g, 15 min, 4°C). Precipitates were suspended in 10 mM Tris-HCl (pH 8.0) and centrifuged (200,000 x g, 2 h, 4°C). After removal of the supernatant by pipetting, LPS pellets were resuspended in 1 ml 1x tricine loading buffer, and 5 µl were analyzed by gel electrophoresis.

Free lipid A was extracted by the Caroff method (El Hamidi et al., 2005), modified as described (Leung et al., 2017), starting with 10 ml of PYE culture grown to OD₆₀₀ = 0.6. Cultures were divided into multiple tubes and centrifuged at 14,000 x g for 2 min. In a fume hood, cell pellets from each culture were resuspended, combined, and transferred to a gasketed microcentrifuge tube using 250 µl 70% (v/v) isobutyric acid + 150 µl 1 M ammonium hydroxide. Samples were incubated in a boiling water bath in a fume hood for 1 h, with vortexing every 15 min. Samples were cooled on ice and centrifuged at 2000 x g for 15 min. In a fume hood, supernatants (~400 µl) were transferred to new gasketed tubes, each containing 400 µl endotoxin-free water. Small holes were punched in the gasketed caps using a syringe needle before the samples were frozen in liquid nitrogen and lyophilized overnight. Methanol (1 ml) was added, and samples were sonicated in a water bath for 5 min. Samples were centrifuged at 10,000 x g for 5 min, and methanol was aspirated. The methanol wash was repeated before lipids were solubilized in 190 µl 3:1.5:0.25 v/v/v chloroform:methanol:endotoxin-free water. After vortexing, samples were centrifuged at 8,000 x g for 5 min. Supernatants were transferred to fresh gasketed tubes, and extracts were dried under a stream of nitrogen before analysis by mass spectrometry (see below) or gel electrophoresis. Samples for gel electrophoresis were resuspended using 100 µl 1x tricine loading buffer, and 10 µl of each sample was analyzed.

All lipid samples were analyzed on 16.5% Mini-PROTEAN Tris-Tricine gels (Bio-Rad). Carbohydrates were stained using Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes; P20495) per manufacturer's instructions. For Western blot analysis of S-

LPS, equal numbers of cells grown in PYE with appropriate additions were pelleted, resuspended in 1x SDS loading buffer, and boiled before analysis on 12% polyacrylamide gels and transfer to Immobilon-P PVDF membranes. Blots were probed with α -S-LPS (1:20,000) (Walker et al., 1994) and horseradish peroxidase-conjugated anti-rabbit antibodies (1:5000) and analyzed using Western Lightning (Perkin Elmer). Stained lipid species were visualized using a Bio-Rad Gel Doc XR.

High performance liquid chromatography-tandem mass spectrometry (HPLC-MSMS) of lipid A extracts. All samples for HPLC-electrospray ionization tandem mass spectrometry were generated by the modified Caroff extraction protocol described above. Each extract was initially dissolved in 100 μ L 1:2 chloroform: methanol before dilution 1:10 with methanol for analysis. A 2-5 μ L aliquot of each solution was injected onto a Phenomenex Jupiter C4 column (2 x 50 mm, 5 μ m, 300 Å) for HPLC-MSMS analysis with a Waters Acquity UPLC system coupled to a Thermo LTQ-Orbitrap Velos Pro mass spectrometer, which was equipped with an atmospheric pressure electrospray ionization source. For lipid detection, the HPLC-MSMS analyses were carried out with full-mass detection over a mass range of m/z 250 to 2000 in the Fourier transform MS mode, with negative-ion detection. The mass resolution was 60,000 FWHM @ m/z 400. Fragmentation product ion masses of the three most intense precursor ions were measured in the ion trap or orbitrap (7500 resolution) mass analyzer using stepped collision-induced dissociation (35% of the normalized collision energy) or Higher energy collision-induced dissociation (35% of the normalized collision energy) activation energies. During data acquisitions, real-time mass calibration was applied with m/z 283.26454 as the lock mass for negative-ion detection. The mobile phase for separation was (A) 1 mM ammonium acetate solution and (B) 90% (1:1 acetonitrile/propanol)/10% water/1 mM ammonium acetate as the binary solvents for the 16-minute gradient elution: 0 to 10 min, 30% to 100%B; 10 to 12 min, 100% B and 12 to 12.1 min at 30% B, followed by column equilibration at 30% B from 12.1 to 16 min. The column flow rate was 0.35 mL/min and the column temperature was maintained at 40°C.

Lipid A structure analysis. MALDI-TOF MS was used to screen lipid extracts. To check structures, tandem MS and ancillary separation techniques were required. These are described below. HPLC-MSMS (above) describes the generation of data for structure determinations in Fig. S3. Notably, the triple deletion strain $\Delta ctpA \Delta fur \Delta sspB$ contained no lipid A with sugars at the terminal (1 and 4') positions but rather contained phosphates, as found in canonical lipid A

structures. The $\Delta lpxC \Delta fur \Delta sspB$ strain contained an ion at 1412 m/z , the structure of which remains unclear. The HPLC-MSMS data of this ion showed no loss of phosphate, as seen in $\Delta ctpA \Delta fur \Delta sspB$, nor loss of sugars, as seen for the NA1000, $\Delta sspB$, or $\Delta fur \Delta sspB$ strains. The fragmentation pattern strongly suggested that something other than lipid A was responsible for the ion at 1412 m/z . Given that cardiolipin is a common microbial membrane lipid, we carried out HILIC-MS (described below) with cardiolipin and lipid A standards. Both standards were retained by HILIC, as expected for hydrophobic molecules, but extracts from the $\Delta lpxC \Delta fur \Delta sspB$ mutant showed no ions at all, suggesting that the species at 1412 m/z is not hydrophobic enough to be retained. Regrettably, there remains no structure identified for the ion at 1412 m/z . Generally, structure analysis was conducted manually according to our prior effort in this field (Yoon et al., 2016).

Hydrophobic interaction liquid chromatography-mass spectrometry (HILIC-MS). A 10- μ L aliquot of each solution was injected into a Waters Atlantis HILIC column (4.6 mm x 150 mm, 5 μ m) to run LC-MS on a Water Acquity UPLC system coupled to a Thermo LTQ-Orbitrap Velos Pro mass spectrometer, which was equipped with an atmospheric pressure electrospray ionization source. For lipid detection, the HILIC-MS runs were carried out with full-mass detection over a mass range of m/z 80 to 2000 in the Fourier transform MS mode, with positive-ion and negative-ion detection, respectively, in two rounds of LC injections. The mass resolution was 60,000 FWHM @ m/z 400. During data acquisitions, real-time mass calibration was applied with m/z 391.28426 as the lock mass for positive-ion detection and with m/z 112.98563 as the lock mass for negative-ion detection. The mobile phase of HILIC was (A) 20 mM ammonium acetate solution (pH adjusted to 4.0 with acetic acid) and (B) methanol as the binary solvents for gradient elution: 0-4 min, 99% B; 4 to 12.5 min, 99% to 20% B and 12.5 to 15 min at 20% B, followed by column equilibration at 99% B for 5 min between injections. The column flow rate was 0.4 mL/min and the column temperature was maintained at 40°C.

Microscopy. Cells were immobilized on agarose pads (1% w/v in reverse osmosis-purified water). Images were taken using a Zeiss EC Plan-Neofluar 100x/1.3 Oil M27 objective on a Zeiss AxioImager M1 microscope with a Hamamatsu Digital CCD Camera (C8484-03G01). Images were acquired using iVision-Mac software (BioVision Technologies) and processed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997-2018).

CryoEM imaging. Cultures (5 mL) of KR4000, KR4102, KR4103, and KR3906 grown to OD_{660} 0.2-0.5 were centrifuged (4°C, 16,000 x g, 15 minutes), and cell pellets were resuspended in 50 µL PYE. For KR3906, cells grown in PYEX were washed twice with PYE, released into PYED at OD_{660} = 0.02, and incubated for 12 hours before harvest. 3 µL of cell suspension, mixed 1:1 with Fiducial markers (10-nm gold particles conjugated to Protein A; Aurion) was applied to glow-discharged quantifoil grids (R2/2) and frozen in liquid ethane using an automatic plunge freezing device (Vitrobot, FEI. 12°C, 8-12s blot time, blot force 8, humidity 100%).

Grids of KR4000 and KR4103 were imaged on a Jeol3100 cryoTEM operating at 300kV with in column omega energy filter and K2 direct electron camera. Grids of KR4102 and KR3906 were imaged on a Krios Cryo TEM (FEI) operating at 300kV with post column energy filter (Quantum, GATAN) and K2 direct electron camera. All data were collected with the automatic data collection program serialEM (Mastonarde, 2005). Square overview images were acquired using a defocus of 80-100 microns at a nominal magnification of 3600-6500x (Krios) or 1200x (Jeol) using the polygon montage operation (specimen pixel size: 33-67Å). Beam intensity was set to 8e⁻/px/s over an empty hole and exposure times ranged from 2-5s depending on ice thickness. Bidirectional tomographic tilt series were collected from ±60° using a defocus of 6-8 µm and at a magnification which provided specimen pixel size of 4-7 Å. Total dose of the tilt series were kept between 60-90 e⁻/Å². All tilt series images were collected in movie mode and the frames aligned using MotionCor2 (Zheng et al., 2017). Aligned frames were compiled into stacks and processed using IMOD (Kremer et al., 1996). Contrast of resulting tomograms was enhanced using a non-linear anisotropic diffusion filter (Frangakis and Hegerl, 2001) and manually segmented using the 3D visualization program AMIRA (ThermoFisher).

RB-Tnseq analysis. A 1 ml aliquot of the RB-Tnseq library in NA1000 (Price et al., 2018) was thawed and grown to OD_{660} = 0.65 in 25 ml PYE medium with kanamycin. Aliquots of this culture were saved for sequencing of pre-challenge barcodes, or were diluted to OD_{660} = 0.02 in PYE medium (set8IT011, set8IT023, and set8IT035) or PYE medium with 2 µg/ml CHIR-090 (set8IT012, set8IT024, and set8IT036). Cultures were grown for 9 hours at 30°C as described (Price et al., 2018) before cells were harvested and post-challenge barcodes were sequenced. Gene fitness (f) and significance (t) scores were calculated as described (Wetmore et al., 2015). Candidate genes examined in this study (*CCNA_01217-01220*) had fitness scores between -1.5 and -3.7, with significance scores between -3.0 and -8.4, for individual trials of library growth in PYE + CHIR-090.

Sphingolipid extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS). *Caulobacter* strains were grown overnight with or without 0.5 mM vanillate (5 ml), and lipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959). Cells were harvested and resuspended in 1 ml of water, 3.75 ml of 1:2 (v/v) chloroform: methanol was added, and the samples were mixed by vortexing. Chloroform (1.25 ml) and water (1.25 ml) were added sequentially with vortexing to create a two-phase system and the samples were centrifuged at 200 x *g* for 5 minutes at room temperature. The bottom, organic phase was transferred to a clean glass tube with a Pasteur pipette and washed twice in “authentic” upper phase. Subsequently, the organic phase containing lipids was collected and dried under argon. Our methods for lipid analysis by normal phase LC/ESI–MS/MS have been described (Guan et al., 2014). Briefly, normal phase LC was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column, 5 µm, 25 cm × 2.1 mm (Sigma-Aldrich, St. Louis, MO) as described. The LC eluent (with a total flow rate of 300 µl/min) was introduced into the ESI source of a high resolution TripleTOF5600 mass spectrometer (Applied Biosystems, Foster City, CA). Instrumental settings for negative ion ESI and MS/MS analysis of lipid species were as follows: ion spray voltage (IS) = –4500 V; curtain gas (CUR) = 20 psi; ion source gas 1 (GSI) = 20 psi; declustering potential (DP) = –55 V; and focusing potential (FP) = –150 V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software (Applied Biosystems, Foster City, CA).

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 9.3.1 was used for statistical analyses. Statistical details of experiments can be found in the figure legends. Statistical analysis of RB-Tnseq data is described in Wetmore *et al.* 2015, and at <https://fit.genomics.lbl.gov>.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit α -S-LPS (recognizes smooth LPS of <i>Caulobacter crescentus</i>)	John Smit, University of British Columbia	Walker <i>et al.</i> , 1994
Bacterial and Virus Strains		
RB-Tnseq library constructed in <i>Caulobacter crescentus</i> NA1000	Adam Deutschbauer, Lawrence Berkeley National Laboratory and Plant & Microbial Biology, UC Berkeley	Price, <i>et al.</i> 2018
Please see Table S6 for a list of strains.		
Chemicals, Peptides, and Recombinant Proteins		
streptonigrin	Sigma-Aldrich	Cat#S1014 CAS 3930-19-6
Q5 High-Fidelity DNA Polymerase	New England Biolabs	Cat#M0491
NEBuilder HiFi DNA Assembly Master Mix	New England Biolabs	Cat#E2621
2,2-dipyridyl	Acros Organics (now Thermo Scientific)	Cat#AC117500100 CAS 366-18-7
CHIR-090	APExBIO	Cat#A3307 CAS 728865-23-4
Bacto Peptone	Becton Dickinson, acquired through our stockroom	Lot# 9239004 Ref#211677
Agar Granulated Bacteriological Grade	Apex BioResearch Products	Cat#20-248 Lot#AB-2010160
PowerUp SYBR Green Master Mix	Thermo Scientific	Cat#A25777
High Capacity cDNA Reverse-Transcription Kit	Thermo Scientific	Cat#4368813
DNeasy Blood & Tissue Kit	Qiagen	Cat#69504
16.5% Mini-PROTEAN Tris-Tricine gels	Bio-Rad	Cat#4563063
RNeasy Kit	Qiagen	Cat#74004
Critical Commercial Assays		
Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit	Thermo Fisher	Cat#P20495

ToxinSensor Chromogenic LAL Endotoxin Assay	GenScript	Cat#L00350
Deposited Data		
RB-Tnseq data	Fitness Browser https://fit.genomics.lbl.gov	<i>Caulobacter crescentus</i> NA1000 library grown in PYE medium: set8IT011, set8IT023, and set8IT035. <i>Caulobacter crescentus</i> NA1000 library grown in PYE medium with 2µg/ml CHIR-090: set8IT012, set8IT024, and set8IT036.
Transcriptomic data for <i>Caulobacter crescentus</i> NA1000 wild-type, Δfur , and wild-type treated with 2,2-dipyridyl		da Silva Neto <i>et al.</i> , 2013
Transcriptomic data for <i>Caulobacter crescentus</i> NA1000 wild-type, Δfur , and wild-type treated with 2,2-dipyridyl		Leaden <i>et al.</i> , 2018
For genome resequencing accession numbers, see Table S6	Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra , BioProject ID PRJNA526705	
Oligonucleotides		
For oligonucleotide sequences see Table S8		
Recombinant DNA		
For plasmids used in this study see Table S7		
Software and Algorithms		
Bowtie2	Langmead and Salzberg, 2013	
FreeBayes	Garrison and Marth, 2012	
iVision-Mac	BioVision Technologies	
ImageJ	Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/ , 1997-2018	
MotionCor2	Zheng, <i>et al.</i> , 2017	
AMIRA	Thermo Fisher	

Analyst TF1.5	Applied Biosystems	
Galaxy	Afgan <i>et al.</i> 2016	
Cutadapt	Martin, 2011	
GraphPad Prism 9.3.1 for statistical analyses	GraphPad Software, LLC	
IMOD	Kremer, <i>et al.</i> , 1996	
serialEM	Mastonarde <i>et al.</i> , 2005	

Supplemental Information Titles and Legends

Movie S1, related to Figure 2. Tomogram of *Caulobacter crescentus* NA1000 grown to mid-exponential phase in PYE.

Movie S2, related to Figure 2. Tomogram of $\Delta ctpA \Delta sspB \Delta fur$ grown to mid-exponential phase in PYE.

Movie S3, related to Figure 2. Tomogram of $\Delta lpxC \Delta sspB \Delta fur$ grown to mid-exponential phase in PYE.

Movie S4, related to Figure 2. Tomogram of $\Delta ctpA \Delta sspB$ + pJS14- P_{xyIX} -*ctpA*-3xFLAG-*ssrA* grown to mid-exponential phase in PYEX, washed twice with PYE, released into PYED at OD₆₆₀ = 0.02, and incubated for 10 hours before harvest.

References

Afgan, E., Baker, D., Beek, M. van den, Blankenberg, D., Bouvier, D., Cech, M., Chilton, J., Clements, D., Coraor, N., Eberhard, C., et al. (2016). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res.* **44**, W3–W10.

Altschul, S. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Andrews, S., Norton, I., Salunkhe, A.S., Goodluck, H., Aly, W.S.M., Mourad-Agha, H., and Cornelis, P. (2013). Control of iron metabolism in bacteria. *Met. Ions Life Sci.* **12**, 203–239.

- Awram, P., and Smit, J. (2001). Identification of lipopolysaccharide O antigen synthesis genes required for attachment of the S-layer of *Caulobacter crescentus*. *Microbiology* 147, 1451–1460.
- Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can J. Biochem. Physiol.* 37, 911–917.
- Blondelet-Rouault, M.H., Weiser, J., Lebrihi, A., Branny, P., and Pernodet, J.L. (1997). Antibiotic resistance gene cassettes derived from the omega interposon for use in *E. coli* and *Streptomyces*. *Gene* 190, 315–317.
- Boll, J.M., Crofts, A.A., Peters, K., Cattior, V., Vollmer, W., Davies, B.W., and Trent, M.S. (2016). A penicillin-binding protein inhibits selection of colistin-resistant, lipooligosaccharide-deficient *Acinetobacter baumannii*. *Proc. Natl. Acad. Sci. USA* 113, E6228–E6237.
- Brown, D.B., Forsberg, L.S., Kannenberg, E.L., and Carlson, R.W. (2012). Characterization of galacturonosyl transferase genes *rgtA*, *rgtB*, *rgtC*, *rgtD*, and *rgtE* responsible for lipopolysaccharide synthesis in nitrogen-fixing endosymbiont *Rhizobium leguminosarum*: lipopolysaccharide core and lipid galacturonosyl residues confer membrane stability. *J. Biol. Chem.* 287, 935–949.
- Brown, D.B., Muszynski, A., and Carlson, R.W. (2013). Elucidation of a novel lipid A alpha-(1,1)-GalA transferase gene (*rgtF*) from *Mesorhizobium loti*: Heterologous expression of *rgtF* causes *Rhizobium etli* to synthesize lipid A with alpha-(1,1)-GalA. *Glycobiology* 23, 546–558.
- Christen, B., Abeliuk, E., Collier, J.M., Kalogeraki, V.S., Passarelli, B., Collier, J.A., Fero, M.J., McAdams, H.H., and Shapiro, L. (2011). The essential genome of a bacterium. *Mol. Syst. Biol.* 7, 528.
- Crosson, S., McGrath, P.T., Stephens, C., McAdams, H.H., and Shapiro, L. (2005). Conserved modular design of an oxygen sensory/signaling network with species-specific output. *Proc. Natl. Acad. Sci. USA* 102, 8018–8023.
- Darveau, R.P., and Hancock, R.E. (1983). Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *J. Bacteriol.* 155, 831–838.
- Davis, M.R.J., and Goldberg, J.B. (2012). Purification and visualization of lipopolysaccharide from Gram-negative bacteria by hot aqueous-phenol extraction. *J. Vis. Exp.* e3916.
- De Castro, C., Molinaro, A., Lanzetta, R., Silipo, A., and Parrilli, M. (2008). Lipopolysaccharide structures from *Agrobacterium* and *Rhizobiaceae* species. *Carbohydr. Res.* 343, 1924–1933.

1219 Dehio, C., and Meyer, M. (1997). Maintenance of broad-host-range incompatibility group P and
 1220 group Q plasmids and transposition of Tn5 in *Bartonella henselae* following conjugal plasmid
 1221 transfer from *Escherichia coli*. J. Bacteriol. 179, 538–540.
 1222
 1223 El Hamidi, A., Tirsoaga, A., Novikov, A., Hussein, A., and Caroff, M. (2005). Microextraction of
 1224 bacterial lipid A: Easy and rapid method for mass spectrometric characterization. J. Lipid Res.
 1225 46, 1773–1778.
 1226
 1227 Ely, B. (1991). Genetics of *Caulobacter crescentus*. Methods Enzymol. 204, 372–384.
 1228
 1229 Evinger, M., and Agabian, N. (1977). Envelope-associated nucleoid from *Caulobacter*
 1230 *crescentus* stalked and swarmer cells. J. Bacteriol. 132, 294–301.
 1231
 1232 Fontenot, C.R., Tasnim, H., Valdes, K.A., Popescu, C.V., and Ding, H. (2020). Ferric uptake
 1233 regulator (Fur) reversibly binds a [2Fe-2S] cluster to sense intracellular iron homeostasis in
 1234 *Escherichia coli*. J. Biol. Chem. 295, 15454–15463.
 1235
 1236 Frangakis, A.S., and Hegerl, R. (2001). Noise reduction in electron tomographic reconstructions
 1237 using nonlinear anisotropic diffusion. J. Struct. Biol. 135, 239–250.
 1238
 1239 Garrison, E., and Marth, G. (2012). Haplotype-based variant detection from short-read
 sequencing.
 1240
 1241 Gilchrist, A., and Smit, J. (1991). Transformation of freshwater and marine caulobacters by
 1242 electroporation. J. Bacteriol. 173, 921–925.
 1243
 1244 Guan, Z., Katzianer, D., Zhu, J., and Goldfine, H. (2014). *Clostridium difficile* contains
 1245 plasmalogen species of phospholipids and glycolipids. Biochim. Biophys. Acta 1842, 1353–
 1246 1359.
 1247
 1248 Hassett, D.J., Britigan, B.E., Svendsen, T., Rosen, G.M., and Cohen, M.S. (1987). Bacteria form
 1249 intracellular free radicals in response to paraquat and streptonigrin. Demonstration of the
 1250 potency of hydroxyl radical. J. Biol. Chem. 262, 13404–13408.
 1251
 1252 Henry, R., Crane, B., Powell, D., Lucas, D.D., Li, Z., Aranda, J., Harrison, P., Nation, R.L.,
 1253 Adler, B., Harper, M., et al. (2015). The transcriptomic response of *Acinetobacter baumannii* to
 1254 colistin and diripenem alone and in combination in an in vitro
 1255 pharmacokinetics/pharmacodynamics model. J. Antimicrob. Chemother. 70, 1303–1313.
 1256
 1257 Hershey, D.M., Fiebig, A., and Crosson, S. (2019). A genome-wide analysis of adhesion in
 1258 *Caulobacter crescentus* identifies new regulatory and biosynthetic components for holdfast
 1259 assembly. mBio 10, 2273.

1260 Jones, M.D., Vinogradov, E., Nomellini, J.F., and Smit, J. (2015). The core and O-
1261 polysaccharide structure of the *Caulobacter crescentus* lipopolysaccharide. Carbohydr. Res.
1262 402, 111–117.

1263

1264 Justino, M.C., Almeida, C.C., Teixeira, M., and Saraiva, L.M. (2007). *Escherichia coli* di-iron
1265 YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. J. Biol. Chem.
1266 282, 10352–10359.

1267

1268 Karbarz, M.J., Kalb, S.R., Cotter, R.J., and Raetz, C.R.H. (2003). Expression cloning and
1269 biochemical characterization of a *Rhizobium leguminosarum* lipid A 1-phosphatase. J. Biol.
1270 Chem 278, 39269–39279.

1271

1272 Kawahara, K., Seydel, U., Matsuura, M., Danbara, H., Rietschel, E.T., and Zahring, U. (1991).
1273 Chemical structure of glycosphingolipids isolated from *Sphingomonas paucimobilis*. FEBS Lett.
1274 292, 107–110.

1275

1276 Kawasaki, S., Moriguchi, R., Sekiya, K., Nakai, T., Ono, E., Kume, K., and Kawahara, K. (1994).
1277 The cell envelope structure of the lipopolysaccharide-lacking Gram-negative bacterium
1278 *Sphingomonas paucimobilis*. J. Bacteriol. 176, 284–290.

1279

1280 Kremer, J.R., Mastonarde, D.N., and McIntosh, J.R. (1996). Computer visualization of three-
1281 dimensional image data using IMOD. J. Struct. Biol. 116, 71–76.

1282

1283 Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat
1284 Methods 9, 357–359.

1285

1286 Leaden, L., Silva, L.G., Ribiero, R.A., Santos, N.M.D., Lorenzetti, A.P.R., Alegria, T.G.P.,
1287 Schultz, M.L., Medeiros, M.H.G., Koide, T., and Marques, M.V. (2018). Iron deficiency
1288 generates oxidative stress and activation of the SOS response in *Caulobacter crescentus*.
1289 Front. Microbiol. 9, 2014.

1290

1291 Leung, L.M., Fondrie, W.E., Doi, Y., Johnson, J.K., Strickland, D.K., Ernst, R.K., and Goodlett,
1292 D.R. (2017). Identification of the ESKAPE pathogens by mass spectrometric analysis of
1293 microbial membrane glycolipids. Sci. Rep. 7, 6403.

1294

1295 Levchenko, I., Seidel, M., Sauer, R.T., and Baker, T.A. (2000). A specificity-enhancing factor for
1296 the ClpXP degradation machine. Science 289, 2354–2356.

1297

1298 Liu, L., Feng, X., Wang, W., Chen, Y., Chen, Z., and Gao, H. (2020). Free rather than total iron
1299 content is critically linked to the Fur physiology in *Shewanella oneidensis*. Front. Microbiol. 11,
1300 593246.

1301

1302 Livak, K.J., and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using
Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. Methods 25, 402–408.

1303 Mamat, U., Meredith, T.C., Aggarwal, P., Kuhl, A., Kirchoff, P., Lindner, B., Hanuszkiewicz, A.,
 1304 Sun, J., Holst, O., and Woodard, R.W. (2008). Single amino acid substitutions in either YhjD or
 1305 MsbA confer viability to 3-deoxy-D-manno-oct-2-ulosonic acid-depleted *Escherichia coli*. *Mol.*
 1306 *Microbiol.* **67**, 633–648.
 1307
 1308 Marks, M.E., Castro-Rojas, C., Teiling, C., Du, L., Kapatral, V., Walunas, T.L., and Crosson, S.
 1309 (2010). The genetic basis of laboratory adaptation in *Caulobacter crescentus*. *J. Bacteriol.* **192**,
 1310 3678–3688.
 1311
 1312 Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing
 1313 reads. *EMBnet j.* **17**, 10.
 1314
 1315 Mastonarde, D.N. (2005). Automated electron microscope tomography using robust prediction
 1316 of specimen movements. *J. Struct. Biol.* **152**, 36–51.
 1317
 1318 McClerren, A.L., Endsley, S., Bowman, J.L., Andersen, N.H., Guan, Z., Rudolph, J., and Raetz,
 1319 C.R. (2005). A slow, tight-binding inhibitor of the zinc-dependent deacetylase LpxC of lipid A
 1320 biosynthesis with antibiotic activity comparable to ciprofloxacin. *Biochemistry* **44**, 16574–16583.
 1321
 1322 Meisenzahl, A.C., Shapiro, L., and Jenal, U. (1997). Isolation and characterization of a xylose-
 1323 dependent promoter from *Caulobacter crescentus*. *J. Bacteriol.* **179**, 592–600.
 1324
 1325 Meredith, T.C., Aggarwal, P., Mamat, U., Lindner, B., and Woodard, R.W. (2006). Redefining
 1326 the requisite lipopolysaccharide structure in *Escherichia coli*. *ACS Chem. Biol.* **1**, 33–42.
 1327
 1328 Moffatt, J.H., Harper, M., Harrison, P., Hale, J.D., Vinogradov, E., Seemann, T., Henry, R.,
 1329 Crane, B., Michael, F.S., Cox, A.D., et al. (2010). Colistin resistance in *Acinetobacter baumannii*
 1330 is mediated by complete loss of lipopolysaccharide production. *Antimicrob. Agents Chemother.*
 1331 **54**, 4971–4977.
 1332
 1333 Moffatt, J.H., Harper, M., and Boyce, J.D. (2019). Mechanisms of polymyxin resistance. *Adv.*
 1334 *Exp. Med. Biol.* 55–71.
 1335
 1336 Nachin, L., El Hassouni, M., Loiseau, L., Expert, D., and Barras, F. (2001). SoxR-dependent
 1337 response to oxidative stress and virulence of *Erwinia chrysanthemi*: the key role of SufC, an
 1338 orphan ABC ATPase. *Mol. Microbiol.* **39**, 960–972.
 1339
 1340 Nagiec, M.M., Skrzypek, M., Nagiec, E.E., Lester, R.L., and Dickson, R.C. (1998). The LCB4
 1341 (YOR171c) and LCB5 (YLR260w) genes of *Saccharomyces* encode sphingoid long chain base
 1342 kinases. *J. Biol. Chem.* **273**, 19437–19442.
 1343
 1344 Nagy, E., Losick, R., and Kahne, D. (2019). Robust suppression of lipopolysaccharide
 1345 deficiency in *Acinetobacter baumannii* by growth in minimal medium. *J. Bacteriol.* **201**, 420.

1346 Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited.
 1347 Microbiol. Mol. Biol. Rev. 67, 593–656.
 1348
 1349 Olea-Ozuna, R.J., Poggio, S., Bergström, E., Quiroz-Rocha, E., García-Soriano, D.A.,
 1350 Sahonero-Canavesi, D.X., Padilla-Gómez, J., Martínez-Aguilar, L., López-Lara, I.M., Thomas-
 1351 Oates, J., et al. (2021). Five structural genes required for ceramide synthesis in *Caulobacter*
 1352 and for bacterial survival. Environ. Microbiol. 23, 143–159.
 1353
 1354 Peng, D., Hong, W., Choudhury, B.P., Carlson, R.W., and Gu, X.-X. (2005). *Moraxella*
 1355 *cararrhalis* bacterium without endotoxin, a potential vaccine candidate. Infect. Immun. 73, 7569–
 1356 7577.
 1357
 1358 Plötz, B.M., Lindner, B., Stetter, K.O., and Holst, O. (2000). Characterization of a novel lipid A
 1359 containing D-galacturonic acid that replaces phosphate residues. The structure of the lipid A of
 1360 the lipopolysaccharide from the hyperthermophilic bacterium *Aquifex pyrophilus*. J. Biol. Chem.
 1361 275, 11222–11228.
 1362
 1363 Price, M.N., Wetmore, K.M., Waters, R.J., Callaghan, M., Ray, J., Liu, H., Kuehl, J.V., Melnyk,
 1364 R.A., Lamson, J.S., Suh, Y., et al. (2018). Mutant phenotypes for thousands of bacterial genes
 1365 of unknown function. Nature 557, 503–509.
 1366
 1367 Qureshi, N., Takayama, K., Mascagni, P., Honovich, J., Wong, R., and Cotter, R.J. (1988).
 1368 Complete structural determination of lipopolysaccharide obtained from deep rough mutant of
 1369 *Escherichia coli*. Purification by high performance liquid chromatography and direct analysis by
 1370 plasma desorption mass spectrometry. J. Biol. Chem. 263, 11971–11976.
 1371
 1372 Radolf, J.D., and Kumar, S. (2018). The *Treponema pallidum* outer membrane. Curr. Top.
 1373 Microbiol. Immuno. 415, 1–38.
 1374
 1375 Rojas, E.R., Billings, G., Odermatt, P.D., Auer, g K., Zhu, L., Miguel, A., Chang, F., Weibel,
 1376 D.B., Theriot, J.A., and Huang, K.C. (2018). The outer membrane is an essential load-bearing
 1377 element in Gram-negative bacteria. Nature 559, 617–621.
 1378
 1379 Samuel, G., and Reeves, P. (2003). Biosynthesis of O-antigens: Genes and pathways involved
 1380 in nucleotide sugar precursor synthesis and O-antigen assembly. Carbohydr. Res. 338, 2503–
 1381 2519.
 1382
 1383 Shapland, E.B., Reisinger, S.J., Bajwa, A.K., and Ryan, K. (2011). An essential tyrosine
 1384 phosphatase homolog regulates cell separation, outer membrane integrity, and morphology in
 1385 *Caulobacter crescentus*. J. Bacteriol. 193, 4361–4370.
 1386
 1387 da Silva Neto, J.F., Braz, V.S., Italiani, V.C.S., and Marques, M.V. (2009). Fur controls iron
 1388 homeostasis and oxidative stress defense in the oligotrophic alpha-proteobacterium
 1389 *Caulobacter crescentus*. Nucleic Acids Res. 37, 4812–4825.

da Silva Neto, J.F., Lourenco, R.F., and Marques, M.V. (2013). Global transcriptional response of *Caulobacter crescentus* to iron availability. *BMC Genomics* 14, 549.

Simpson, B.W., Nieckarz, M., Pinedo, V., McLean, A.B., Cava, F., and Trent, M.S. (2021). *Acinetobacter baumannii* can survive with an outer membrane lacking lipooligosaccharide due to structural support from elongasome peptidoglycan synthesis. *mBio* 12, e0309921.

Skerker, J.M., Prasol, M.S., Perchuk, B.S., Biondi, E.G., and Laub, M.T. (2005). Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: A systems-level analysis. *PLoS Biol.* 3, e334.

Smit, J., Kaltashov, I.A., Cotter, R.J., Vinogradov, E., Perry, M.B., Haider, H., and Qureshi, N. (2008). Structure of a novel lipid A obtained from the lipopolysaccharide of *Caulobacter crescentus*. *Innate Immun.* 14, 25–37.

Stankeviciute, G., Guan, Z., Goldfine, H., and Klein, E.A. (2019). *Caulobacter crescentus* adapts to phosphate starvation by synthesizing anionic glycolipids and a novel glycosphingolipid. *mBio* 10, 107.

Stankeviciute, G., Tang, P., Ashley, B., Chamberlain, J.D., Hansen, M.E.B., Coleman, A., D’Emilia, R., Fu, L., Mohan, E.C., Nguyen, H., et al. (2021). Convergent evolution of bacterial ceramide synthesis. *Nat. Chem. Biol.* DOI: 10.1038/s41589-021-00948-7.

Steeghs, L., Hartog, R. den, Boer, A. den, Zomer, B., Roholl, P., and Ley, P. van der (1998). Meningitis bacterium is viable without endotoxin. *Nature* 392, 449–450.

Thanbichler, M., Iniesta, A.A., and Shapiro, L. (2007). A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in *Caulobacter crescentus*. *Nucleic Acids Res.* 35, e137.

Toh, E., Jr, H.D.K., and Brun, Y.V. (2008). Characterization of the *Caulobacter crescentus* holdfast polysaccharide biosynthesis pathway reveals significant redundancy in the initiating glycosyltransferase and polymerase steps. *J. Bacteriol.* 190, 7219–7131.

Velkov, T., Thompson, P.E., Nation, R.L., and Li, J. (2010). Structure-activity relationships of polymyxin antibiotics. *J. Med. Chem.* 53, 1898–1916.

Walker, S.G., Karunaratne, D.N., Ravenscroft, N., and Smit, J. (1994). Characterization of mutants of *Caulobacter crescentus* defective in surface attachment of the paracrystalline surface layer. *J. Bacteriol.* 176, 6313–6323.

1432 Wang, X., Ribeiro, A.A., Guan, Z., McGrath, S.C., Cotter, R.J., and Raetz, C.R.H. (2006).
 1433 Structure and biosynthesis of free lipid A molecules that replace lipopolysaccharide in
 1434 *Francisella tularensis* subsp. novicida. *Biochemistry* **45**, 14427–14440.
 1435
 1436 Westphal, O., and Jann, K. (1965). Bacterial lipopolysaccharides extraction with phenol-water
 1437 and further applications of the procedure. *Methods Carbohydr. Chem.* **5**, 83–91.
 1438
 1439 Wetmore, K.M., Price, M.N., Waters, R.J., Lamson, J.S., He, J., Hoover, C.A., Blow, M.J.,
 1440 Bristow, J., Butland, G., Arkin, A.P., et al. (2015). Rapid quantification of mutant fitness in
 1441 diverse bacteria by sequencing randomly bar-coded transposons. *mBio* **6**, e00306–e00315.
 1442
 1443 Whitfield, C., and Trent, M.S. (2014). Biosynthesis and export of bacterial lipopolysaccharides.
 1444 *Annu. Rev. Biochem.* **83**, 99–128.
 1445
 1446 Wofford, J.D., Bolaji, N., Dziuba, N., Outten, F.W., and Lindahl, P.A. (2019). Evidence that a
 1447 respiratory shield in *Escherichia coli* protects a low-molecular-mass Fe(II) pool from O₂-
 1448 dependent oxidation. *J. Biol. Chem.* **294**, 50–62.
 1449
 1450 Yeowell, H.N., and White, J.R. (1982). Iron requirement in the bactericidal mechanism of
 1451 streptonigrin. *Antimicrob. Agents Chemother.* **22**, 961–968.
 1452
 1453 Yoon, S.H., Liang, T., Schneider, T., Oyler, B.L., Chandler, C.E., Ernst, R.K., Yen, G.S., Huang,
 1454 Y., Nilsson, E., and Goodlett, D.R. (2016). Rapid lipid a structure determination via surface
 1455 acoustic wave nebulization and hierarchical tandem mass spectrometry algorithm. *Rapid*
 1456 *Commun. Mass Spectrom.* **30**, 2555–2560.
 1457
 1458 Zhang, G., Meredith, T.C., and Kahne, D. (2013). On the essentiality of lipopolysaccharide to
 1459 Gram-negative bacteria. *Curr. Opin. Microbiol.* **16**, 779–785.
 1460
 1461 Zheng, S.Q., Plaovcak, E., Armache, J.P., Verba, K.A., Cheng, Y., and Agard, D.A. (2017).
 1462 MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron
 1463 microscopy. *Nat. Methods* **14**, 331–332.
 1464
 1465 Zhou, B., Schrader, J.M., Kalogeraki, V.S., Abeliuk, E., Dinh, C.B., Pham, J.Q., Cui, Z.Z., Dill,
 1466 D.L., McAdams, H.H., and Shapiro, L. (2015). The global regulatory architecture of transcription
 1467 during the *Caulobacter* cell cycle. *PLoS Genet.* **11**, e1004831.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit α -S-LPS (recognizes smooth LPS of <i>Caulobacter crescentus</i>)	John Smit, University of British Columbia	Walker <i>et al.</i> , 1994
Bacterial and Virus Strains		
RB-Tnseq library constructed in <i>Caulobacter crescentus</i> NA1000	Adam Deutschbauer, Lawrence Berkeley National Laboratory and Plant & Microbial Biology, UC Berkeley	Price, <i>et al.</i> 2018
Please see Table S6 for a list of strains.		
Chemicals, Peptides, and Recombinant Proteins		
streptonigrin	Sigma-Aldrich	Cat#S1014 CAS 3930-19-6
Q5 High-Fidelity DNA Polymerase	New England Biolabs	Cat#M0491
NEBuilder HiFi DNA Assembly Master Mix	New England Biolabs	Cat#E2621
2,2-dipyridyl	Acros Organics (now Thermo Scientific)	Cat#AC117500100 CAS 366-18-7
CHIR-090	APExBIO	Cat#A3307 CAS 728865-23-4
Bacto Peptone	Becton Dickinson, acquired through our stockroom	Lot# 9239004 Ref#211677
Agar Granulated Bacteriological Grade	Apex BioResearch Products	Cat#20-248 Lot#AB-2010160
PowerUp SYBR Green Master Mix	Thermo Scientific	Cat#A25777
High Capacity cDNA Reverse-Transcription Kit	Thermo Scientific	Cat#4368813
DNeasy Blood & Tissue Kit	Qiagen	Cat#69504
16.5% Mini-PROTEAN Tris-Tricine gels	Bio-Rad	Cat#4563063
RNeasy Kit	Qiagen	Cat#74004
Critical Commercial Assays		
Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit	Thermo Fisher	Cat#P20495
ToxinSensor	GenScript	Cat#L00350

Chromogenic LAL Endotoxin Assay		
Deposited Data		
RB-Tnseq data	Fitness Browser https://fit.genomics.lbl.gov	<i>Caulobacter crescentus</i> NA1000 library grown in PYE medium: set8IT011, set8IT023, and set8IT035. <i>Caulobacter crescentus</i> NA1000 library grown in PYE medium with 2µg/ml CHIR-090: set8IT012, set8IT024, and set8IT036.
Transcriptomic data for <i>Caulobacter crescentus</i> NA1000 wild-type, Δfur , and wild-type treated with 2,2-dipyridyl		da Silva Neto <i>et al.</i> , 2013
Transcriptomic data for <i>Caulobacter crescentus</i> NA1000 wild-type, Δfur , and wild-type treated with 2,2-dipyridyl		Leaden <i>et al.</i> , 2018
For genome resequencing accession numbers, see Table S6	Sequence Read Archive, https://www.ncbi.nlm.nih.gov/sra , BioProject ID PRJNA526705	
Oligonucleotides		
For oligonucleotide sequences see Table S8		
Recombinant DNA		
For plasmids used in this study see Table S7		
Software and Algorithms		
Bowtie2	Langmead and Salzberg, 2013	
FreeBayes	Garrison and Marth, 2012	
iVision-Mac	BioVision Technologies	
ImageJ	Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/ , 1997-2018	
MotionCor2	Zheng, <i>et al.</i> , 2017	
AMIRA	Thermo Fisher	
Analyst TF1.5	Applied Biosystems	
Galaxy	Afgan <i>et al.</i> 2016	
Cutadapt	Martin, 2011	

GraphPad Prism 9.3.1 for statistical analyses	GraphPad Software, LLC	
IMOD	Kremer, <i>et al.</i> , 1996	
serialEM	Mastonarde <i>et al.</i> , 2005	

Figure 1

Figure 1

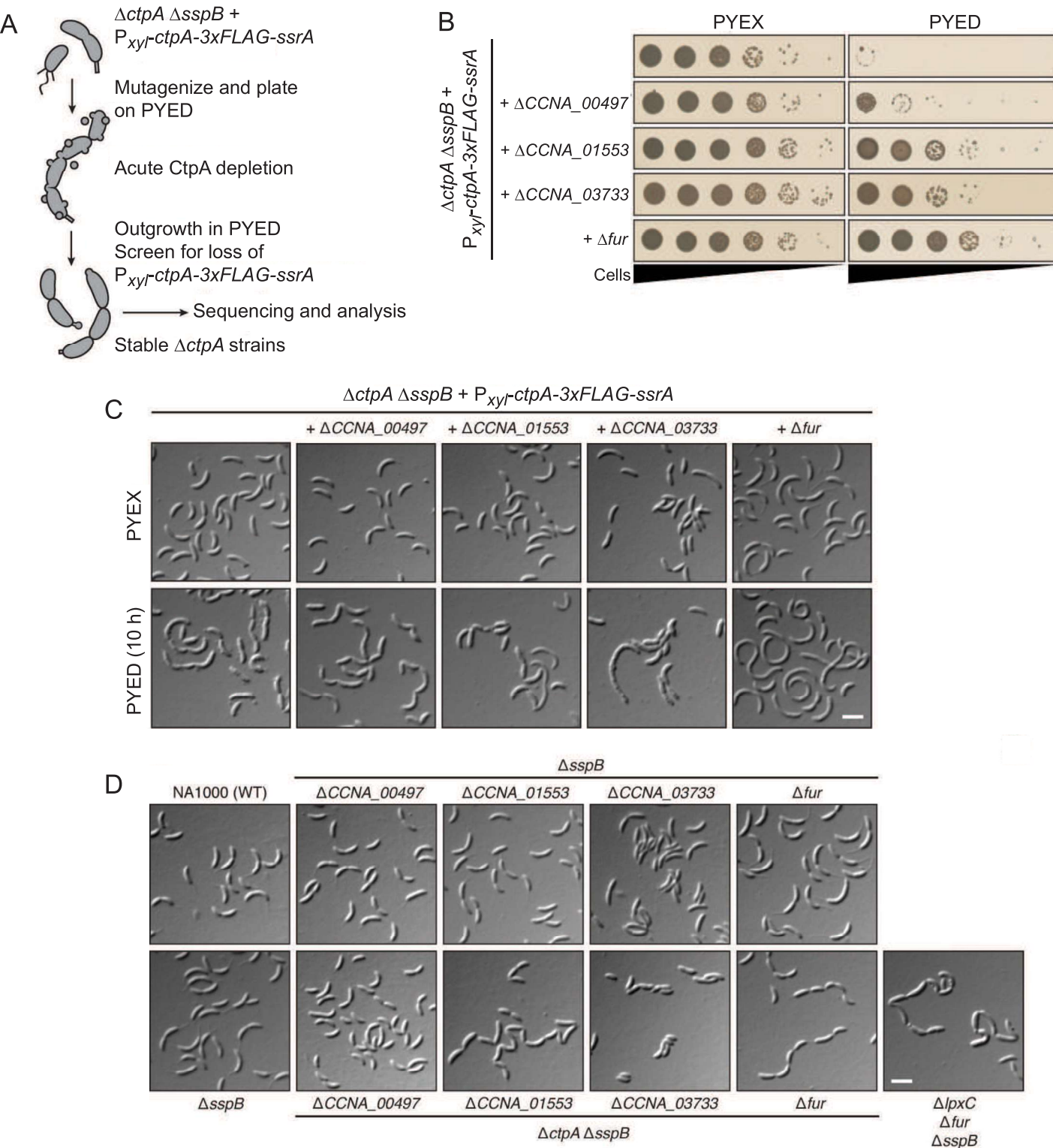


Figure 2

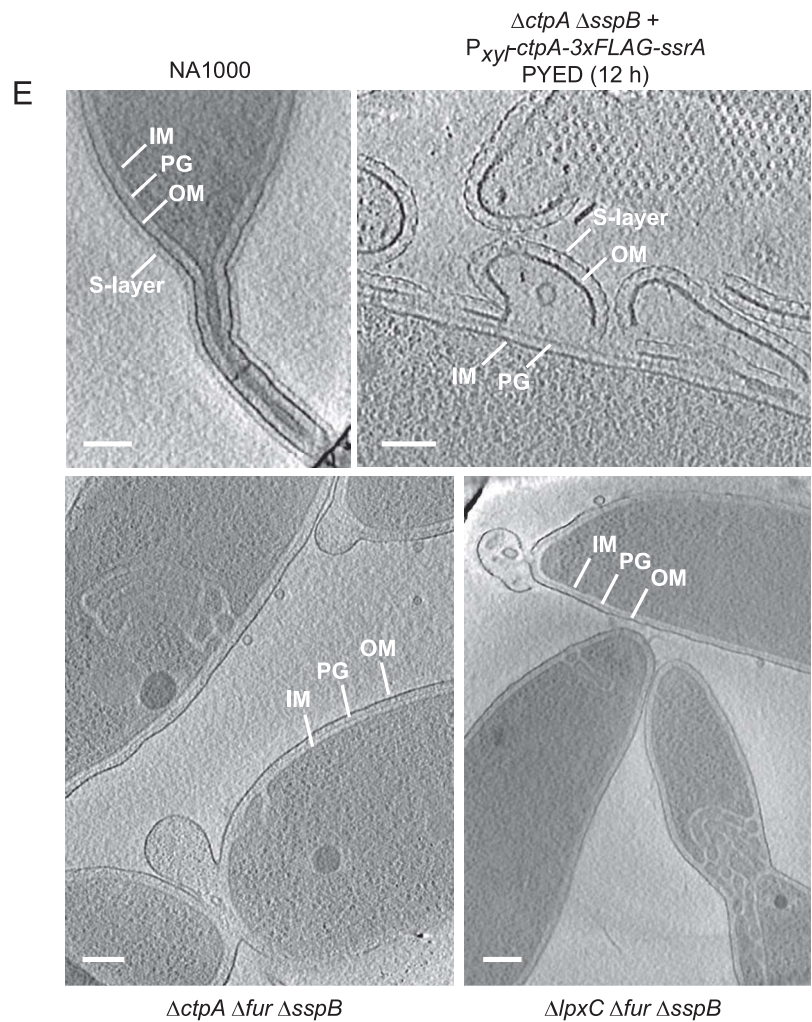
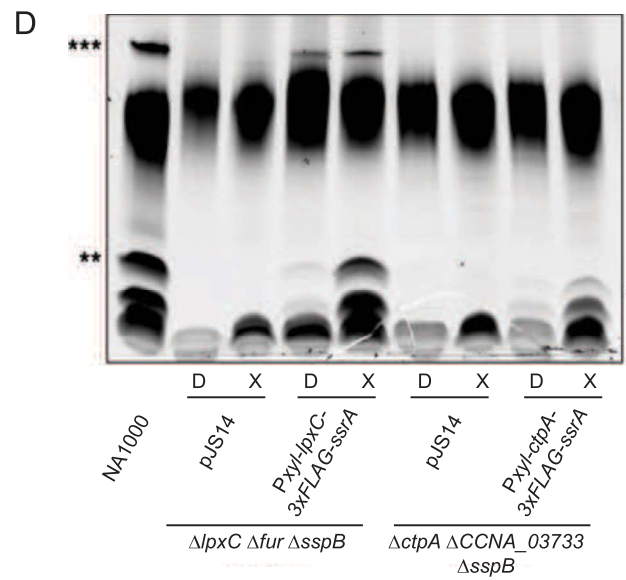
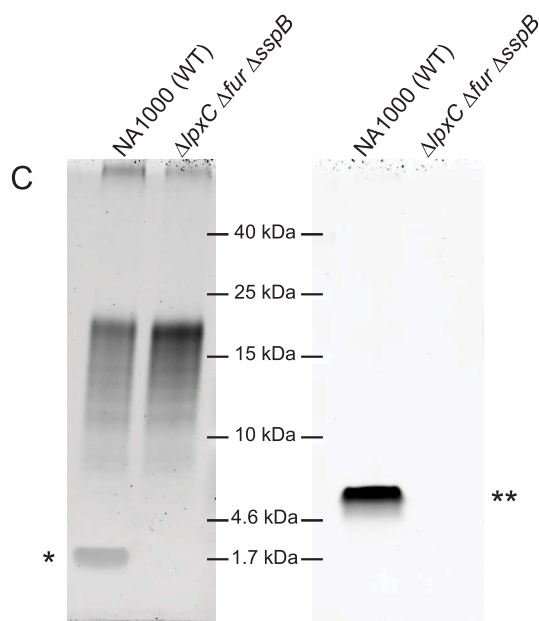
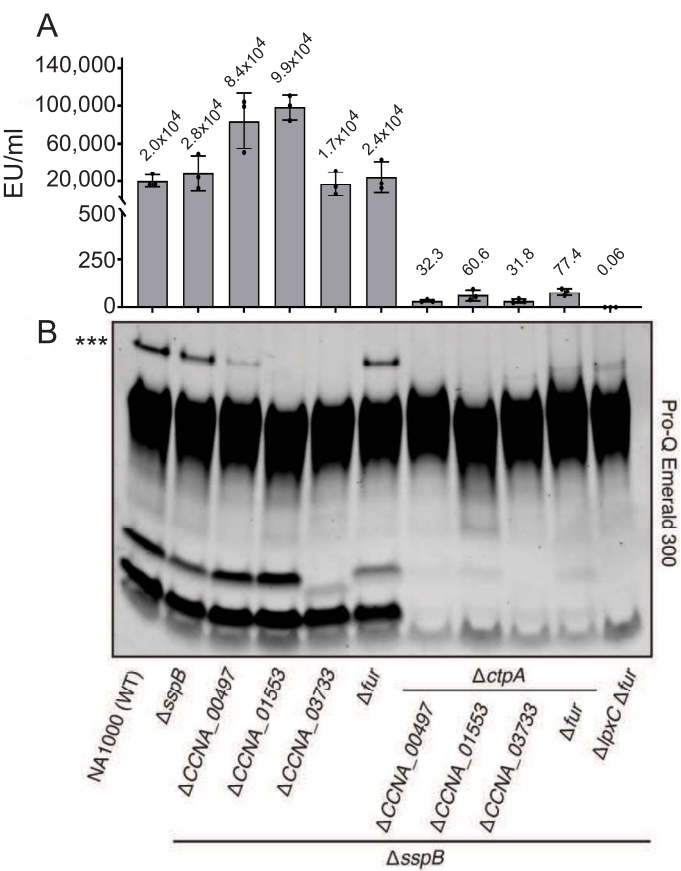


Figure 2

Figure 3

Figure 3

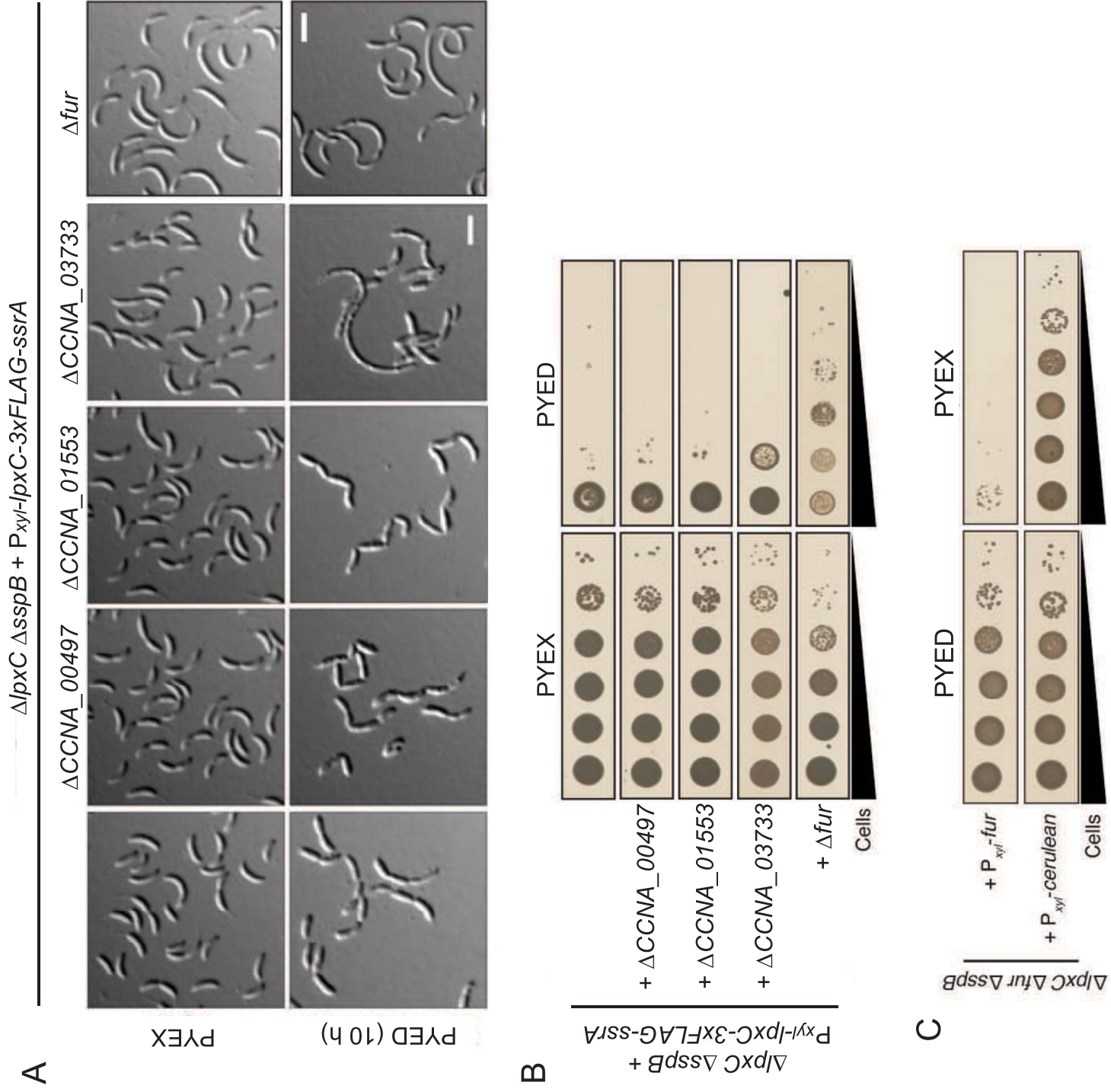


Figure 4

Figure 4

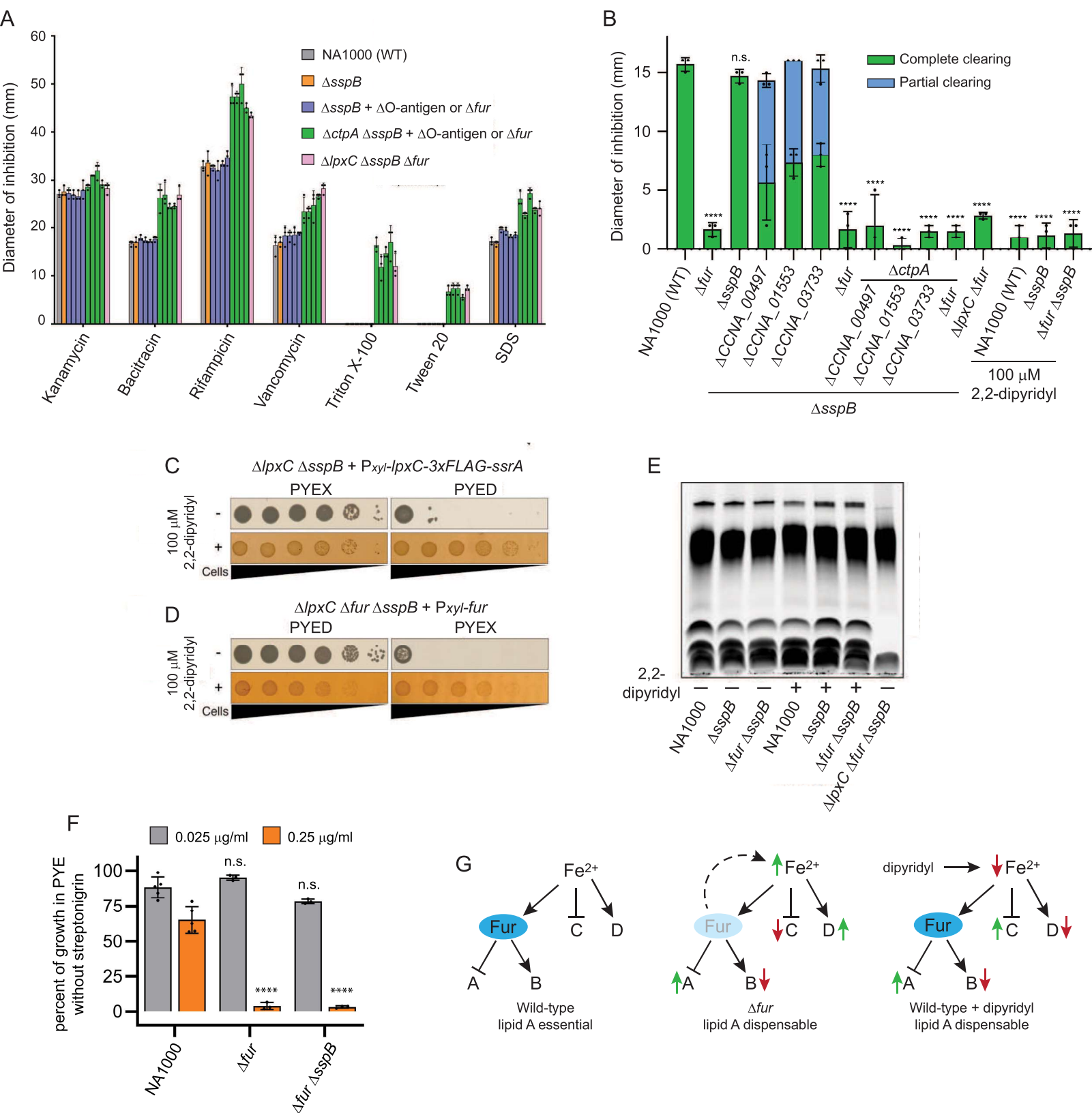


Figure 5

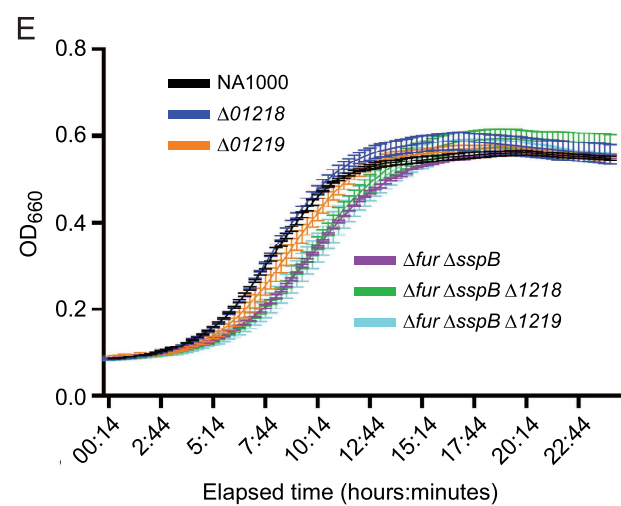
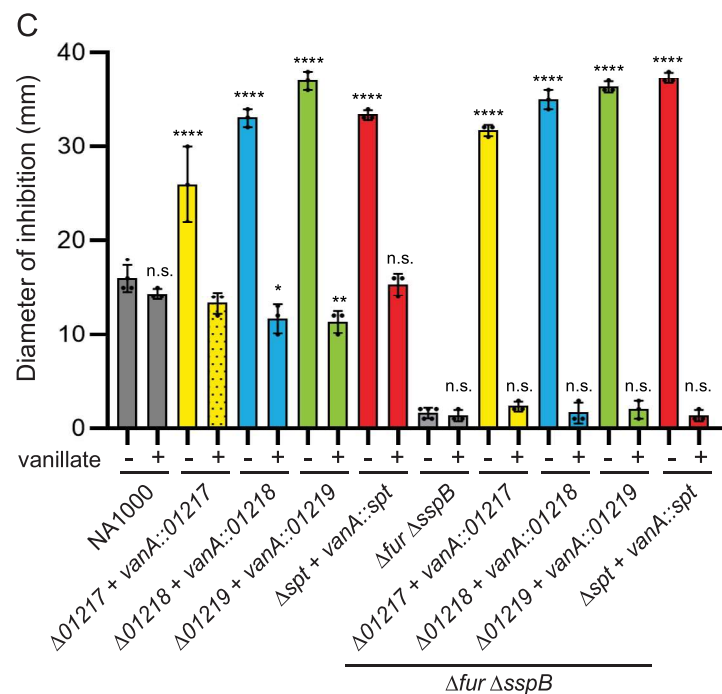
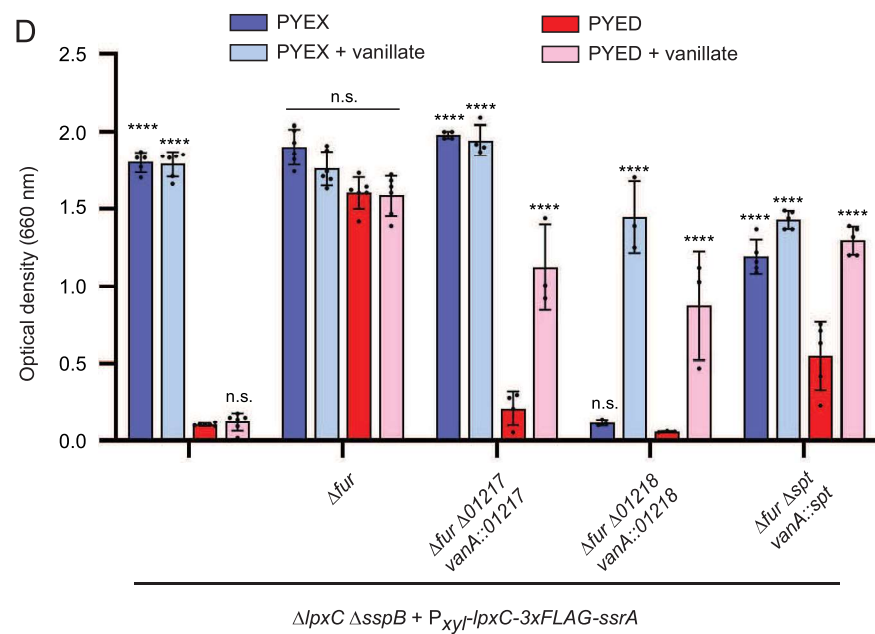
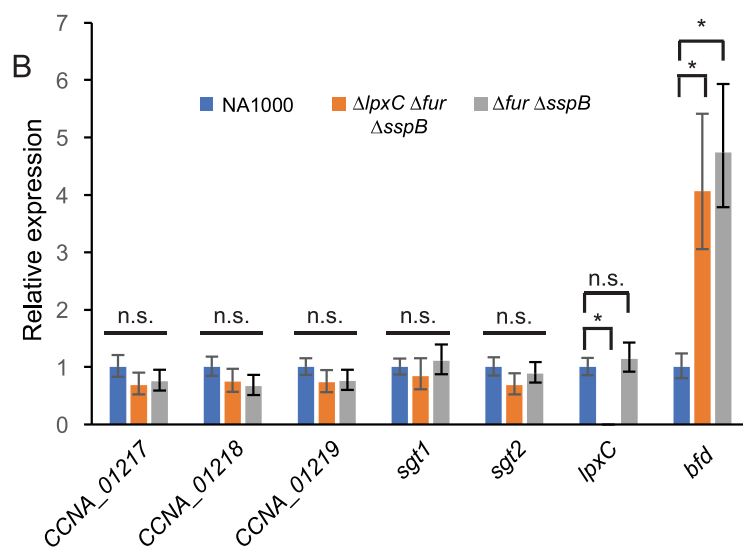
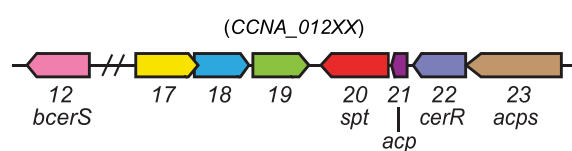
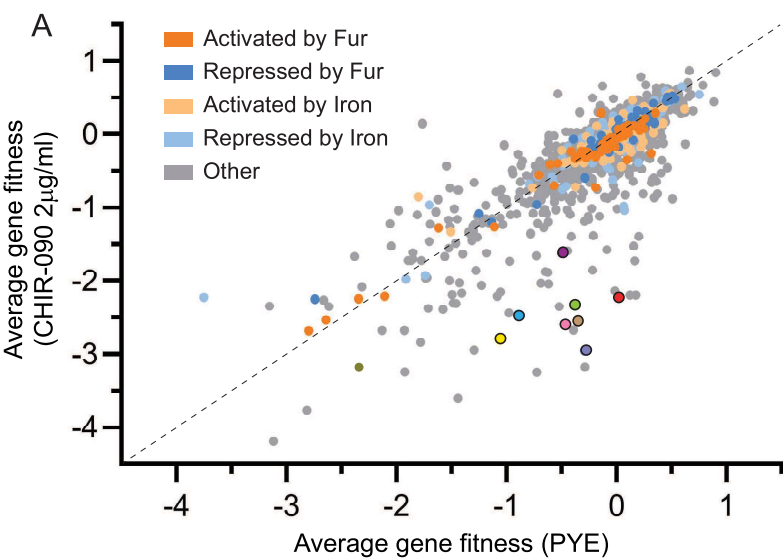


Figure 5

Figure 6

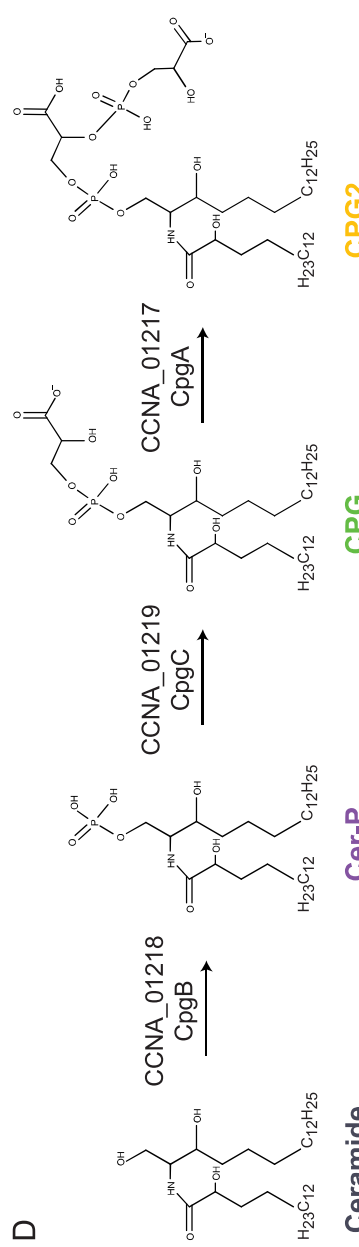
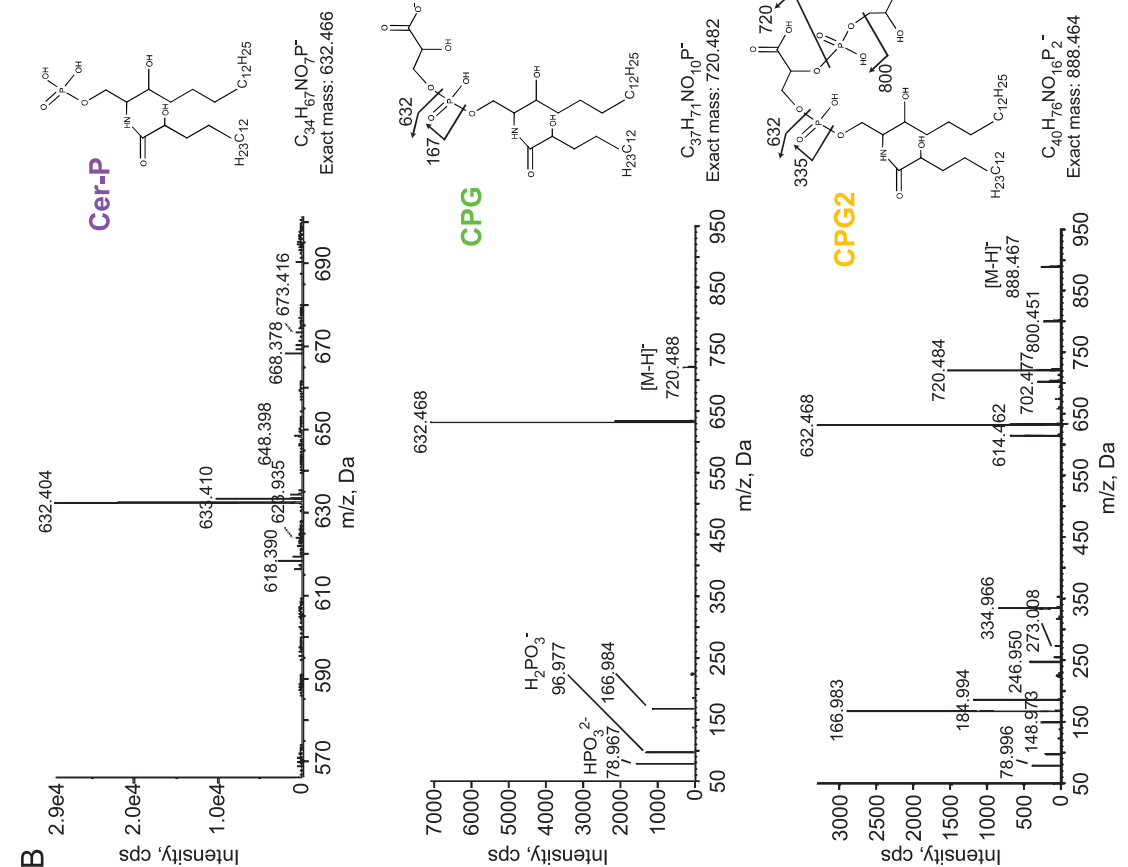
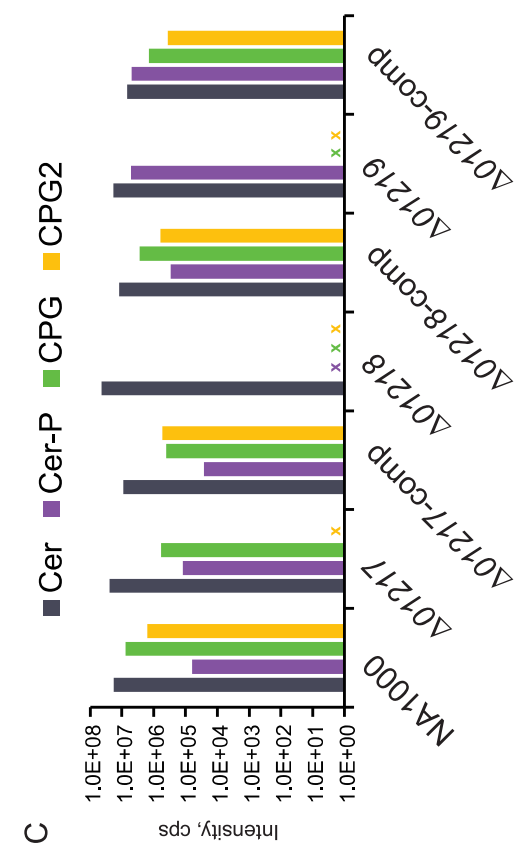
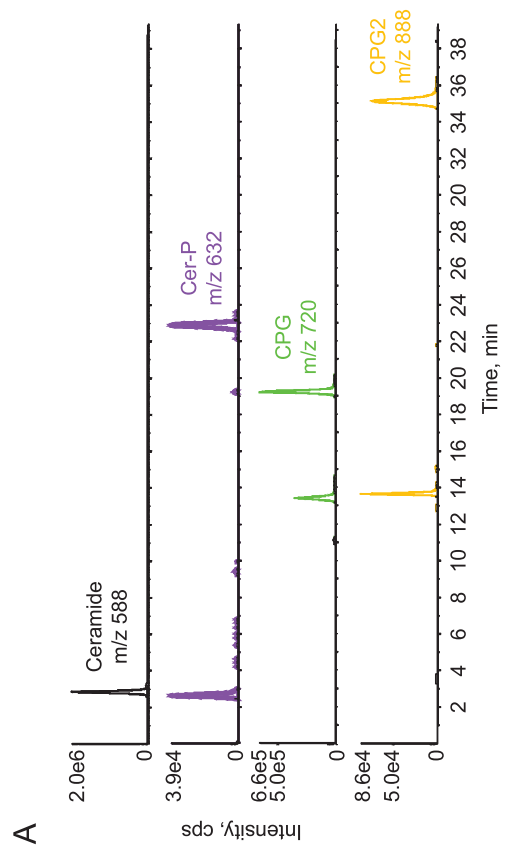
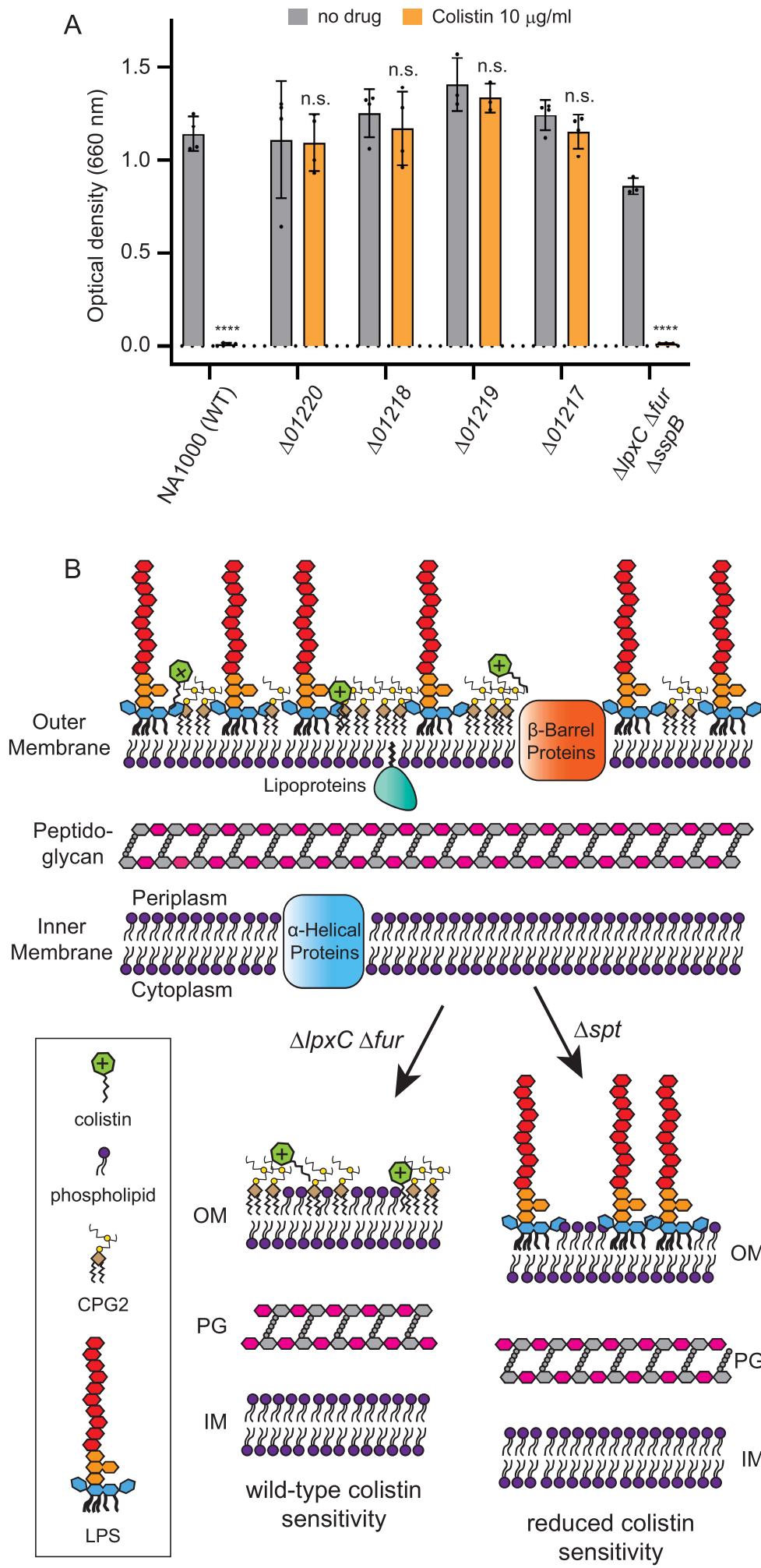


Figure 7

Figure 7



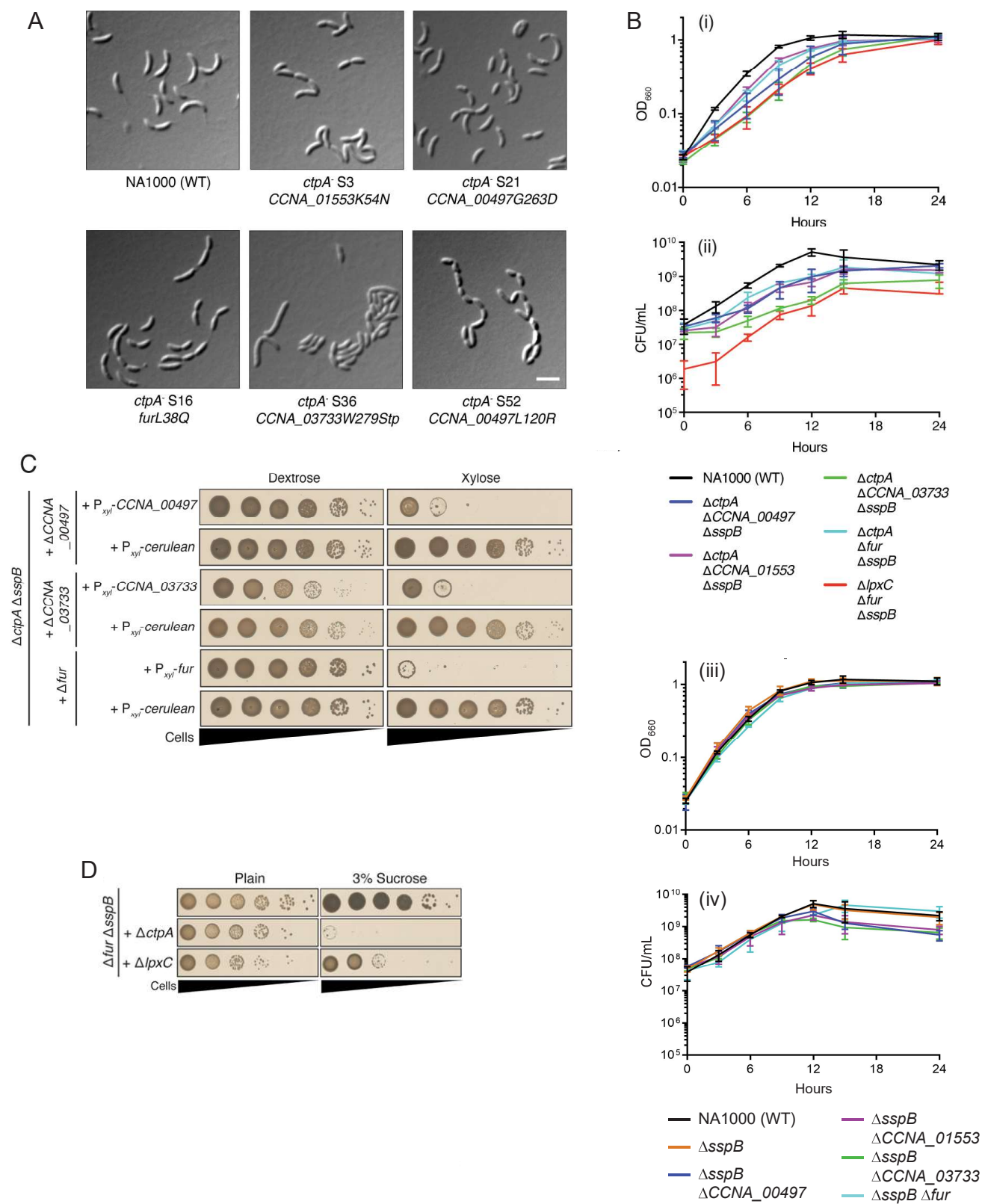


Figure S1, related to Fig. 1: Morphology, growth, and complementation of strains in which suppressor mutations permit the loss of *ctpA*. (A) Isolates from the $\Delta ctpA$ suppressor screen show variations in morphology. DIC images of selected suppressor isolates confirmed to have lost the *ctpA* covering plasmid. Putative suppressor mutations identified by whole-genome resequencing are indicated. Scale bar, 3 μm . (B) Growth curves of the indicated strains in PYE showing (i or iii) OD_{660} and (ii or iv) colony-forming units (CFU) per mL (mean $\pm$ S.D., $N=3$). (C) Viability assays of $\Delta ctpA$ suppressor mutants, each harboring a vector for xylose-driven expression of the corresponding suppressor gene or the *cerulean* gene as a control. Kanamycin was included in media to retain expression vectors. (D) Viability of the indicated strains on PYE medium (Plain) or PYE medium + 3% sucrose.

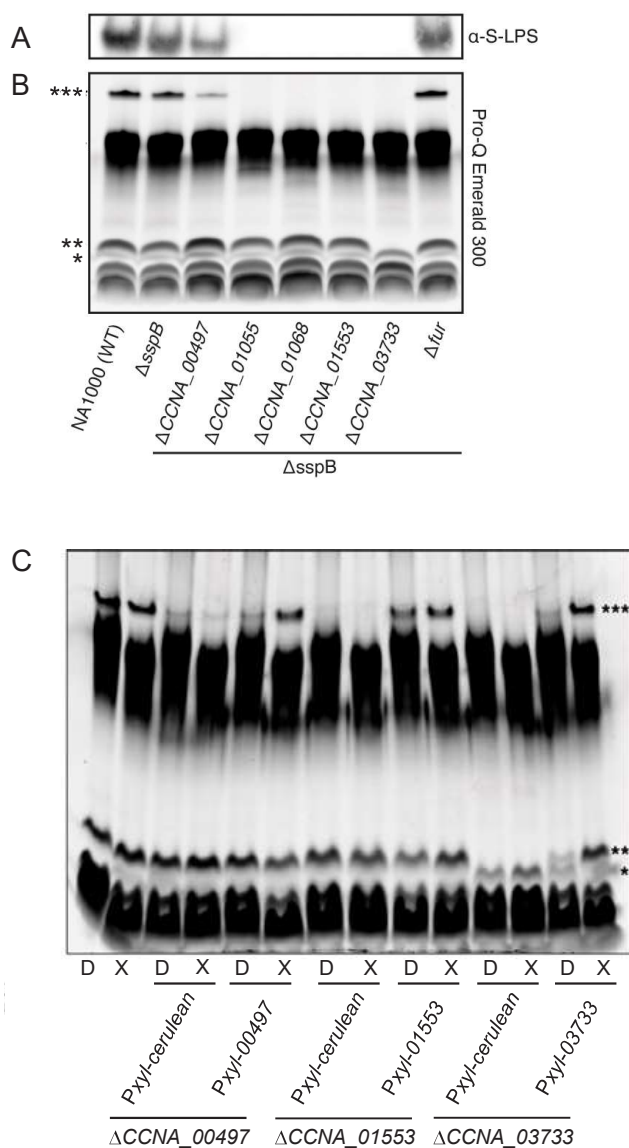


Figure S2, related to Fig.1: A subset of *ctpA* suppressor mutations impair or block S-LPS production. (A) α -S-LPS-probed immunoblot and (B) Pro-Q Emerald 300-stained gel of Proteinase K-treated whole-cell lysates of the indicated strains. (C) Complementation of O-antigen biosynthesis using plasmid-borne genes driven by a xylose-inducible promoter. Pro-Q Emerald 300-stained polyacrylamide gel of Proteinase K-treated whole-cell lysates of the indicated strains grown in either PYED (D) or PYEX (X). Samples were normalized by OD660. *** = S-LPS, ** = putative full-length lipid A-core polysaccharide, * = putative incomplete lipid A-core species in cells lacking *manC* activity (*CCNA_03733*).

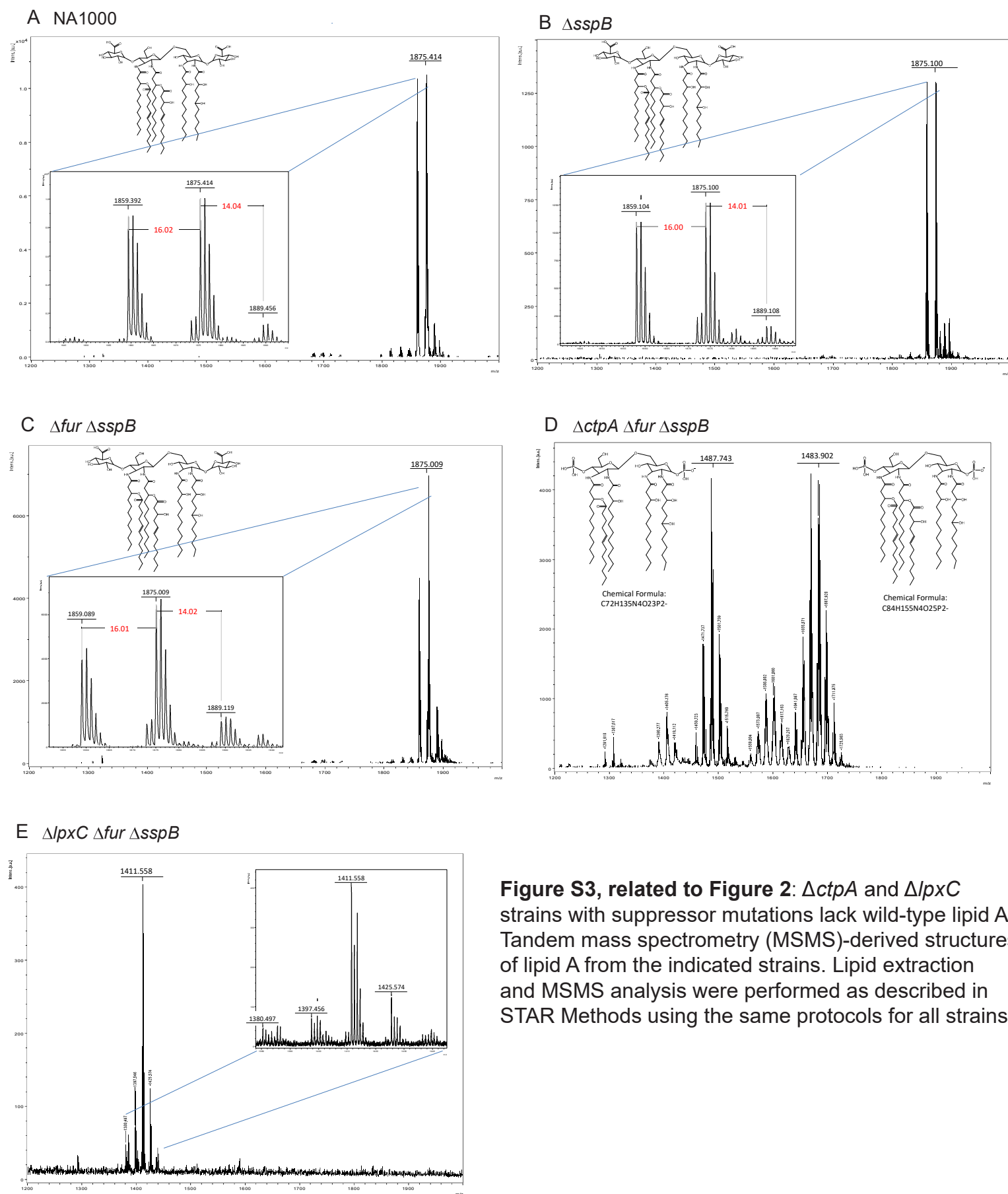


Figure S4

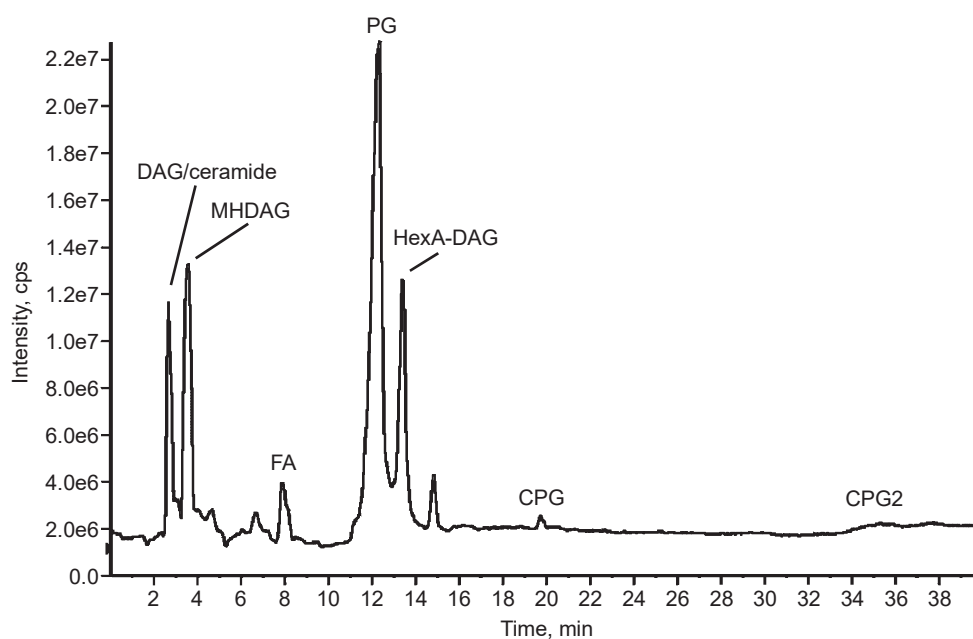


Figure S4, related to Figure 6: The total ion chromatogram of the *C. crescentus* lipidome shows the major lipid species present. DAG: diacylglycerol; MHDAG: mono-hexosyl diacylglycerol; FA: fatty acids; PG: phosphatidylglycerol; HexA-DAG: hexuronic acid-diacylglycerol; CPG: ceramide phosphoglycerate.

Figure S5

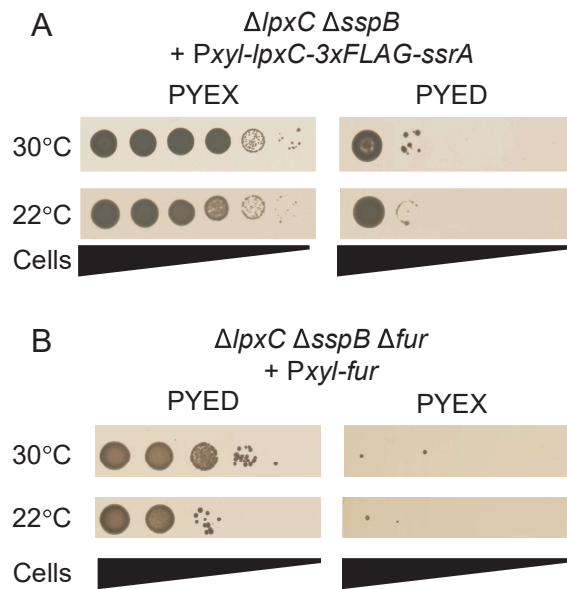


Figure S5, related to Figure 3. Slow growth at a reduced temperature in complex medium is insufficient for viability of $\Delta lpxC$ strains. (A) Viability of the *LpxC* depletion strain grown in inducing (PYEX) or depleting (PYED) conditions, at the indicated temperatures. (B) Viability of the stable $\Delta lpxC \Delta fur \Delta sspB$ strain harboring a $P_{xyl-fur}$ plasmid, grown in noninducing (PYED) or inducing (PYEX) conditions, at the indicated temperatures. Cells were plated from 10-fold serially diluted suspensions normalized to OD₆₆₀ = 0.1. Plates were incubated for 3 days (30°C) or 6 days (22°C) and are representative of at least three independent trials. Plates in B included kanamycin to retain the *fur* expression vector.

Supplementary Tables

Table S1, related to Figure 1: Single-nucleotide polymorphisms and indels in *ΔctpA* suppressors^a

Strain	Sequence position ^b	Gene ^c	Annotation	Base Change	Amino Acid Substitution
S3	1668695	01553	Undecaprenyl-phosphate beta-N-acetyl-D-fucosaminephosphotransferase ^d	G>T	K54N
S3	2383967	02235	SGNH hydrolase family protein	G>A	G109D
S8	3492550	03316	UDP-N-acetylglucosamine 4,6-dehydratase/UDP-D-quinovosamine 4-dehydrogenase ^d	AG>ATG	E487Frameshift
S16	58496	00055	Ferric uptake regulation protein	A>T	L38Q
S21	515085	00497	Smooth LPS biosynthesis glycosyltransferase ^d	G>A	G263D
S21	1378399	01250	FecCD-family transporter protein	GCC>GC	A340Frameshift
S32	1157376	01056	Methyltransferase ^e	A>AG	S31Framshift
S32	2164248	02016	nuoMNADH-quinone oxidoreductase chain M	C>T	M7I
S32	2949436	02792	TonB-dependent outer membrane receptor	A>G	S516G
S36	3901011	03733	Mannose-1-phosphate guanylyltransferase ^d	C>T	W279Stop
S38	726779	00669	Glycosyltransferase family 99 protein WbsX ^d	CTG>CG	Q476Frameshift
S40	378822	00362	Zinc uptake regulation protein	A>T	C156S
S40	2868272	-	-	G>C	-
S40	3913883	03744	dTDP-glucose 4,6-dehydratase ^d	T>A	I269F
S43	1377138	01249	ABC-transporter substrate binding protein	C>T	A169V
S43	1668861	01553	Undecaprenyl-phosphate beta-N-acetyl-D-fucosaminephosphotransferase ^d	CC>GA	P110E
S43	2976030	02820	TadG-family protein	C>T	Silent
S44	727833	00669	Glycosyltransferase family 99 protein WbsX ^d	A>C	L125R
S47	537654	00524	Conserved hypothetical cytosolic protein	A>AG	L369Frameshift
S47	1173251	01068	Glycosyltransferase ^d	GCC>GC	R293Frameshift
S52	514656	00497	Smooth LPS biosynthesis glycosyltransferase ^d	T>G	L120R
S52	810384	00752	3-hydroxybutyryl-CoA dehydrogenase	T>A	Stop>Y
S52	3797776	-	-	T>C	-

S53	58379	00055	Ferric uptake regulation protein	G>T	S77Stop
S53	1778110	01656	Endonuclease/exonuclease/phosphatase family protein	A>C	L4R
S53	3900664	03733	Mannose-1-phosphate guanylyltransferase ^d	G>A	Q395Stop
S54	1377127	01249	ABC-transporter substrate binding protein	CGG>CG	G166Frameshift
S54	3492771	03316	UDP-N-acetylglucosamine 4,6-dehydratase/UDP-D-quinovosamine 4-dehydrogenase ^d	G>A	E561K
S57	1492703	01378	Protein-L-isoaspartate O-methyltransferase	G>GC	G59Frameshift
S57	2487074	02347	Phosphomannomutase/ phosphoglucomutase ^d	G>A	G266D
S111	295119	00283	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	A>T	L284Q
S111	1173965	01068	Glycosyltransferase ^d	TGC>T	R55Frameshift
S112	311705	00297	Two-component response regulator	C>T	W64Stop
S112	315570	00301	Phosphotransferase family protein	T>G	I319S
S112	2487307	02347	Phosphomannomutase/ phosphoglucomutase ^d	G>A	E344K
S112	3569520	03399	Flavin prenyltransferase UbiX	C>T	A159T

^aIn addition to the indicated mutations, each strain is also $\Delta ctpA \Delta sspB$.

^bSequence positions refer to the genome of *Caulobacter crescentus* NA1000 (NC_011916.1).

^cThe prefix for each gene number is CCNA_.

^dGenes known or predicted to be involved in O-antigen synthesis.

^eWe speculate that the frameshift in CCNA_01056 affects the expression of the downstream gene CCNA_01055, annotated as a GT1 family glycosyltransferase.

Table S2, related to Figure 3: Single-nucleotide polymorphisms and indels identified in $\Delta lpxC$ suppressors^a

Strain	Sequence position ^b	Gene ^c	Annotation	Base Change	Amino Acid Substitution
S1	58446	00055	Ferric uptake regulation protein Fur	A>T	Y55N
	4000704	03835	3-oxoacyl-(Acyl-carrier-protein) synthase	C>T	G396S
S5	58436	00055	Ferric uptake regulation protein Fur	A>T	V58E

^aIn addition to the indicated mutations, each strain is also $\Delta lpxC \Delta sspB \Delta 00497$.

^bSequence positions refer to the genome of *Caulobacter crescentus* NA1000 (NC_011916.1).

^cThe prefix for each gene number is *CCNA_*.

Table S3, related to Fig. 2: Fur or iron regulation of genes predicted to participate in lipid synthesis.

Gene ^a	Name ^b	Regulation by Fur and/or iron ^c
CCNA_00363	<i>pgpB</i>	None
CCNA_01382	<i>fadD</i>	None
CCNA_01431	<i>plsX</i>	None
CCNA_01432	<i>fabH</i>	None
CCNA_01746	<i>fabD</i>	None
CCNA_01747	<i>fabG</i>	None
CCNA_01750	<i>fabF</i>	None
CCNA_01932	<i>plsC</i>	None
CCNA_01960	<i>accB</i>	None
CCNA_01961	<i>accC</i>	Downregulated by iron limitation only (Leaden, <i>et al.</i> , 2018)
CCNA_01977	<i>plsB</i>	None
CCNA_01989	<i>fabZ</i>	None
CCNA_01995	<i>cdsA</i>	None
CCNA_02001	<i>pgpC</i>	None
CCNA_02040	<i>pgpC</i>	None
CCNA_02302	<i>plsC</i>	None
CCNA_03002	<i>pgsA</i>	None
CCNA_03090	<i>accA</i>	None
CCNA_03656	<i>accD</i>	Upregulated by iron limitation only (Leaden, <i>et al.</i> , 2018)
CCNA_03833	<i>fabI</i>	Downregulated by iron limitation only (Leaden, <i>et al.</i> , 2018)
CCNA_03835	<i>fabB</i>	None
CCNA_03836	<i>fabA</i>	None

^aGene numbers refer to the genome of *Caulobacter crescentus* NA1000 (NC_011916.1).

^bNames are derived from annotations in NCBI associated with NC_011916.1 or from BLAST or PSI-BLAST searches of the *Caulobacter crescentus* NA1000 genome initiated with the indicated *E. coli* amino acid sequence ((Altschul, 1997; Altschul et al., 1990).

^cGene regulation was assessed by inspection of results (Leaden et al., 2018; da Silva Neto et al., 2013).

Table S4, related to Figure 5: Genes whose average fitness scores were lower in CHIR-090-treated cultures than in control cultures by >1.

Gene ^a	Average fitness PYE ^b	Average fitness PYE + 2 µg/ml CHIR-090 ^c	Net average fitness ^d	Annotation
CCNA_03609	-0.287	-3.176	-2.889	Outer membrane protein
CCNA_01222	-0.290	-2.953	-2.662	NADH-ubiquinone oxidoreductase (cerR)
CCNA_03983	-0.726	-3.246	-2.520	HicA-related toxin-antitoxin protein
CCNA_01060	0.175	-2.191	-2.365	Type I protein secretion ATP-binding protein, RsaD
CCNA_01638	0.122	-2.195	-2.317	Beta-lactamase family protein
CCNA_01162	-0.391	-2.668	-2.277	Beta-D-glucoside glucohydrolase
CCNA_01220	0.019	-2.222	-2.240	BioF-family ceramide biosynthesis protein CcbF (spt)
CCNA_01223	-0.354	-2.549	-2.195	Acyl-CoA synthetase (acps)
CCNA_03026	-1.443	-3.605	-2.162	Two-component response regulator, PetR
CCNA_01817	-0.016	-2.139	-2.123	Nitrogen assimilatory regulatory protein, NtrX
CCNA_01212	-0.470	-2.589	-2.120	dATP pyrophosphohydrolase (bcerS)
CCNA_01219	-0.383	-2.337	-1.955	Putative cytosolic protein (cpgC)
CCNA_01067	0.164	-1.730	-1.894	Type I secretion outer membrane protein RsaFa
CCNA_00252	-0.523	-2.345	-1.822	Multimodular transpeptidase-transglycosylase PbpX
CCNA_01217	-1.063	-2.808	-1.746	Phosphatidylglycerophosphate synthase (cpgA)
CCNA_02127	0.155	-1.521	-1.676	Bifunctional lysylphosphatidylglycerol flippase/synthetase MprF
CCNA_00525	-1.296	-2.941	-1.645	Prolipoprotein diacylglycerol transferase
CCNA_03027	-0.219	-1.855	-1.636	Two-component sensor histidine kinase
CCNA_03321	0.202	-1.390	-1.593	VanZ superfamily protein
CCNA_01218	-0.894	-2.458	-1.564	Sphingosine kinase/diacylglycerol kinase-related protein (cpgB)
CCNA_03864	-0.087	-1.572	-1.485	DUF3576 domain-containing protein
CCNA_00001	-0.980	-2.436	-1.456	Pyruvate, phosphate dikinase regulatory protein
CCNA_01427	-0.619	-2.040	-1.421	Beta-barrel assembly machine protein BamE
CCNA_03876	0.509	-0.880	-1.389	Transcription termination factor rho
CCNA_00512	-0.689	-2.035	-1.345	GTP-binding protein, probable translation factor
CCNA_00050	-1.925	-3.242	-1.317	Apolipoprotein N-acyltransferase Lnt

CCNA_00190	-0.624	-1.885	-1.261	Acyl-CoA hydrolase
CCNA_02386	-0.650	-1.875	-1.225	O-antigen ligase related enzyme
CCNA_00080	0.341	-0.862	-1.203	LexA-related transcriptional repressor
CCNA_00924	0.373	-0.813	-1.186	MarR/EmrR family transcriptional regulator
CCNA_00354	-0.212	-1.384	-1.172	NlpC/P60 CHAP domain amidase protein
CCNA_00027	0.109	-1.045	-1.154	2OG-Fe(II) oxygenase
CCNA_01324	-0.445	-1.588	-1.143	LSU ribosomal protein L30P
CCNA_01226	-1.084	-2.210	-1.126	OstA family protein
CCNA_01221	-0.499	-1.623	-1.124	acyl carrier protein (acp)
CCNA_00851	0.073	-1.030	-1.103	periplasmic multidrug efflux lipoprotein precursor
CCNA_03782	-1.055	-2.153	-1.098	cytochrome c biogenesis ATP-binding export protein CcmA
CCNA_03336	-3.118	-4.188	-1.071	Tol-Pal system periplasmic component YbgF
CCNA_03820	-1.783	-2.837	-1.055	LolA-family outer membrane lipoprotein carrier protein
CCNA_03260	0.243	-0.810	-1.054	queuosine biosynthesis protein QueD
CCNA_00850	0.065	-0.980	-1.045	cation/multidrug efflux pump acrB2
CCNA_02081	0.006	-1.013	-1.019	Sec-independent protein translocase protein tatB
CCNA_00735	0.361	-0.644	-1.005	beta-barrel assembly machine protein BamF

^aGene numbers refer to the genome of *Caulobacter crescentus* NA1000 (NC_011916.1).

^bAverage fitness in PYE was calculated from fitness values in set8IT011, set8IT023, and set8IT035.

^cAverage fitness in PYE + 2 µg/ml CHIR-090 was calculated from fitness values in set8IT012, set8IT024, and set8IT036.

^dNet average fitness = (Average fitness PYE + 2 µg/ml CHIR-090)-(Average fitness PYE).

Table S5, related to Figure 3: Growth rates in PYE medium at 22°C.

	Doubling time +/- standard deviation (hours) ^a	
Strain	PYE 30°C	PYE 22°C
NA1000	1.61 +/- 0.04	2.50 +/- 0.17
$\Delta sspB$	1.51 +/- 0.04	2.44 +/- 0.09
$\Delta fur \Delta sspB$	1.77 +/- 0.08	3.16 +/- 0.21

^aDoubling times were calculated from optical density measurements (660 nm) during exponential growth.

Table S6, related to STAR Methods: Strains used in this study.

Strain Number	Description ^a	NCBI BioSample Accession ^b or Note	Reference
KR4000	Wild-type <i>Caulobacter</i> NA1000		(Evinger and Agabian, 1977)
KR3180	NA1000 pJS14		This study
KR1499	Δ sspB::aadA		(Shapland et al., 2011)
KR3877	Δ CCNA_00497::hyg		This study
KR4198	Δ CCNA_00497::hyg pJS14		This study
KR3871	Δ CCNA_01553::hyg		This study
KR4197	Δ CCNA_01553::hyg pJS14		This study
KR4076	Δ fur::hyg		This study
KR4199	Δ fur::hyg pJS14		This study
KR3953	Δ CCNA_00497::hyg Δ sspB::aadA		This study
KR4115	Δ CCNA_01055::hyg Δ sspB::aadA		This study
KR4116	Δ CCNA_01068::hyg Δ sspB::aadA		This study
KR3954	Δ CCNA_01553::hyg Δ sspB::aadA		This study
KR3955	Δ CCNA_03733::hyg Δ sspB::aadA		This study
KR4077	Δ fur::hyg Δ sspB::aadA		This study
KR4153	Δ sspB::aadA pXCERN-2		This study
KR4154	Δ CCNA_00497::hyg Δ sspB::aadA pXCERN-2		This study
KR4155	Δ CCNA_01553::hyg Δ sspB::aadA pXCERN-2		This study
KR4156	Δ CCNA_03733::hyg Δ sspB::aadA pXCERN-2		This study
KR4157	Δ fur::hyg Δ sspB::aadA pXCERN-2		This study
KR4158	Δ CCNA_00497::hyg Δ sspB::aadA pZIK172		This study
KR4159	Δ CCNA_01553::hyg Δ sspB::aadA pZIK173		This study
KR4160	Δ CCNA_03733::hyg Δ sspB::aadA pZIK174		This study
KR4161	Δ fur::hyg Δ sspB::aadA pZIK175		This study
KR4269	Δ fur::hyg Δ sspB::aadA pZIK197		This study
KR3906	Δ ctpA::tetAR Δ sspB::aadA pAB6	Restock of KR2423 from Shapland 2011	(Shapland et al., 2011)
KR4111	Δ ctpA::tetAR Δ sspB::aadA Δ CCNA_00497::hyg pAB6	SAMN12568762	This study
KR4112	Δ ctpA::tetAR Δ sspB::aadA Δ CCNA_01553::hyg pAB6	SAMN12568763	This study
KR4092	Δ ctpA::tetAR Δ sspB::aadA Δ CCNA_03733::hyg pAB6	SAMN12568761	This study
KR4090	Δ ctpA::tetAR Δ sspB::aadA Δ fur::hyg pAB6	SAMN12568759	This study
KR4007	Δ lpxC::tetAR Δ sspB::aadA pZIK133		This study
KR4008	Δ lpxC::tetAR Δ sspB::aadA Δ CCNA_00497::hyg pZIK133		This study
KR4223	Δ lpxC::tetAR Δ CCNA_00497::hyg pZIK133		This study
KR4009	Δ lpxC::tetAR Δ sspB::aadA Δ CCNA_01553::hyg pZIK133		This study
KR4010	Δ lpxC::tetAR Δ sspB::aadA Δ CCNA_03733::hyg pZIK133		This study
KR4091	Δ lpxC::tetAR Δ sspB::aadA Δ fur::hyg pZIK133	SAMN12568760	This study

KR4113	<i>ΔctpA::tetAR ΔsspB::aadA ΔCCNA_00497::hyg</i>	SAMN12568767	This study
KR4114	<i>ΔctpA::tetAR ΔsspB::aadA ΔCCNA_01553::hyg</i>	SAMN12568768	This study
KR4104	<i>ΔctpA::tetAR ΔsspB::aadA ΔCCNA_03733::hyg</i>	SAMN12568766	This study
KR4102	<i>ΔctpA::tetAR ΔsspB::aadA Δfur::hyg</i>	SAMN12568764	This study
KR4103	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg</i>	SAMN12568765	This study
KR4176	<i>ΔctpA::tetAR ΔsspB::aadA Δfur::hyg pZIK175</i>		This study
KR4270	<i>ΔctpA::tetAR ΔsspB::aadA Δfur::hyg pZIK197</i>		This study
KR4177	<i>ΔctpA::tetAR ΔsspB::aadA Δfur::hyg pXCERN-2</i>		This study
KR4178	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg pZIK175</i>		This study
KR4271	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg pZIK197</i>		This study
KR4179	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg pXCERN-2</i>		This study
KR4180	<i>ΔctpA::tetAR ΔsspB::aadA ΔCCNA_03733::hyg pZIK174</i>		This study
KR4181	<i>ΔctpA::tetAR ΔsspB::aadA ΔCCNA_03733::hyg pXCERN-2</i>		This study
KR4182	<i>ΔctpA::tetAR ΔsspB::aadA ΔCCNA_00497::hyg pZIK172</i>		This study
KR4183	<i>ΔctpA::tetAR ΔsspB::aadA ΔCCNA_00497::hyg pXCERN-2</i>		This study
KR4148	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg pZIK133</i> Plasmid re-introduced for complementation		This study
KR4149	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg pJS14</i>		This study
KR4150	<i>ΔctpA::tetAR ΔsspB::aadA ΔCCNA_03733::hyg pAB6</i> Plasmid re-introduced for complementation		This study
KR4151	<i>ΔctpA::tetAR ΔsspB::aadA ΔCCNA_03733::hyg pJS14</i>		This study
KR4264	NA1000 pZIK200		This study
KR4147	NA1000 pZIK171		This study
KR4170	NA1000 pZIK179		This study
KR4489	<i>ΔCCNA_01217</i>		This study
KR4549	<i>ΔCCNA_01217 vanA::01217::FLAG</i>	pKR438 integrated	This study
KR4450	<i>ΔCCNA_01218</i>		This study
KR4505	<i>ΔCCNA_01218 vanA::01218::FLAG</i>	pKR435 integrated	This study
KR4430	<i>ΔCCNA_01219</i>		This study
KR4501	<i>ΔCCNA_01219 vanA::01219::FLAG</i>	pKR436 integrated	This study
EK720	<i>ΔCCNA_01220</i> also known as KR4431		(Stankeviciute et al., 2019)
KR4530	<i>ΔCCNA_01220 vanA::01220::FLAG</i>	pKR437 integrated	This study
KR4517	<i>Δfur::hyg ΔsspB::aadA ΔCCNA_01217</i>		This study
KR4551	<i>Δfur::hyg ΔsspB::aadA ΔCCNA_01217 vanA::01217::FLAG</i>	pKR438 integrated	This study
KR4442	<i>Δfur::hyg ΔsspB::aadA ΔCCNA_01218</i>		This study
KR4503	<i>Δfur::hyg ΔsspB::aadA ΔCCNA_01218 vanA::01218::FLAG</i>	pKR435 integrated	This study
KR4438	<i>Δfur::hyg ΔsspB::aadA ΔCCNA_01219</i>		This study
KR4513	<i>Δfur::hyg ΔsspB::aadA ΔCCNA_01219 vanA::01219::FLAG</i>	pKR436 integrated	This study
KR4439	<i>Δfur::hyg ΔsspB::aadA ΔCCNA_01220</i>		This study
KR4532	<i>Δfur::hyg ΔsspB::aadA ΔCCNA_01220 vanA::01220::FLAG</i>	pKR437 integrated	This study
KR4509	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg ΔCCNA_01217</i> pZIK133		This study

KR4555	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg ΔCCNA_01217 vanA::01217::FLAG</i> pZIK133	pKR438 integrated	This study
KR4451	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg ΔCCNA_01218</i> pZIK133		This study
KR4527	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg ΔCCNA_01218 vanA::01218::FLAG</i> pZIK133	pKR435 integrated	This study
KR4441	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg ΔCCNA_01220</i> pZIK133		This study
KR4545	<i>ΔlpxC::tetAR ΔsspB::aadA Δfur::hyg ΔCCNA_01220 vanA::01220::FLAG</i> pZIK133	pKR437 integrated	This study
KR4205	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #3	SAMN11107060	This study
KR4206	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #8	SAMN11107061	This study
KR4207	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #16	SAMN11107062	This study
KR4208	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #21	SAMN11107063	This study
KR4209	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #32	SAMN11107064	This study
KR4210	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #36	SAMN11107065	This study
KR4211	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #38	SAMN11107066	This study
KR4212	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #40	SAMN11107067	This study
KR4213	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #43	SAMN11107068	This study
KR4214	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #44	SAMN11107069	This study
KR4215	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #47	SAMN11107070	This study
KR4216	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #52	SAMN11107071	This study
KR4217	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #53	SAMN11107072	This study
KR4218	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #54	SAMN11107073	This study
KR4219	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #57	SAMN11107074	This study
KR4220	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #111	SAMN11107075	This study
KR4221	<i>ΔctpA::tetAR ΔsspB::aadA</i> suppressor isolate #112	SAMN11107076	This study
KR4224	<i>ΔlpxC::tetAR ΔsspB::aadA</i> suppressor isolate #1	SAMN11107077	This study
KR4225	<i>ΔlpxC::tetAR ΔsspB::aadA</i> suppressor isolate #5	SAMN11107078	This study

^a For all strains designated “suppressor isolate,” additional genotype information is available in Table S1 or Table S2.

^bBioSample Accession numbers refer to genome resequencing data for the indicated strains in the Sequence Read Archive at <https://www.ncbi.nlm.nih.gov/sra>, under BioProject ID PRJNA526705

Table S7, related to STAR Methods: Plasmids used in this study.

Name	Description	Reference
pJS14	Broad host range cloning vector; high copy; chlor ^R ; pBBR1MCS derivative with unique EcoRI site	(J. Skerker, unpublished)
pNPTS138	kan ^R ; sacB-containing integration vector	(M.R. Alley, unpublished)
pXCERN-2	For integration at P _{xyiX} ; encodes xylose-inducible <i>cerulean</i> that can be exchanged for gene of interest; kan ^R	(Thanbichler et al., 2007)
pMCS-2	For integration at locus specified by insert sequence; kan ^R	(Thanbichler et al., 2007)
pAB6	pJS14-P _{xyiX} - <i>ctpA</i> -3xFLAG- <i>ssrA</i>	(Shapland et al., 2011)
pZIK133	pJS14-P _{xyiX} - <i>lpxC</i> -3xFLAG- <i>ssrA</i>	This study
pZIK172	pXCERN-2-P _{xyiX} -CCNA_00497	This study
pZIK173	pXCERN-2-P _{xyiX} -CCNA_01553	This study
pZIK174	pXCERN-2-P _{xyiX} -CCNA_03733	This study
pZIK175	pXCERN-2-P _{xyiX} -CCNA_00055 (<i>fur</i>)	This study
pZIK78	pNPTS138-CCNA_00497::hyg; for replacing CCNA_00497 with hygromycin resistance cassette	This study
pZIK82	pNPTS138-CCNA_01055::hyg; for replacing CCNA_01055 with hygromycin resistance cassette	This study
pZIK81	pNPTS138-CCNA_01068::hyg; for replacing CCNA_01068 with hygromycin resistance cassette	This study
pZIK73	pNPTS138-CCNA_01553::hyg; for replacing CCNA_01553 with hygromycin resistance cassette	This study
pZIK80	pNPTS138-CCNA_03733::hyg; for replacing CCNA_03733 with hygromycin resistance cassette	This study
pZIK161	pNPTS138-CCNA_00055::hyg; for replacing <i>fur</i> with hygromycin resistance cassette	This study
pZIK134	pNPTS138-CCNA_02064::tetAR; for replacing <i>lpxC</i> with tetracycline resistance cassette	This study
pHP45Ω-hyg	For isolating <i>hyg</i> fragment; hyg ^R ; amp ^R	(Blondelet-Rouault et al., 1997)
pKOC3	Contains <i>tetAR</i> flanked by EcoRI sites; amp ^R ; tet ^R	(Skerker et al., 2005)
pVMCS-4	For amplification of <i>aacC1</i> ; gent ^R	(Thanbichler et al., 2007)
pMCS-4	For single integration to disrupt genomic loci; gent ^R	(Thanbichler et al., 2007)
pVGFP-2	Complementation vector for expressing target genes from <i>vanA</i> locus; kan ^R	(Thanbichler et al., 2007)

pVGFPC-4	Complementation vector for expressing target genes from <i>vanA</i> locus; gent ^R	(Thanbichler et al., 2007)
pVCHYC-5	Complementation vector for expressing target genes from <i>vanA</i> locus; tet ^R	(Thanbichler et al., 2007)
pFLGC-1	Vector for adding FLAG tag to the C-terminus of open reading frames; spec ^R	(Thanbichler et al., 2007)
pGS74	pNPTS138-based plasmid for markerless deletion of <i>CCNA_01217</i>	This study
pEK406	pVCHYC-5-based complementing vector, <i>vanA::CCNA_01217-FLAG</i>	This study
pKR438	pVGFPC-4-based complementing vector, <i>vanA::CCNA_01217-FLAG</i>	This study
pKR429	pNPTS138-based plasmid for markerless deletion of <i>CCNA_01218</i>	This study
pKR432	<i>CCNA_01218::FLAG</i> in pFLGC-1	This study
pKR435	pVGFPC-2-based complementation vector, <i>vanA::CCNA_01218-FLAG</i>	This study
pGS76	pNPTS138-based plasmid for markerless deletion of <i>CCNA_01219</i>	This study
pKR433	<i>CCNA_01219::FLAG</i> in pFLGC-1	This study
pKR436	pVGFPC-2-based complementation vector, <i>vanA::CCNA_01219-FLAG</i>	This study
pEK722	pNPTS138-based plasmid for markerless deletion of <i>CCNA_01220</i>	(Stankeviciute et al., 2019)
pKR434	<i>CCNA_01220::FLAG</i> in pFLGC-1	This study
pKR437	pVGFPC-2-based complementation vector, <i>vanA::CCNA-01220-FLAG</i>	This study
pKR438	pVGFPC-4-based complementation vector, <i>vanA::CCNA-01217-FLAG</i>	This study

Table S8, related to STAR Methods: Primers used in this study.

Name	Sequence (5'-3')
pJS14-PxylX	AGAACTAGTGGATCCTCACATGGTCTCGAA
PxylX-lpxC R	ACCCGAAGCCGACACGGCGTCGTCTCCCA
PxylX-lpxC F	TGGGGAGACGACGCCGTGTCGGCTTCGGGT
lpxC-3xFLAG R	GTAGTCCATGGATCCAACCGCTTCTGCAAG
lpxC-3xFLAG F	CTTGCAAGAAGCGTTGGATCCATGGACTAC
ssrA-pJS14	CTTGATATCGAATTCTCACGCAGCGACGGC
pVCERN-2 00497 F	ACGCATATGAACAGCATTCTCCCG
pVCERN-2 00497 R	CCGGAGCTCCTAGATCGGCCGGCC
pVCERN-2 01553 F	ACGCATATGAAGCGTATGTTTGAT
pVCERN-2 01553 R	CCGGAGCTCCTAACGGGTGACGCC
pVCERN-2 03733 F	ACGCATATGGCTGCGATCTATCCG
pVCERN-2 03733 R	CCGGAGCTCTCAACGCGGCTTCGT
Pvan-fur	GAGGAAACGCATATGGATCGACTCGAAAAG
fur-pVCERN	AATTCTCCGGAGCTCTTACTCCTCCAGCGG
3xFLAG-F	cgccggcgatccatggactacaaag
3xFLAG-JS14	CTTGATATCGAATTCTCACTTGTCATCGTCATC
00497::hyg UpF	CTCACTAGTAGGACGACGCCATA
00497::hyg UpR	ATCCCCGGGGGCAAGGGTCGAGAC
00497::hyg DownF	ATCCCCGGGCGCCCGCTGCTGTGG
00497::hyg DownR	AGCGAATTCACCGAGGATTTGGTT
01055::hyg UpF	CTCACTAGTGCTGGCGCTGGAAGA
01055::hyg UpR	ACCGGATCCTTGAGCGCCATGGGC
01055::hyg DownF	ACCGGATCCGATGGACGAGCGCAG
01055::hyg DownR	AGCGAATTCTACGATGACGAGTCG
01068::hyg UpF	CTCACTAGTGCGCGAGGACACCGT
01068::hyg UpR	ACCGGATCCGACGCCTGGGTGGCG
01068::hyg DownF	ACCGGATCCCAGGCGGCGCCATAT
01068::hyg DownR	AGCGAATTCTTGACCTGCTTGAGC
01553 UpF	AAACTAGTATCGAGCAGGGCGTC
01553 UpR	GGCCCCGGGATCAAACATACGCTT
01553 DownF	ATCCCCGGGCCGGTGCTGACCGCA
01553 DownR	AAAGAATTCTATGCCGCCAAGCT
03733::hyg UpF	CTCACTAGTGACGCTGGCCCTTGT
03733::hyg UpR	ACCGGATCCGAAGGCGTGATCGAG

03733::hyg DownF	ACCGGATCCGCCGCCACACAGGAT
03733::hyg DownR	AGCGAATTCATGCTCAAGGACCTC
fur UpF	CTCACTAGTAAGAGGGTGACCTCG
fur UpR	ACCGGATCCGAGCTCTACGGGATG
fur DownF	ACCGGATCCTTCGATACAGGCCTT
fur DownR	AGCGAATTCTATATGCAGGCCTTC
lpxC KO F	CTTCGAAACGGCCGATGATG
lpxC KO R	GATCAACAACGCCGATGACG
lpxC UpF	CTCACTAGTTCAGATAGGCTTCGA
lpxC UpR	CATGAATTCCTCAATAACGCCGTG
lpxC DownF	CTTGAATTCCGTGTGCTGAAAATA
lpxC DownR	GACGCATGCTGGCCGCAAGCCGCG
aacC1 EcoRI F	CGCGAATTCgaattgacataagcc
aacC1 EcoRI R	GGGGAATTCgaattggccgcggcg
ctpA KO F	GAAGAAGCGCGGGATCAAGA
ctpA KO R	GTTGCCATGCTTGATGTGCA
EK897	AAGCTTGGCGCCAGCCGG
EK898	GAATTCGCTAGCTTCGGC
EK1047	gctggcgccaagcttAGGCGCTCGATCTGATCTTG
EK1048	caggaagatCGGCCCTTTTTGAACTTCAC
EK1049	aagggccgATCTTCCTGGCCCTCTTC
EK1050	cgaagctagcgaattcCTTGATCGTCGGGTTCTC
EK1055	gctggcgccaagcttGGCTTCTTCCACAACTTTG
EK1056	accttgagGAGGACCCCGGACATATC
EK1057	gggtcctcCTCAAGGTCTGGAGAGAG
EK1058	cgaagctagcgaattcCGTCATCTGTCGTCCCTAC
01218 up_fwd	attgaagccggctggcgccaTGGCACGGCCATTTCCGC
01218 up_rev	tgagcccaaaGCCGTCGATCACCAGCAAG
01218 down_fwd	gatcgacggcTTTGGGCTCAAGCCGTTCTG
01218 down_rev	cgtcacggccgaagctagcgGCCGTTGACAAGCCGCG
EK1357	tactcatATGAGTAGTGAAGTTCAAAAAGGGCCG
EK1358	tactgctagcTTActgtcatgctcatcctgtagtcTTTCGCCAGCCAGGACTGG
EK S216	ACCCCGTCTGATAAGGCTTC
EK S217	GCGAGACCGTGATCGACT
EK S238	GTAGGCAGGGTCCGACAGT
EK S239	ACCGCAAAGTTGTGGAAGAA

EK S240	ATGACCTTCCTCGACACG
EK S241	GAGTCGATCACGGTCTCG
EK S242	AGGGCTTCTTCTTTGGCATT
EK S243	ACTTCATCGTCGGCACCTT
RecUni-1	ATGCCGTTTGTGATGGCTTCCATGTCG
RecVan-2	CAGCCTTGGCCACGGTTTCGGTACC
Nde-01218	TATATTCATATGCTTCGTCTGTCACGCCATCC
01218-Mlu	ATTATACGCGTTCCGACCAGGAACCGCAAGGC
Nde-01219	TATATTCATATGAGCCGCCTGCGCGGCCTC
01219-Mlu	TAATAACGCGTTGCGGCTTGCCGCCGCTCTC
Nde-01220	TATATTCATATGGGGCTATTTGATAAGCACCTGGCC
01220-Mlu	AATAACGCGTGGCGCGGGCGCGCTTGAG
01217-FLAG F	CCACGATGCGAGGAAACGCATATGAGTAGTGAAGTTCAAAAAG
01217-FLAG R	AATTAAGGCGCCTGCAGGCAGCTAGCTCACTTGTCATC
01218-FLAG F	CCACGATGCGAGGAAACGCATATGCTTCGTCTGTCACG
01218-FLAG R	AATTAAGGCGCCTGCAGGCAGCTAGCTTACTTGTCATCGTC
01219-FLAG F	CCACGATGCGAGGAAACGCATATGAGCCGCCTGCGCGG
01219-FLAG R	AATTAAGGCGCCTGCAGGCAGCTAGCTTACTTGTCATCGTCATCCTTGTAG
01220-FLAG F	CCACGATGCGAGGAAACGCATATGGGGCTATTTGATAAG
01220-FLAG R	AATTAAGGCGCCTGCAGGCAGCTAGCTTACTTGTCATC

The following primer pairs were used for qRT-PCR analyses of the indicated genes.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>CCNA_03142 rpoD</i>	CTCTATGCGATCAACAAGCG	ATAGGCCTTGAGGAACTCGC
<i>CCNA_03372 bfd</i>	GTCTGCAACTGTAACGGCATC	CAGCCCTTGTGACGGAAG
<i>CCNA_00793 sgt1</i>	AGCCTTTGTCAGGACCAGAA	CACCACCTCGTCGAGAATTT
<i>CCNA_00792 sgt2</i>	CCACGAGCTGTTTCGTCATC	TGTAGTAGTTGGCGTCAAACG
<i>CCNA_01217 cpqA</i>	GTCACCTTCATCGGCTTTGT	CGAGGAAGGTCATGATCCAG
<i>CCNA_01218 cpqB</i>	GAGAGAACCCGACGATCAAG	CAGGTTGGTGGCCTTCAC
<i>CCNA_01219 cpqC</i>	CCGGGGTCCTCGACTACTAC	CCGTGCTCAAACCTCGTGATA
<i>CCNA_02064 lpxC</i>	TTGTTGATCACCGTGCCGAG	CCGACATGGGGATCGTCTTT