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Five structural genes required for ceramide synthesis in *Caulobacter* and for bacterial survival

Roberto Jhonatan Olea-Ozuna,[1] Sebastian Poggio,[2] Ed Bergström,[3] Elva Quiroz-Rocha,[1] Daniela A. García-Soriano,[1] Diana X. Sahonero-Canavesi,[1] Jonathan Padilla-Gómez,[1] Lourdes Martínez-Aguilar,[1] Isabel M. López-Lara,[1] Jane Thomas-Oates,[3] and Otto Geiger[1*]

[1]*Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Avenida Universidad s/n, Cuernavaca, Morelos, CP 62210, Mexico;* [2]*Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico;* [3]*Centre of Excellence in Mass Spectrometry and Department of Chemistry, University of York, Heslington, York, YO10 5DD, United Kingdom.*

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[*]For correspondence. E-mail otto@ccg.unam.mx; Tel. (+52) 777 3290815; Fax (+52) 777 3134035

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Originality-Significance Statement

Sphingolipids are important structural components in membranes of *Eukarya* and participate in numerous cellular functions. In the *Bacteria* domain, sphingolipids seem to be limited to some members of the phyla *Bacteroidetes* and *Proteobacteria*. Ceramide is the lipidic anchor of sphingolipids attaching them to the membrane and although the structural genes for ceramide biosynthesis are known in *Eukarya*, only the structural gene for serine palmitoyltransferase (*spt*) has been reported in *Bacteria*.

Using data from genome-wide studies of genes required for fitness and genes showing cofitness with *spt*, we have been able to narrow down candidate genes that might act in the same biosynthesis pathway as Spt. Here we report for the α-proteobacterium *Caulobacter crescentus* that, in addition to *spt,* at least four more structural genes are required for the formation of dihydroceramide, a more reduced form of ceramide. Furthermore, we show that all five genes, including *spt*, are required to survive elevated temperatures, or treatment with mild detergents. Also, all five genes need to be functional for sensitivity of *C. crescentus* towards the antibiotic polymyxin B. Whereas in *Eukarya*, Spt forms the biosynthetic intermediate 3-oxo-sphinganine, in *C. crescentus* the expression of two additional genes, encoding a predicted acyl carrier protein and a predicted acyl-CoA synthetase, is required for 3-oxo-sphinganine formation. In this work, an experimentally supported proposal for the bacterial dihydroceramide biosynthesis pathway is presented as well as several phenotypes that are altered when sphingolipids are absent in *C. crescentus*.

Summary

Sphingolipids are essential and common membrane components in eukaryotic organisms, participating in many important cellular functions. Only a few bacteria are thought to harbour sphingolipids in their membranes, among them the well-studied α-proteobacterium *Caulobacter crescentus*, a model organism for asymmetric cell division and cellular differentiation.

Here we report that *C. crescentus* wild type produces several molecular species of dihydroceramides, which are not produced in a mutant lacking the structural gene for serine palmitoyltransferase (*spt*). Whereas growth of a *spt*-deficient mutant and wild type are indistinguishable during the exponential phase of growth, survival of the *spt*-deficient mutant is much reduced, in comparison with wild type, during stationary phase of growth, especially at elevated temperatures. The structural gene for *spt* is located within a genomic cluster, comprising another 16 genes and which, like *spt*, are important for fitness of *C. crescentus*. Mutants deficient in genes linked to *spt* by high cofitness were unable to produce dihydroceramide or to survive in stationary phase of growth at elevated temperatures. At least five structural genes are required for dihydroceramide biosynthesis in *C. crescentus* and sphingolipid biosynthesis is needed for survival of this bacterium and the integrity of its outer membrane.

Introduction

Besides glycerophospholipids and sterols, sphingolipids (SphLs) are essential structural components of eukaryotic membranes. In addition, members of the SphL family of lipids, including sphingoid bases, sphingoid base phosphates, ceramides, and complex SphLs, play crucial roles in the formation of lipid rafts, cell signaling, inflammation, regulation of cell division, differentiation, migration, programmed cell death, and other processes (Nelson and Cox, 2017). As bacteria were thought to be devoid of SphLs, the discovery of SphLs in some bacterial genera was considered so unusual that initially these bacteria received the prefix "Sphingo" to their genus name, i.e. in *Sphingomonas* and *Sphingobacterium*.

Many Gram-negative bacteria possess two bilayered membranes in their envelope, the inner or cytoplasmic membrane (IM) and the outer membrane (OM). The IM is composed of (glycero)phospholipids (PLs) and proteins and contributes to the maintenance of the cellular electrochemical gradient. In contrast, the OM harbors a different set of proteins, with PLs at its inner leaflet, and lipopolysaccharides (LPS) with their lipid A moiety at its outer leaflet (Raetz and Dowhan, 1990) and this structural organization is thought to deny hydrophobic compounds easy access to the bacterial cell. However, since the early 1990s, it has been clear that some Gram-negative bacteria, such as *Sphingomonas* species and other members of the α-4 subclass of proteobacteria, lack LPS and seem to have SphLs instead in the outer leaflet of their OM (Kawasaki *et al.*, 1994; Kawahara *et al.*, 1999). Other bacteria, i.e. of the genus *Bacteroides,* are predominantly in the human intestine and many *Bacteroides* species have in addition to LPS (Weintraub *et al.*, 1989) distinct phospho-SphLs, glyco-SphLs, and deoxy-SphLs (Brown *et al.*, 2019). Also the genus *Acetobacter* may have both LPS and SphLs in their OM. In *Acetobacter malorum*, an increase in

ceramide has been observed in acidic growth conditions or at elevated temperatures (Ogawa *et al.*, 2010), which suggests that under these conditions of stress, LPS might be at least partially replaced by SphLs.

In eukaryotes, the biosynthesis of SphLs takes place in five stages (Nelson and Cox, 2017). Initially, serine is condensed with a fatty acyl-CoA to form 3-oxo-sphinganine (3-ketosphinganine)(stage 1), followed by its reduction to sphinganine (stage 2), acylation to *N*-acylsphinganine (dihydroceramide)(stage 3), and modification of the long-chain base (LCB), i.e. by desaturation to obtain ceramide in mammals or through hydroxylation at C4 of the LCB part to form phytosphingosine in yeast (stage 4)(reviewed by Rego *et al.*, 2014). In stage 5, ceramide is modified with different polar groups to form the enormous diversity of SphLs. Although the eukaryotic genes involved in SphL biosynthesis are known (reviewed by Rego *et al.*, 2014), little is known in bacteria. An exception is SphL biosynthesis step 1 catalyzed by serine palmitoyltransferase (Spt). Based on the phylogenetic distribution of bacterial Spts (Geiger *et al.*, 2010; Geiger *et al.*, 2019), SphLs might be formed in members of two major phyla of *Bacteria*, the *Bacteroidetes* and the *Proteobacteria*.

In a recent contribution, Stankeviciute *et al.* (2019) reported that *C. crescentus* forms ceramides in a Spt-dependent manner. Upon phosphate limitation, a glyco-SphL, termed GSL-2, was formed that required the sequential action of two glycosyltransferases. First, glycosyltransferase Sgt1 (CC_0756) transfers an uronic acid to ceramide and subsequently glycosyltransferase Sgt2 (CC_0755) connects a hexose to the uronic acid residue in order to obtain GSL-2 (Stankeviciute *et al.,* 2019). In a study of *Bacteroides*-derived SphLs (Brown *et al.,* 2019), 3-oxo-sphinganine (3-ketosphinganine), sphinganine, and dihydroceramide were detected in different *Bacteroides* wild types but not in their

respective *spt*-deficient mutants, suggesting that the early steps of sphingolipid biosynthesis in bacteria up to dihydroceramide were via the same intermediates (3-oxo-sphinganine and sphinganine) as had been reported for eukaryotes.

We now report that a Spt-deficient mutant of *C. crescentus* is unable to survive at elevated temperatures of growth. Like the Spt-deficient mutant, mutants deficient in four other genes cannot form dihydroceramide, are sensitive to elevated temperatures or deoxycholate and are resistant towards the antibiotic polymyxin B. Finally, we propose biochemical functions for the five structural genes essential for dihydroceramide biosynthesis in *C. crescentus* and we show that in caulobacterial SphL synthesis, besides Spt, the expression of structural genes for a predicted acyl carrier protein (ACP) and for a predicted acyl-CoA synthetase are needed for efficient 3-oxo-sphinganine formation.

Results and Discussion

Serine palmitoyltransferase gene required for dihydroceramide formation in C. crescentus

Employing a P-BLAST search using the serine palmitoyltransferase (Spt) sequence from *Sphingomonas wittichii* RW1 (Swit_3900)(Raman *et al.,* 2010), we identified several homologues encoded by the *C. crescentus* genome. The best match was encountered with CC_1162 (also termed CCNA_01220), which displayed 73% similarity, 53% identity and an E value of 2×10^{-153} with Swit_3900, suggesting that CC_1162 might encode a caulobacterial Spt. A deletion mutant (DAGS01) was generated in which most of the ORF for the presumptive serine palmitoyltransferase

(CC_1162) had been eliminated. Thin-layer chromatography (TLC) analysis of radiolabeled lipids reveals that *C. crescentus* wild type forms at least two compounds which are absent in mutant DAGS01 and which migrate similarly to a ceramide standard (Fig. 1). These compounds from the wild type are resistant to mild alkali treatment, suggesting that they do not contain carboxylic acid esters. Expression of the presumptive serine palmitoyltransferase (*spt*) gene in *trans* in mutant DAGS01 restores the formation of both alkali-resistant compounds. These compounds are not formed in a DAGS01 mutant harboring the empty plasmid (Fig. 1). Quantification of radiolabeled compounds suggests that the Spt-dependent signals amount to about 3% of total lipids in a wild type chloroform extract.

In order to identify the compounds for which an intact CC_1162 is required, lipids were isolated from *C. crescentus* wild type and the CC_1162-deficient mutant, treated with mild alkali, separated by TLC, extracted from silica gel, and subjected to electrospray ionization (ESI) mass spectrometry in the negative mode. A series of ions at even *m/z* values, consistent with sphingolipids, was observed in the spectrum of the alkali-treated lipid extract of the wild type (Fig. 2), and these were not detected in the spectra of the mutant extracts. Three main species were detected, with two species differing from highest mass species by one and two CH_2 groups (Fig. 2A). The most intense signal was observed at *m/z* 526.484052 for a species with elemental composition $C_{32}H_{64}NO_4$. On collision induced dissociation (CID) product ion analysis fragment ions were observed consistent with a dihydroceramide species with a saturated C18 long-chain base (LCB) with two OH groups, and a hydroxylated C14 saturated fatty acid (Fig. 2B). A slightly less intense signal is observed in the mass spectrum of the extracted lipids at *m/z* 512.468389 ($C_{31}H_{62}NO_4$) for the species that is one CH_2 group

smaller than that at *m/z* 526.484052. On CID product ion analysis, fragmentation of this species suggests that the missing CH_2 group was located either on the LCB or the fatty acid, with the product ion spectrum consistent with the presence of a mixture of these two isomers (Fig. 2C). The least intense of the three signals (*m/z* 498.452708) ($C_{30}H_{60}NO_4$) was also shown on CID product ion analysis to be due to a pair of isomers; one has a C17 LCB and a C13 fatty acid and the other gives product ions consistent with a structure with a C18 LCB and a C12 fatty acid (Fig. 2D). Although the *m/z* value of this pair of isomers would also be consistent with an isomeric structure containing a C16 LCB and a C14 fatty acid, product ions consistent with this structure were not observed. Low intensity signals for additional dihydroceramide species were detected at *m/z* 540.49985 and 538.48427 consistent with components with elemental formulae $C_{33}H_{66}NO_4$ and $C_{33}H_{64}NO_4$, respectively (Fig. 2A). It is noteworthy that odd carbon number C15 and C13 fatty acids are present in *C. crescentus* (Chow and Schmidt, 1974), and would be required to form a dihydroceramide species with a C17 LCB and a C13 fatty acid. In contrast to the structures reported here by us, Stankeviciute *et al.* (2019) detected a C16 ceramide in *C. crescentus* after this bacterium had been cultivated on a minimal medium with low concentrations of inorganic phosphate (1 μM). The reported ceramide is different from the dihydroceramides described here, as it contains a C18:1 LCB and a C16 fatty acid.

Although bacteria usually do not possess mammalian-like ceramides with a 4-5 *trans* unsaturation in their LCB, SphLs of the myxobacterium *Sorangium cellulosum* contain LCBs with a 4-5 *trans* unsaturation (Keck *et al.*, 2011). However, unsaturations at other positions of the LCB have also been reported for the α-4 subclass of proteobacteria (Kawahara *et al.*, 1999) and the mass spectrometric studies employed by Stankeviciute *et al.* (2019) do not permit the assignment of the

position of a C=C unsaturation within the LCB. Clearly, future studies need to clarify whether *C. crescentus* forms both dihydroceramide and ceramide, and whether ratios of SphLs with saturated or unsaturated LCB are altered under distinct growth conditions.

Serine palmitoyltransferase required for survival of C. crescentus *at elevated temperatures*

Although *spt* (CCNA_01220; CC_1162) is not an essential gene for *C. crescentus*, it is important for fitness of this bacterium (Christen *et al.,* 2011; Data Set 1: Supplemental Data Table DT2_Essential_ORFs). In order to study whether dihydroceramide formation in *C. crescentus* was required for growth and survival at different temperatures, *C. crescentus* wild type and *spt*-deficient mutant were cultivated at 30, 37, or 42ºC and optical density at 600 nm (OD_{600}) as well as colony-forming units (CFU) of the cultures were recorded over time (Fig. 3). At the normal growth temperature of *C. crescentus* (30ºC), OD_{600} as well as CFU are essentially indistinguishable between the wild type and the *spt*-deficient mutant (Fig. 3A). At 37ºC, comparison of OD_{600} values between the wild type and the *spt*-deficient mutant indicate that they are nearly the same throughout the experiment and upon entry into the stationary phase of growth (8 h), CFU are still the same in wild type and mutant (about 1.2 x 10^9 CFU per ml)(Fig. 3B). However, continued exposure to stationary phase conditions led to a much more dramatic decrease of replicable cells in the case of the mutant (to 2.2 x 10^4 CFU per ml at 24 h and no survivor at 48 h) than in the wild type (to 4 x 10^8 CFU per ml at 24 h and 2 x 10^5 CFU per ml at 48 h)(Fig. 3B). At 42ºC, OD_{600} values encountered for the mutant were slightly less than for the wild type at all time points and this effect is more pronounced in stationary phase of growth (Fig. 3C). Whereas the wild type reached 1.1 x 10^9 CFU per ml at the

end of exponential growth phase, in the mutant only 1.2 x 10^8 CFU per ml are detectable. After incubation of the mutant for 24 h in stationary phase conditions, only 7 x 10^2 CFU per ml were detected, whereas a density of 4 x 10^5 CFU per ml was detected in the case of the wild type.

In complementation experiments, the *spt*-deficient mutant DAGS01 carrying plasmid pRJ08, from which expression of Spt can be induced in a xylose-inducible fashion, was compared with the *spt*-deficient mutant carrying the empty plasmid pRXMCS-2. At 42ºC, OD_{600} values encountered for the complemented mutant are always higher than for the mutant harboring the empty vector and this effect is pronounced in stationary phase of growth where the complemented mutant reaches a maximal OD_{600} of 1.44 whereas with the mutant harboring the empty vector only an OD_{600} of 0.45 is obtained (Fig. 3D). Whereas 4.0 x 10^8 CFU per ml are reached by the complemented mutant at the end of exponential growth phase, in the mutant harboring the empty vector only 8.3 x 10^7 CFU per ml are detectable. After incubation of the complemented mutant for 24 h in stationary phase conditions, only 6.5 x 10^5 CFU per ml were detected, whereas no survivors of the mutant carrying the empty vector were detected at this time point. Clearly, expression of the *spt* gene is required for survival of *C. crescentus* at elevated temperatures, especially in stationary phase of cultivation (Fig. 3B, C, D).

The serine palmitoyltransferase gene is part of a genomic region required for fitness of C. crescentus

The extensive study performed by Christen *et al.* (2011) permitted definition of the essentiality of each gene of the *C. crescentus* genome. In the course of that study, barcoded Tn*5*-tagged mutant

strains were cultivated on complex medium-containing PYE agar for 7 d and their relative abundance at the end of this cultivation period was used to define fitness. Based on the conditions reported, several growth phases, not only the exponential one, must have been run through during cultivation of the Tn*5*-tagged strains. It is interesting to note that the *spt* gene and most of the other 16 genes localized within the region comprising from CC_1152 (CCNA_01210) to CC_1168 (CCNA_01226) are required for high fitness, whereas none of the 25 genes neighbouring this region upstream or downstream are essential or required for high fitness (Christen *et al.*, 2011). The *spt* gene and the surrounding other 16 genes are organized into 6 operons (Schrader *et al.*, 2014) and might constitute a fitness island (Fig. 4). However, analysis of the *C. crescentus* genome employing IslandViewer 4 (Bertelli *et al.*, 2017) does not identify this 6 operon-comprising region as a genomic island. Although the function of *spt* is known, for the remaining 16 genes of this region there are only vague suggestions for possible functions based on similarities of the encoded proteins to other proteins with known functions. In a more recent study, Price *et al.* (2018) studied the abundance of barcoded Tn*5*-tagged strains before and after growth for a few generations in different liquid medium cultures. Although close to 200 different culture conditions were studied for the growth of *C. crescentus*, in most cases a *spt*-deficient mutant grew as well or even better than the wild type strain. Remarkably, the *spt*-deficient mutant grew better than wild type at many culture conditions, i.e. in the presence of polymyxin B, or when using α-cyclodextrin as the carbon source. A genome-wide analysis of *C. crescentus* in which mutants behaved similarly to the *spt*-deficient mutant in these growth experiments, made it possible to define a cofitness coefficient. A high cofitness value (close to 1) means that the two mutants behaved in a very similar way in most growth experiments, suggesting that they might be involved in the same biochemical pathway (Price *et al.,* 2018). High cofitness with

a *spt* (CC_1162; CCNA_01220)-deficient mutant was observed for mutants in 8 other genes within this fitness-providing region (Price *et al.* (2018) and visualized in Fig. 4). In order to identify structural genes involved in sphingolipid biosynthesis in *C. crescentus*, we decided to generate mutants in genes that were correlated by high cofitness (> 0.5) with *spt*, i.e. mutants deficient in CC_1154, CC_1158, CC_1159-CC_1160, CC_1163, CC_1164, CC_1165, or CC_1167, and study their phenotypes.

Mutants affected in CC_1154, CC_1159-CC_1160, CC_1163, CC_1164, or CC_1165 are altered in dihydroceramide formation

In order to study dihydroceramide formation, *C. crescentus* wild type and mutants deficient in CC_1154, CC_1158, CC_1159-CC_1160, *spt* (CC_1162), CC_1163, CC_1164, CC_1165 or CC_1167 were radiolabeled *in vivo* with ^{14}C-acetate, lipids were extracted, separated by TLC and visualized by phosphorimaging (Fig. 5). Whereas dihydroceramide amounted to about 3% of the total lipid fraction in the wild type, mutants deficient in CC_1154, *spt*, CC_1163, CC_1164, or CC_1165 did not produce detectable dihydroceramide (Fig. 5), though the mutant deficient in CC_1164 formed at least five other compounds of so-far unknown nature. Surprisingly, polar or non-polar deletion mutants lacking CC_1159 and CC_1160 accumulated much more dihydroceramide (about 28% of the total lipid fraction) than the wild type (Fig. 5), suggesting that further metabolism of dihydroceramide might be impaired in those mutants. Mutants deficient in CC_1158 or CC_1167 seem to produce similar levels of dihydroceramide as the wild type. As mutants deficient in CC_1154, CC_1163, CC_1164, or CC_1165 did not produce detectable dihydroceramide, similarly

to the *spt*-deficient mutant DAGS01 (Figs. 1, 5), they might lack proteins/enzymes required for dihydroceramide biosynthesis in bacteria. In the course of this study these mutants were characterized in more detail.

CC_1154, CC_1163, CC_1164, and CC_1165 are required for survival of C. crescentus *at elevated temperatures*

Mutants deficient in the four structural genes required for dihydroceramide formation (CC_1154, CC_1163, CC_1164, or CC_1165) as well as *C. crescentus* wild type were cultivated at 37ºC or at 42ºC and OD_{600} as well as CFU of the cultures were recorded over time (Fig. 6). At 37ºC, comparison of OD_{600} values between the wild type and the mutants indicate that they are nearly the same throughout the experiment and during exponential phase of growth (initial 4 h), CFU are about the same in wild type and mutant cultures (about 6 x 10^8 CFU per ml)(Fig. 6A). However, continued exposure to stationary phase conditions led to a much more dramatic decrease in the case of the mutants (between 10^3 and 2 x 10^4 CFU per ml at 24 h and no survivors detected at 48 h) than in the wild type (to 4 x 10^8 CFU per ml at 24 h and 2 x 10^5 CFU per ml at 48 h)(Fig. 6A). At 42ºC, OD_{600} values encountered for the mutants are always slightly less than in the wild type and the maximal OD_{600} values encountered for the mutants (between 0.64 and 0.68) are considerably less than in the wild type (1.3) when entering stationary phase of growth (Fig. 6B). At mid-exponential phase (4 h) wild type cultures contain only slightly more live cells (4 x 10^8 CFU per ml) than mutants (1.3-1.5 x 10^8 CFU per ml). While replicable cells reached 1.1 x 10^9 CFU per ml in the wild type at the end of exponential growth (8 h), living cells in the mutants had already decreased and only about 1.1-1.5 x

10^7 CFU per ml were detectable. After incubation of the mutants for 24 h in stationary phase conditions, only between 2 x 10^2 and 10^3 CFU per ml were detected (Fig. 6B), whereas a density of 4 x 10^5 CFU per ml were detected in the case of the wild type. Clearly, the mutants deficient in structural genes encoding CC_1154, CC_1163, CC_1164, or CC_1165 behaved very similar to a mutant deficient in *spt* (CC_1162)(Fig. 3) in growth and survival experiments and therefore these four structural genes are also required for survival at elevated temperatures.

Serine palmitoyltransferase, CC_1154, CC_1163, CC_1164, and CC_1165 required for sensitivity of C. crescentus *towards polymyxin B*

A surprising finding in the study of Price *et al.* (2018) was that *spt*-deficient mutants grew better than the wild type strain in the presence of the antibiotic polymyxin B, a result recently confirmed by Stankeviciute *et al.* (2019). We investigated whether our mutants deficient in *spt*, CC_1154, CC_1163, CC_1164, or CC_1165 grew better than wild type in the presence of polymyxin B (Fig. 7). As shown in Fig. 7A, the mutant deficient in *spt* grows without delay when subcultivated in complex medium in the presence of polymyxin B and reached OD_{600} values of 0.80 and 1.32 after 8 h and 24 h of cultivation, respectively. Determination of CFU indicated that upon initiation of the experiment at OD_{600} = 0.05, CFU for wild type and *spt* mutant were essentially the same (about 9 x 10^7 CFU per ml)(Fig. 7A). Whereas CFU for the mutant increased to 1.6 x 10^9 CFU per ml within 8 h of cultivation, in the case of the wild type only 8 x 10^4 CFU per ml were detected (Fig. 7A) at this time point. Growth of the wild type only partially recuperated after a delay of 12 h, suggesting that, at least during the initial phases of growth, the wild type was sensitive to the antibiotic and lost viability

while the mutant was not. Similarly, mutants deficient in CC_1154, CC_1163, CC_1164, or CC_1165 grew without delay when subcultivated in complex medium in the presence of polymyxin B and reached OD_{600} values between 0.67 and 0.82 after 8 h or 1.3 after 24 h of cultivation, respectively (Fig. 7B). Again, wild type growth only recuperated slightly after a delay of 12 h, confirming that it was sensitive to the antibiotic (Fig. 7B). The mechanistic details of this delayed increase in living cells in the wild type were not studied further during this work. Although many aspects of the mechanism of action of the cyclic cationic peptide polymyxin B are still unclear, it is thought that polymyxin B interacts with the 4´-phosphate group of the lipid A portion of the lipopolysaccharide (LPS)-containing outer membrane of Gram-negative bacteria (Breazeale *et al.,* 2005), replacing bivalent cations and thereby weakening interactions between LPS molecules. In a self-promoting uptake mechanism, polymyxin B is thought to cross the outer membrane and after interacting with the inner membrane provokes lysis and cell death (Trimble *et al.,* 2016). Like some other α-proteobacteria (Ingram *et al.,* 2010), *Caulobacter crescentus* seems to be devoid of phosphate residues on the lipid A part of its LPS (Smit *et al.,* 2008) and therefore LPS might not be the main target for polymyxin B in this bacterium. The fact that five structural genes for dihydroceramide synthesis are required to confer polymyxin sensitivity on *Caulobacter*, makes it tempting to speculate that in *Caulobacter* the target for polymyxin B might be an as yet unidentified negatively charged phospho-SphL in the outer membrane.

Dihydroceramide synthesis required for outer membrane stability in Caulobacter

Mutants unable to synthetize dihydroceramide are impaired in growth at elevated temperatures and this phenotype might be due to a reduced stability of the outer membrane. We tested the sensitivity of *C. crescentus* wild type and the *spt*-deficient mutant to a variety of detergents, antibiotics, and the chelator EDTA (Table S1). Mutants of *C. crescentus* deficient in *spt* were more sensitive to the nonionic detergent Triton X-100, the anionic detergent deoxycholate, and to the antibiotics chloramphenicol, carbenicillin, and rifampicin, than the wild type (Table S1). The *spt* mutation in *Caulobacter* causes hypersensitivity to antibiotics which affect disparate processes in different cellular compartments, consistent with a role for SphLs in maintaining the outer membrane permeability barrier.

As the *spt*-deficient mutant was much more sensitive to deoxycholate, we also investigated whether mutants deficient in CC_1154, CC_1163, CC_1164, or CC_1165 were more sensitive to deoxycholate than wild type. Although some bacteria, i.e. *Salmonella enterica*, can grow in the presence of sodium deoxycholate concentrations (5% = 120 mM) (Villareal *et al.*, 2014), which are well above the critical micelle concentration (2-4 mM) for deoxycholate (Neugebauer *et al.*, 1990), *C. crescentus* is more sensitive to this detergent and experiments were performed at 1 mg/ml (2.5 mM). As shown in Fig. 8A, the wild type grows without delay when subcultivated in complex medium in the presence of deoxycholate and reached OD_{600} values of 0.10 and 0.23 after 2 h and 4 h of cultivation, respectively. Determination of CFU indicated that upon initiation of the experiment at OD_{600} = 0.05, CFU for wild type and *spt* mutant were essentially the same (about 8 x 10^7 CFU per ml)(Fig. 8A). Whereas CFU for the wild type increased to 1.5 x 10^8 CFU per ml within 2 h of cultivation, in the case of the *spt*-deficient mutant only 1.5 x 10^4 CFU per ml were detected (Fig. 8A)

at this time point. Growth of the *spt*-deficient mutant only recuperated after a delay of 4 h, suggesting that, at least during the initial phases of growth, the *spt*-deficient mutant was sensitive to the deoxycholate and lost viability while the wild type was not. Similarly, mutants deficient in CC_1154, CC_1163, CC_1164, or CC_1165 lost viability when subcultivated in complex medium in the presence of deoxycholate and OD_{600} values between 0.023 and 0.027 after 2 h and 0.015 and 0.023 after 4 h of cultivation were determined, respectively (Fig. 8B). The reduction in living cells in addition to the reduction in OD_{600} in mutant cell cultures suggests that SphL-deficient mutants of *C. crescentus* are subject to lysis in the presence of deoxycholate (Fig. 8). As resistance of Gram-negative bacteria to deoxycholate usually requires a functionally intact OM (Villareal *et al.*, 2014), we suggest that the OM in SphL-deficient *C. crescentus* mutants is severely impaired and does not provide an efficient protection against deoxycholate.

Dihydroceramide-deficient mutants complemented with intact genes in trans *restore dihydroceramide formation, temperature resistance, polymyxin B sensitivity, and deoxycholate resistance*

In order to demonstrate that observed mutant phenotypes were due to the inactivation of an individual gene, mutants were provided with the intact gene or combinations of genes *in trans* or with the empty vector. Complementation of the mutants defective in CC_1154, *spt*, CC_1164, and CC_1165 restored dihydroceramide synthesis although to different levels than those observed in the wild type, which is probably due to different expression levels (Fig. 9). This complementation was not observed when the empty vector was present in these mutants. In contrast, the mutant deficient in

CC_1163, that carries an in frame deletion of 150 bp coding for amino acid residues 11-60 of a predicted special acyl carrier protein, required the simultaneous expression of the downstream encoded *spt* gene for dihydroceramide formation. Expression of CC_1163 or *spt* alone in the CC_1163-deficient mutant did not restore formation of detectable dihydroceramide (Fig. 9). This clearly indicates that the predicted special ACP is required for efficient dihydroceramide synthesis but also that an unexpected polar effect of the deletion is affecting the expression of the *spt* gene. Also, expression of the wild type genes *in trans* reestablished resistance to elevated temperatures (42ºC) to the mutant deficient in CC_1154 (Fig. S1A), the mutant deficient in CC_1164 (Fig. S1C), and the mutant deficient in CC_1165 (Fig. S1D), which was not case when the mutants harboured the empty vector (Fig. S1). Again, temperature resistance for the mutant deficient in CC_1163 was only restored when both, CC_1163 and *spt*, were expressed *in trans*, but not when either CC_1163 or *spt* were expressed alone (Fig. S1B). Similarly, expression of their intact genes *in trans* reestablished sensitivity to polymyxin B (Fig. S2A) and resistance to deoxycholate (Fig. S3A) to the mutant deficient in CC_1154, the mutant deficient in CC_1164, and the mutant deficient in CC_1165, which was not case when the mutants harboured the empty vector. Polymyxin B sensitivity was restored for the mutant deficient in CC_1163 when CC_1163 together with *spt* were expressed *in trans*. Surprisingly, a minor polymyxin B sensitivity was achieved when CC_1163 only was expressed in the mutant deficient in CC_1163 (Fig. S2B). This latter result suggests that the mutant deficient in CC_1163 can be partially complemented by the expression of CC_1163 *in trans*, however, only to a very minor level and is therefore only detectable in the very sensitive polymyxin B assay. In order to obtain resistance to deoxycholate (Fig. S3B) for the mutant deficient in CC_1163 both, CC_1163 and *spt*, needed to be expressed *in trans*. These results confirm that besides the structural gene for *spt*,

CC_1154, CC_1163, CC_1164 and CC_1165 are also necessary for the formation of dihydroceramide, for temperature resistance, for polymyxin B sensitivity, and for deoxycholate resistance in *C. crescentus*.

Likely intermediates of dihydroceramide biosynthesis, 3-oxo-sphinganine and sphinganine, formed in C. crescentus

In our search for potential intermediates of SphL biosynthesis in *C. crescentus*, lipid extracts from distinct strains were analyzed by ESI mass spectrometry in the positive ion mode. In spectra of extracts of mutant DAGS01 (Δ*spt*) which had been complemented with the *spt*-expressing plasmid pRJ08, a weak signal at *m/z* 300.28889 was detected for $C_{18}H_{38}NO_2$ consistent with $M+H^+$ for 3-oxo-sphinganine that was not observed in those of mutant DAGS01 harbouring the empty vector. In spectra of extracts of mutant SPG07 (Δ*1154*) that harboured the *spt*-expressing plasmid pRJ08 *in trans*, ions were observed at *m/z* 300.28972 ($C_{18}H_{38}NO_2$) consistent with $M+H^+$ for 3-oxo-sphinganine, and *m/z* 302.30534 ($C_{18}H_{40}NO_2$) for sphinganine. Due to the excellent mass accuracies the elemental formulae are assignable and fully consistent with C18 3-oxo-sphinganine and C18 sphinganine. In this lipid extract, there are also signals for species with elemental compositions corresponding to 3-oxo-sphinganine with one additional and one fewer CH_2 group although these signals are about an order of magnitude less intense than those signals for the C18 versions. Therefore, like in *Bacteroides* (Brown *et al.*, 2019), the long-chain bases 3-oxo-sphinganine and sphinganine are the likely intermediates for SphL biosynthesis in *C. crescentus*.

Reconstitution of caulobacterial dihydroceramide synthesis in E. coli

The lipid profiles of *E. coli* strains in which individual genes or combinations of genes required for caulobacterial dihydroceramide synthesis had been expressed were analyzed by TLC. If individual genes CC_1163, CC_1165, the operon CC_1163 + *spt*, or the combination CC_1163 + CC_1165 were expressed, the lipid profile of those samples was similar to the profile obtained from an *E. coli* strain harbouring empty vectors pET9a and pCDFDuet-1 (Fig. 10). Interestingly, when the individual *spt* gene was expressed from a pET9a vector, a so-far unknown compound was formed that migrated slightly less than ceramide (Fig. 10). The combined expression of CC_1163, *spt*, and CC_1165 led to the formation of a compound that migrated like 3-oxo-sphinganine (Fig. 10, Fig. S4). Mass spectrometric analysis of a lipid extract from *E. coli* in which CC_1163, *spt*, and CC_1165 had been expressed indicates the presence of a compound with *m/z* = 300.28953 expected for a C18-3-oxo-sphinganine. The signal at *m/z* = 300 was absent in spectra of extracts of *E. coli* carrying the empty vectors. Interestingly, a minor compound had also been formed in this strain, that migrated like a sphinganine standard (Fig. S4). When CC_1164 had been expressed together with CC_1165, CC_1163, and *spt* in *E. coli*, the lipid profile did not change. However, expression of CC_1154 together with CC_1165, CC_1163, and *spt* in *E. coli* led to the formation of intense, fast-migrating compounds. When all five genes (CC_1165, CC_1164, CC_1163, *spt,* and CC_1154), required for dihydroceramide formation in *Caulobacter,* were expressed in the *E. coli* host an even more intense formation of fast-migrating compounds can be observed after radiolabeling for 4 or 12 h (Fig. S4). Notably, increased formation of a compound that migrates like ceramide is observed.

Model for dihydroceramide formation in C. crescentus

Our studies show that five structural genes coding for Spt (CC_1162), CC_1165, CC_1164, CC_1163, and CC_1154 are required for the formation of dihydroceramide in *C. crescentus*. Also, for synthesis of an elevated level of a dihydroceramide-like compound in *E. coli*, these five genes are required. For the formation of 3-oxo-sphinganine in *E. coli*, the combined expression of *spt*, CC_1163 and CC_1165 is required. As CC_1163 is a predicted acyl carrier protein (ACP) and CC_1165 a predicted acyl-CoA synthetase one might propose that CC_1165 is a specific acyl-ACP synthetase which selectively acylates a specialized ACP (CC_1163)(Fig. 11). In the case of *C. crescentus,* the specialized acyl-ACP CC_1163 is expected to be the preferred thioester substrate for Spt when condensing it with serine, decarboxylating the latter and forming 3-oxo-sphinganine (Fig. 11). In all eukaryotic or bacterial organisms known to date, only one enzyme, Spt, was necessary to catalyze the committed step of SphL biosynthesis by condensing palmitoyl-CoA with serine to obtain 3-oxo-sphinganine. In contrast, our results show that for an efficient formation of 3-oxo-sphinganine in *C. crescentus*, three proteins are needed (Spt, a predicted acyl carrier protein (ACP) and a predicted acyl-CoA synthetase). Presently, it is not known why *C. crescentus* and most likely other members of the α-, β-, and γ-proteobacteria employ this more complex pathway for 3-oxo-sphinganine synthesis.

Mutant SPG05 deficient in CC_1164 (Δ*1164*) of *C. crescentus* is unable to form dihydroceramide (Fig. 5), but accumulates 3-oxo-sphinganine and other so-far unknown compounds. As 3-oxo-sphinganine contains an oxo (keto) as well as a primary amino group it is likely to undergo Schiff reactions with itself or other compounds containing oxo or primary amino groups if unprotected. Expression of CC_1164 in mutant Δ*1164* eliminates the accumulation of the other unknown compounds and slightly increases the formation of dihydroceramide. As CC_1164 encodes a

predicted epimerase/dehydrogenase that depends on NAD(P)H, CC_1164 might be the dehydrogenase that reduces 3-oxo-sphinganine to sphinganine, i.e. the bacterial ketosphinganine reductase. The predicted *N*-acyltransferase CC_1154 might transfer an acyl group from an activated acyl-thiol intermediate to sphinganine resulting in the formation of dihydroceramide (Fig. 11). Clearly, biochemical assays that confirm and refine this proposed pathway will have to be developed in future work. The dihydroceramides identified by us in *C. crescentus* possess an extra hydroxyl group on the amide-linked fatty acyl residue when compared with the dihydroceramide species shown here (Fig. 11). This additional modification might be due to an as yet unknown hydroxylase activity in *C. crescentus*.

Bacterial sphingolipids may interfere in symbiosis, pathogenesis, contribute to fitness and seem to be essential in some bacteria

A prominent member of the human gut microbiota, *Bacteroides fragilis*, produces different classes of SphLs, such as dihydroceramide, a phosphorylethanolamine derivative and α-galactosyldihydroceramide (Wieland Brown *et al.*, 2013), which are absent in a *spt*-deficient deletion mutant. A *spt*-deficient mutant of *B. fragilis* grows slightly slower than the respective wild type strain (An *et al.*, 2014). Notably, *B. fragilis* glyco-SphLs inhibit activation of intestinal natural killer T cells and regulate their homeostasis in the host (An *et al.*, 2014).

Like other members of the *Bacteroidetes*, the oral anaerobe *Porphyromonas gingivalis* can synthesize distinct species of dihydroceramides. Studies comparing a *spt*-deficient mutant of *P. gingivalis* with its wild type suggest that SphLs play an essential role in long-term survival of the organism as well as in its resistance to oxidative stress (Moye *et al.,* 2016). In this dental pathogen,

also the display of major virulence determinants, such arginine and lysine gingipains or polysaccharides, on its cell surface depends on sphingolipid synthesis (Moye *et al.,* 2016).

The finding that a *spt* (*ccbF*)-deficient mutant of *C. crescentus* is more sensitive to phage ΦCr30 led to the discovery that phage adsorption and rapid multiplication in this bacterium depended on intact genes for *spt* and *sgt1* suggesting that mature GSL-2 is required to inhibit phage adsorption (Stankeviciute *et al.*, 2019). Although it is known that the phage ΦCr30 attaches to *C. crescentus* by binding to the RsaA protein of the cell envelope S-layer (Edwards and Smit, 1991), S-layer or exopolysaccharide production in an *spt*-deficient mutant seem not to be different from wild type (Stankeviciute *et al.*, 2019).

Our studies presented here clarify that besides *spt,* four other genes are required for the formation of dihydroceramide in *C. crescentus*, for survival of this bacterium at elevated temperatures, for its sensitivity to polymyxin B, and its resistance to the detergent deoxycholate. Therefore, the ability to form SphLs makes *C. crescentus* more resistant to distinct kinds of stress. Although all five dihydroceramide synthesis genes are required for high fitness in *C. crescentus* (Christen *et al.*, 2011; Fig. 4), their close orthologues in *Sphingomonas koreensis* (Table S2) are essential (Price *et al.*, 2018), supporting the idea that in the absence of LPS, glyco-SphLs act as functional replacements in α-4 proteobacteria (Kawahara *et al.*, 1999) and that therefore the synthesis of the glyco-SphL precursor dihydroceramide is essential for these bacteria.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used and their relevant characteristics are shown in Table 1. The construction of caulobacterial mutants deficient in putative sphingolipid biosynthesis genes is described in Table S3. Strains of *C. crescentus* were grown either in complex peptone yeast extract (PYE) medium (Ely, 1991) usually at 30°C or other indicated temperatures on a gyratory shaker at 250 rpm. For growth experiments, strains were first grown on PYE plates. Then, cells were resuspended at cell densities of 5 x 10^7 cells/ml in liquid PYE medium and grown for 20 h during such a first growth cycle. During a second subcultivation in PYE medium, again inoculating with 5 x 10^7 cells/ml, growth of *C. crescentus* wild type and of dihydroceramide-deficient mutants was followed by determining OD_{600} and CFU per ml. For studying growth and survival at different temperatures, each culture contained 100 ml of medium in a 250 ml Erlenmeyer flask, whereas cultures with 15 ml medium in 125 ml Erlenmeyer flasks were employed for growth and survival experiments in the presence of polymyxin B or deoxycholate.

E. coli strains were cultured on Luria-Bertani (LB) medium (Miller, 1972) at 37°C or at 30°C when caulobacterial genes were expressed. Antibiotics were added to media in the following concentrations (μg/ml) when required: kanamycin 5 (20 in solid media), spectinomycin 15 (100 in solid media), polymyxin B 10, in the case of *C. crescentus*, and kanamycin 50, spectinomycin 100, chloramphenicol 20 in the case of *E. coli*.

Plasmids pRXMCS-2, pNPTS138, and their derivatives were moved into *C. crescentus* by transformation employing electroporation or by conjugation.

In the case of transformation, for the preparation of electrocompetent *C. crescentus* cells, a modified protocol developed for *Zymomonas mobilis* (Gliessman *et al.*, 2017) was followed. *C.*

crescentus was grown to mid-exponential phase (OD_{600} = 0.45) in 100 ml PYE medium, harvested by centrifugation, washed three times in 10 ml of ice-cold 10% glycerol and resuspended in 1 ml ice-cold 10% glycerol. Aliquots of 50 µl were stored at -80°C. Electroporation was carried out with a BioRad MicroPulse electroporator, using the manufacturer´s preprogrammed "Ec1" setting (1.8 kV), in 1 mm electroporation cuvettes. Electroporated cells were allowed to recover in 5 ml PYE for at least 18 h at 30°C without shaking before plating onto selective media.

In the case of conjugation, aliquots of exponentially growing cultures of a donor strain of *E. coli* S17-1, harbouring the plasmid of interest (200 µl), and of the receiver strain *C. crescentus* CB15N (1 ml) were mixed. The mixed cell suspension was centrifuged and washed three times with fresh PYE medium, resuspended in 100 µl of PYE medium and applied in drops onto PYE agar. After most of the liquid had evaporated, the cell mixture was incubated at 30°C for 16 h and potential transconjugants were selected on PYE agar containing nalidixic acid (25 µg/ml) and kanamycin (25 µg/ml).

DNA manipulations

Recombinant DNA techniques were performed according to standard protocols (Sambrook *et al.*, 2001). Commercial sequencing of amplified genes by Eurofins Medigenomix (Martinsried, Germany) corroborated the correct DNA sequences. The DNA regions containing *cc_1168-cc_1152* were analyzed using the NCBI (National Center for Biotechnology Information) BLAST network server (Altschul *et al.*, 1997).

Construction of expression plasmids

Using PCR and a pair of specific oligonucleotides (Table S4) genes or combinations of genes encoding potential structural genes for dihydroceramide biosynthesis were amplified from *C. crescentus* genomic DNA. Suitable restriction sites for cloning of the genes were introduced by PCR with oligonucleotides. After restriction with the respective enzymes the PCR-amplified DNA fragments were cloned into a pET9a, pCDFDuet-1 or a pRXMCS-2 vector as detailed in Table S5. Expression of proteins in *E. coli* BL21(DE3) from genes cloned in pET9a or pCDFDuet-1 was achieved by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM to cultures at an OD_{600} = 0.30. Expression of proteins in *C. crescentus* from pRXMCS-2 derivatives was achieved by adding xylose to a final concentration of 10 mM to cultures at an OD_{600} = 0.05.

Mild alkali hydrolysis

Whereas carboxylic acid ester linkages of glycerophospholipids are especially sensitive to treatment with mild alkali, the amide-containing ceramide part of sphingolipids is resistant to such treatment. For treatment, extracted lipids were dried with nitrogen and incubated with 0.5 M KOH in methanol for 4 h at 30ºC. Subsequently, the solution was neutralized with 1 M HCl and lipids were extracted according to Bligh and Dyer (1959).

In vivo labeling of bacterial strains with [^{14}C]acetate

The lipid composition of bacterial strains was determined after labeling with [1-^{14}C]acetate (60 mCi/mmol; Perkin Elmer). Cultures (1 ml) of wild type or mutant strains were inoculated from precultures grown in the same medium in order to obtain an initial cell density of 2 x 10^8 cells/ml. After the addition of 0.5 µCi [1-^{14}C]acetate to each culture, they were incubated for 24 h or shorter times as indicated. At the end of the respective incubation periods, cells were harvested by centrifugation, and resuspended in 100 µl of water. Lipids were extracted according to the method of Bligh and Dyer (1959) and the chloroform phase was separated by one-dimensional TLC on high performance TLC aluminum sheets (silica gel 60; Merck Poole, United Kingdom) and developed with chloroform/methanol/ammonium hydroxide (40:10:1; v/v) as the mobile phase. Radioactive lipids were visualized by phosphorimaging using a Typhoon FLA 9500 and quantification was performed with Image Quant TL (Amersham Biosciences).

Assays for sensitivity to detergents, EDTA, and antibiotics

Membrane integrity of wild type *C. crescentus* and the *spt*-deficient mutant strain was evaluated by examining the sensitivity to detergents, EDTA, and antibiotics similarly as described by Ryan *et al.* (2010). Petri dishes were prepared which contained 20 ml of PYE agar and which were overlaid with 5 ml of PYE soft agar containing 10^8 cells of wild type or mutant strain. After agar solidification, disk diffusion tests were performed by placing sterile 8-mm filter paper disks onto the soft agar and applying 10 µl of the respective dissolved compound to each disk. Diameters of the inhibition zones were determined after growth for 24 h at 30ºC.

FTICR Mass Spectrometry

High resolution mass spectra were acquired with a solariX XR Fourier transform ion cyclotron resonance mass spectrometer (FTICR-MS) equipped with a 9.4 T superconducting magnet (Bruker Daltonics, Bremen, Germany). Spectra were acquired in positive and negative ion mode using electrospray ionization (ESI); lipid samples were dissolved in methanol or in chloroform/methanol (1:1; v/v) for detection of sphinganine and 3-oxo-sphinganine in *C. crescentus* and sample solutions were introduced into the ion source by syringe infusion with a flow rate of 2 µl min^{-1}. The drying gas flow was 4 l min^{-1} and temperature 160°C. The spectra were externally calibrated on sodium trifluoroacetate clusters with a 10 µg ml^{-1} solution in 50% propan-2-ol using a linear calibration and then internally recalibrated with fatty acids in the negative ion mode and diethylhexyl phthalate in the positive ion mode. This procedure results in a mass accuracy better than 1 ppm for the signals reported. The spectra were acquired with 1 M data points over the *m/z* range 100-2000 (transient of 0.367 s) resulting in a resolving power of 88 000 at *m/z* 300 (53 000 at *m/z* 500). Some negative mode spectra were acquired with 4 M data points over the *m/z* range 200-2000 (transient of 2.94 s) resulting in a resolving power of 700 000 at *m/z* 300 (420 000 at *m/z* 500). In negative ion mode in-source excitation (50 V) was applied to increase the relative intensity of the sphingolipid $[M-H]^-$ signals and reduce the relative intensity of the corresponding $[M+HCOO]^-$ signals. CID was performed by isolation of the precursor ions in the quadrupole and then storage in the hexapole collision cell with excitation voltage in the range 5 – 23 V. The ion accumulation time in the ion source was set to 0.05-0.2 for each scan depending on the signal-to-noise in a single scan. A total of 8 scans for mass spectra and 32 scans for product ion spectra were added for each mass spectrum.

Spectra visualisation and formula calculation was performed with DataAnalysis 5.0 (Bruker Daltonics).

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Figure Legends

Fig. 1. Formation of an alkali-resistant lipid in *C. crescentus* requires serine palmitoyltransferase gene. Different *C. crescentus* strains [wild type (wt), *spt*-deficient mutant DAGS01 (Δ*spt*), DAGS01 harbouring the *spt*-complementing plasmid pRJ08 (Δ*spt* x *spt*), and DAGS01 harboring the empty vector pRXMCS-2 (Δ*spt* x vec)] were cultivated on complex medium in the presence of ^{14}C-acetate. For strains harbouring the pRXMCS-2 vector or derivatives of it, xylose and kanamycin were added to the cultivation medium. After harvesting cells, lipids were extracted, half of the lipid sample was treated with mild alkali (+) and reextracted, whereas the other half was not subjected to any treatment (-). Untreated and alkali-treated lipid samples were separated by TLC and developed chromatograms were analyzed by phosphorimaging. As reference compound, *N*-palmitoyl-D-sphingosine (Cer) was developed in the same TLC and visualized by iodine staining. Two arrows indicate potential dihydroceramides formed by *C. crescentus*.

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Fig. 3. Expression of serine palmitoyltransferase required for survival of *C. crescentus* at elevated temperatures. Growth and survival of *C. crescentus* wild type or *spt*-deficient mutant DAGS01 was determined at 30ºC (A), 37ºC (B), and 42ºC (C) on complex medium. Growth of *C. crescentus* wild type (●) or *spt*-deficient mutant (▼) was followed by measuring OD_{600} whereas survival of *C. crescentus* wild type (○) or *spt*-deficient mutant (▽) was quantified by determining CFU per ml. Also, growth (filled symbols) and survival (open symbols) were determined for the *spt*-deficient mutant DAGS01 expressing intact *spt in trans* from pRJ08 (●,○) or for the *spt*-deficient mutant

DAGS01 harbouring an empty vector pRXMCS-2 (▼,▽) at 42ºC (D). Strains harbouring the pRXMCS-2 or pRJ08 were cultivated in the presence of xylose and kanamycin. Data and bars represent the average and standard errors obtained from at least three independent experiments.

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Fig. 5. Dihydroceramide formation in mutants deficient in genes demonstrating high cofitness with *spt*. Different *C. crescentus* strains [wild type (wt), mutant SPG07 deficient in CC_1154 (Δ*1154*), mutant DAGS01 deficient in CC_1162 (Δ*spt*), mutant SPG01 deficient in CC_1163 (Δ*1163*), mutant SPG05 deficient in CC_1164 (Δ*1164*), mutant SPG03 deficient in CC_1165 (Δ*1165*), a markerless deletion mutant SPG09 deficient in CC_1159/CC_1160 (Δ*1159-1160*), a deletion mutant SPG10 deficient in CC_1159/CC_1160 harbouring a resistance cassette replacement (Δ*1159-1160* spec), mutant SPG11 deficient in CC_1158 (Δ*1158*), and mutant SPG13 deficient in CC_1167 (Δ*1167*)] were cultivated on complex medium in the presence of ^{14}C-acetate. After harvesting cells, lipids were extracted, lipid samples were separated by TLC and developed chromatograms were analyzed by phosphorimaging. Filled arrows indicate potential dihydroceramides formed by *C. crescentus* whereas empty arrows indicate compounds accumulated in mutant SPG05 deficient in CC_1164 (Δ*1164*).

Fig. 6. Genes required for dihydroceramide formation are required for survival of *C. crescentus* at elevated temperatures. Growth and survival of *C. crescentus* wild type or dihydroceramide-deficient mutants was determined at 37ºC (A) and 42ºC (B) on complex medium for *C. crescentus* wild type (●,○) or mutants deficient in CC_1154 (▼,▽), CC_1163 (■,□), CC_1164 (♦,◊), or CC_1165 (▲,Δ). For growth, OD_{600} (filled symbols) was measured, whereas survival (open symbols) of *C. crescentus* wild type or dihydroceramide-deficient mutants was quantified by determining CFU per ml. Data and bars represent the average and standard errors obtained from at least three independent experiments.

Fig. 7. Genes for dihydroceramide formation are required for sensitivity of *C. crescentus* to polymyxin B. Growth (OD_{600}, filled symbols) and survival (CFU per ml, empty symbols) of *C. crescentus* wild type (●,○) or *spt*-deficient mutant DAGS01 (▼,▽) was determined at 30ºC on complex medium in the presence of polymyxin B (10 µg/ml) (A), whereas other dihydroceramide-deficient mutants [mutants deficient in CC_1154 (▼), CC_1163 (■), CC_1164 (♦), or CC_1165 (▲)] were analyzed and compared with wild type (●) only for growth (OD_{600}) in the presence of polymyxin B (B). Data and bars represent the average and standard errors obtained from at least three independent experiments.

Fig. 8. Genes for dihydroceramide formation are required for resistance of *C. crescentus* to deoxycholate. Growth (OD_{600}, filled symbols) and survival (CFU per ml, empty symbols) of *C. crescentus* wild type (●,○) or *spt*-deficient mutant DAGS01 (▼,▽) was determined at 30ºC on complex medium in the presence of deoxycholate (1 mg/ml) (A), whereas other dihydroceramide-deficient mutants [mutants deficient in CC_1154 (▼), CC_1163 (■), CC_1164 (♦), or CC_1165 (▲)] were analyzed and compared with wild type (●) only for growth (OD_{600}) in the presence of deoxycholate (B). Data and bars represent the average and standard errors obtained from at least three independent experiments.

Fig. 9. Complementation of dihydroceramide-deficient *C. crescentus* mutants by their native genes

in trans. Mutants of *C. crescentus* deficient in CC_1154, *spt* (CC_1162), CC_1163, CC_1164, or CC_1165 carrying the respective intact gene in the xylose-inducible plasmid pRXMCS-2 (Δ*1154* x *1154*, Δ*spt* x *spt,* Δ*1163* x *1163,* Δ*1164* x *1164,* Δ*1165* x *1165*) or carrying the empty pRXMCS-2 vector (vec) *in trans* as well as the mutant deficient in CC_1163 expressing *spt* (Δ*1163* x *spt*) or *1163* together with *spt* (Δ*1163* x *1163 spt*) were radiolabeled with ^{14}C-acetate for 16 h. At the end of the labeling period, cells were harvested, lipids were extracted, separated by TLC and developed chromatograms were subjected to autoradiography. Filled arrows indicate potential dihydroceramides formed by *C. crescentus* whereas empty arrows indicate compounds accumulated in the mutant deficient in CC_1164 (Δ*1164*) harbouring the empty vector pRXMCS-2 (vec).

Fig. 10. Reconstitution of caulobacterial 3-oxo-sphinganine synthesis in *E. coli*. Combined expression of three structural genes from *C. crescentus* in *E. coli* BL21(DE3) leads to the formation of a compound that migrates like 3-oxosphinganine in TLC. Radiolabeling with ^{14}C-acetate was performed on complex medium at 30ºC for 4 h (transition of exponential to stationary phase of growth) after induction with IPTG at an OD_{600} = 0.3 with *E. coli* BL21(DE3) pLysS expressing different caulobacterial SphL biosynthesis genes. Strains of *E. coli* BL21(DE3) pLysS employed harboured the empty vectors pET9a and pCDFDuet-1 (Empty vectors), vectors pDG01 and pCDFDuet-1 (*1163*), vectors pJPG08 and pCDFDuet-1 (*spt*), pRJ02 and pCDFDuet-1 (*1163 spt*), vectors pET9a and pDG06 (*1165*), vectors pDG01 and pDG06 (*1163 1165*), vectors pJPG08 and pDG06 (*spt 1165*), or vectors pRJ02 and pDG06 (*1163 spt 1165*). At the end of the labeling period, cells were harvested, lipids were extracted, separated by TLC and developed chromatograms were

subjected to autoradiography. The arrow indicates a compound migrating like 3-oxo-sphinganine. Reference compounds 3-oxo-sphinganine (3-oxo), sphinganine (Sph), and *N*-palmitoyl-D-sphingosine (Cer) were developed in the same TLC and visualized by iodine staining.

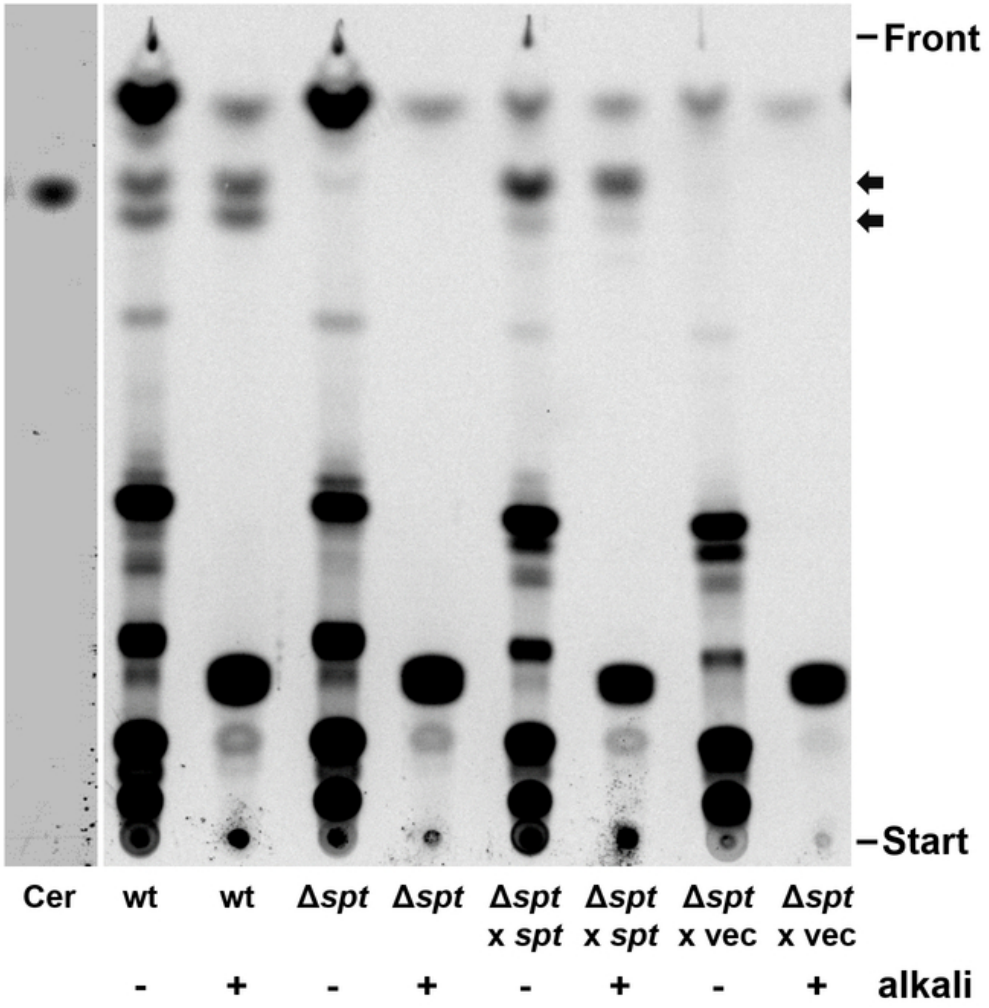
Fig. 11. Model for dihydroceramide biosynthesis in *C. crescentus*. A special acyl-ACP synthetase (CC_1165) links a free fatty acid, i.e. palmitate, in a thioester bond to a special acyl carrier protein (ACP) CC_1163 thereby activating it. The special acyl-ACP is used by serine palmitoyltransferase (Spt, CC_1162) to condense palmitate to serine thereby decarboxylating the latter resulting in the formation of 3-oxo-sphinganine. The ketosphinganine reductase step is probably catalyzed the NAD(P)H-dependent dehydrogenase CC_1164 converting 3-oxo-sphinganine to sphinganine. Finally, CC_1154 is thought to be the *N*-acyltransferase which attaches a shorter-chain fatty acyl residue, i. e. myristate, to sphinganine forming dihydroceramide (*N*-acylsphinganine).

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Reference
Caulobacter crescentus		
C. crescentus		
CB15N	wild type used throughout this study, synchronizable derivative of CB15	Evinger and Agabian, 1977
CB15N derivatives		
DAGS01	*CC_1162::deletion*	This study
SPG01	*CC_1163::deletion*	This study
SPG03	*CC_1165::deletion*	This study
SPG05	*CC_1164::deletion*	This study
SPG07	*CC_1154::deletion*	This study
SPG09	*CC_1159/1160::deletion*	This study
SPG10	*CC_1159/1160::*Ω^{Sp}*, insertion* Sp^R	This study
SPG11	*CC_1158::deletion*	This study
SPG13	*CC_1167::deletion*	This study
E. coli		
DH5α	*recA*1, ϕ80 *lacZ*ΔM15, host for cloning	Hanahan, 1983
TOP10	host for cloning	Invitrogen
S17-1	*thi, pro*, *recA*, *hsdR, hsdM+*, RP4Tc::Mu, Km::Tn*7*;Tp^R, Sm^R, Sp^R	Simon *et al.*, 1983
BL21(DE3)	expression strain	Studier *et al.*, 1990
Plasmids		
pET9a	expression vector, Km^R	Novagen
pCDFDuet-1	vector for coexpression, Sp^R	Novagen
pLysS	production of lysozyme for repression of T7 polymerase, Cm^R	Studier *et al.*, 1990
pNPTS138	suicide vector, Km^R	MRK Alley
pRXMCS-2	expression vector, Km^R,	Thanbichler *et al.*, 2007
pDG01	pET9a carrying *CC_1163*	This study
pDG06	pCDFDuet-1 carrying *CC_1165* in MCS-2	This study
pJPG08	pET9a carrying *CC_1162*	This study
pRJ03	pET9a carrying *CC_1163/CC_1162*	This study
pRJ05	pCDFDuet-1 carrying *CC_1165/CC_1164* in MCS-2	This study
pRJ06	pCDFDuet-1 carrying *CC_1165* in MCS-2 and *CC_1154* in MCS-1	This study

pRJ07	pCDFDuet-1 carrying *CC_1165/CC_1164* in MCS-2 and *CC_1154* in MCS-1	This study
pRJ08	pRXMCS-2 carrying *CC_1162*	This study
pRJ09	pRXMCS-2 carrying *CC_1163*	This study
pRJ10	pRXMCS-2 carrying *CC_1163/CC_1162*	This study
pRJ11	pRXMCS-2 carrying *CC_1164*	This study
pRJ12	pRXMCS-2 carrying *CC_1165*	This study
pRJ13	pRXMCS-2 carrying *CC_1154*	This study

[a]Tp^R, Km^R, Sp^R, Sm^R, Cb^R, Cm^R: trimethoprim, kanamycin, spectinomycin, streptomycin, carbenicillin, chloramphenicol resistance, respectively.



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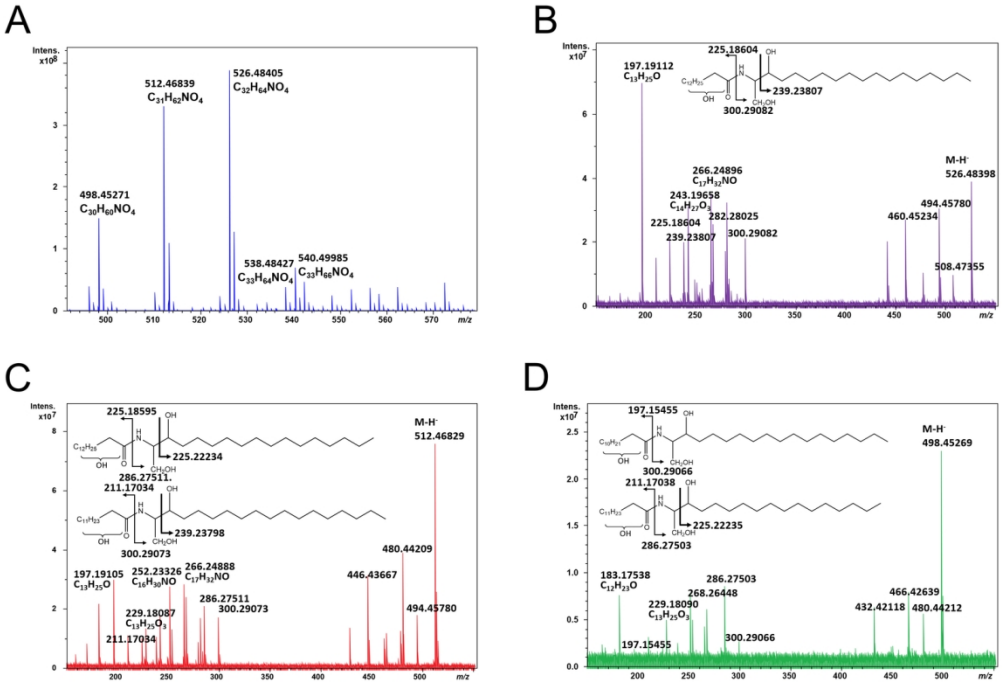

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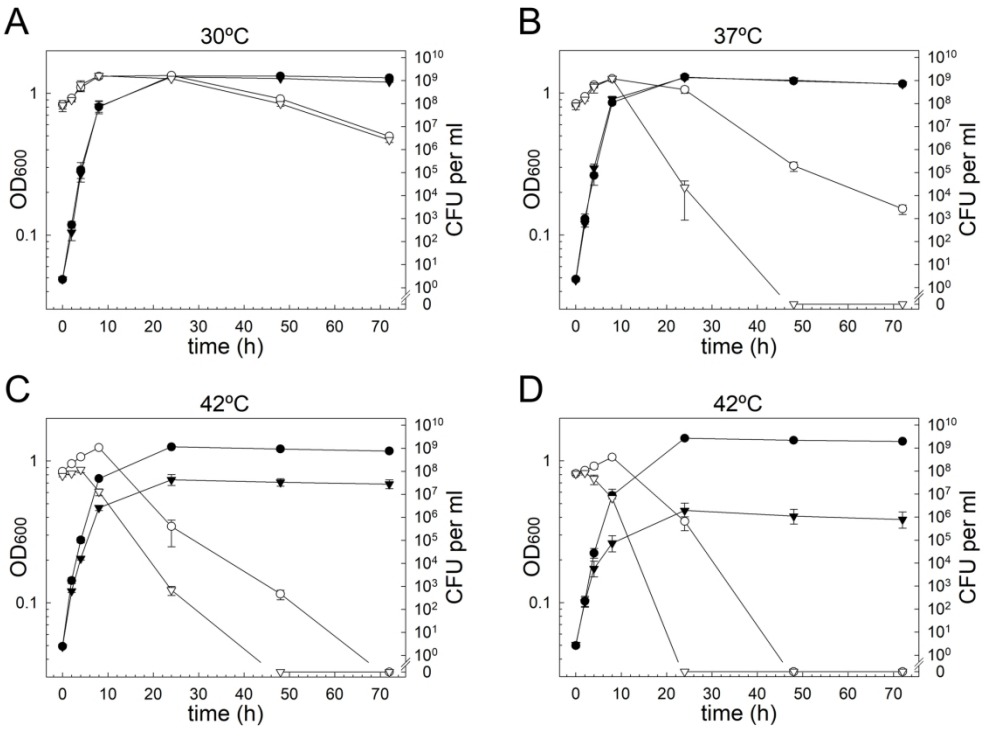

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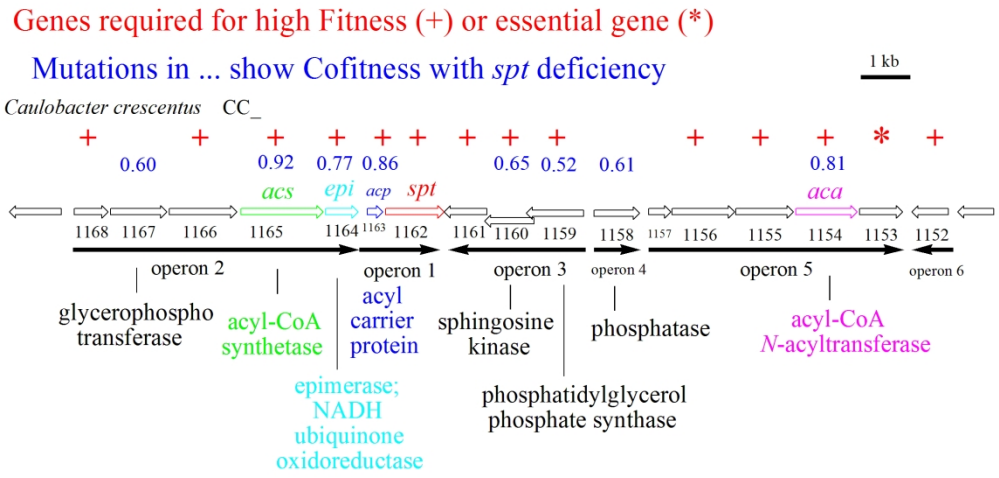

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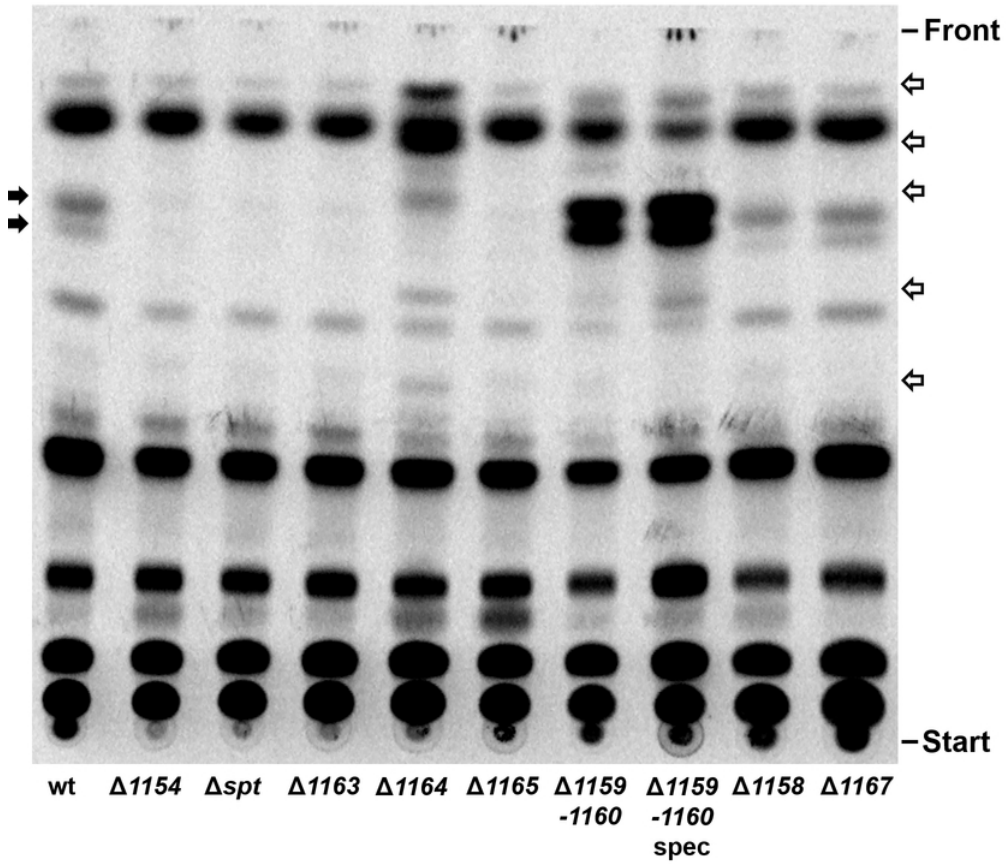

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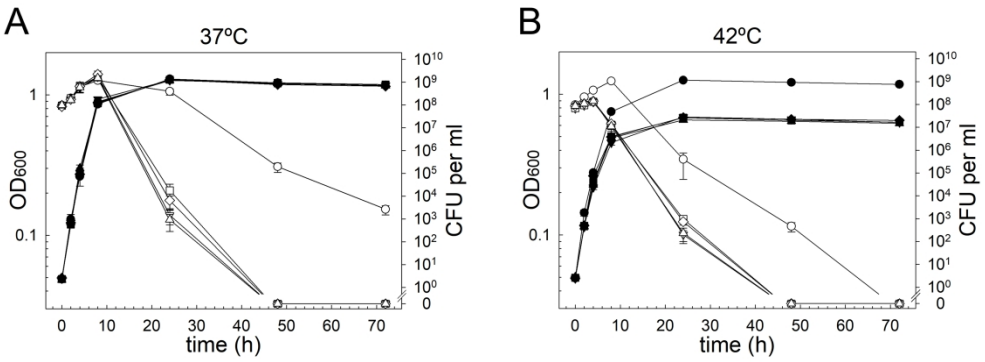

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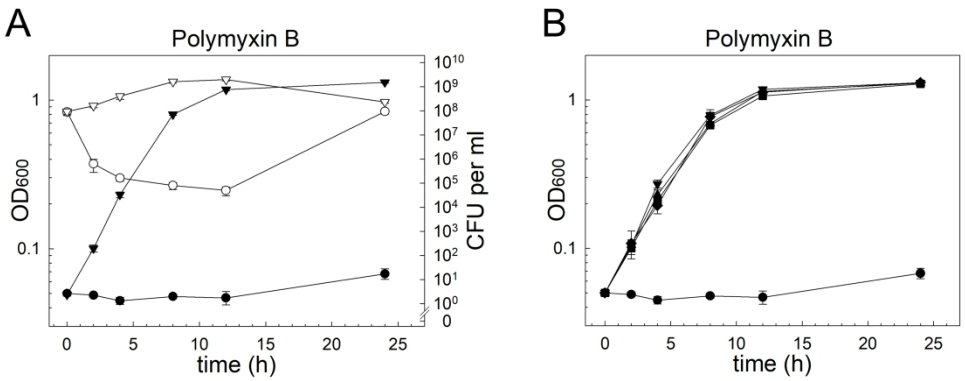

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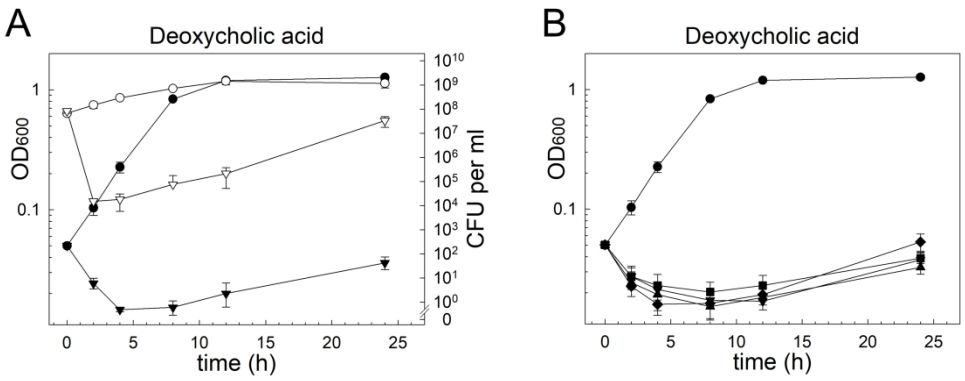

Fig. 8. Genes for dihydroceramide formation are required for resistance of *C. crescentus* to deoxycholate. Growth (OD_{600}, filled symbols) and survival (CFU per ml, empty symbols) of *C. crescentus* wild type (●,○) or *spt*-deficient mutant DAGS01 (▼,▽) was determined at 30ºC on complex medium in the presence of deoxycholate (1 mg/ml) (A), whereas other dihydroceramide-deficient mutants [mutants deficient in CC_1154 (▼), CC_1163 (■), CC_1164 (♦), or CC_1165 (▲)] were analyzed and compared with wild type (●) only for growth (OD_{600}) in the presence of deoxycholate (B). Data and bars represent the average and standard errors obtained from at least three independent experiments.

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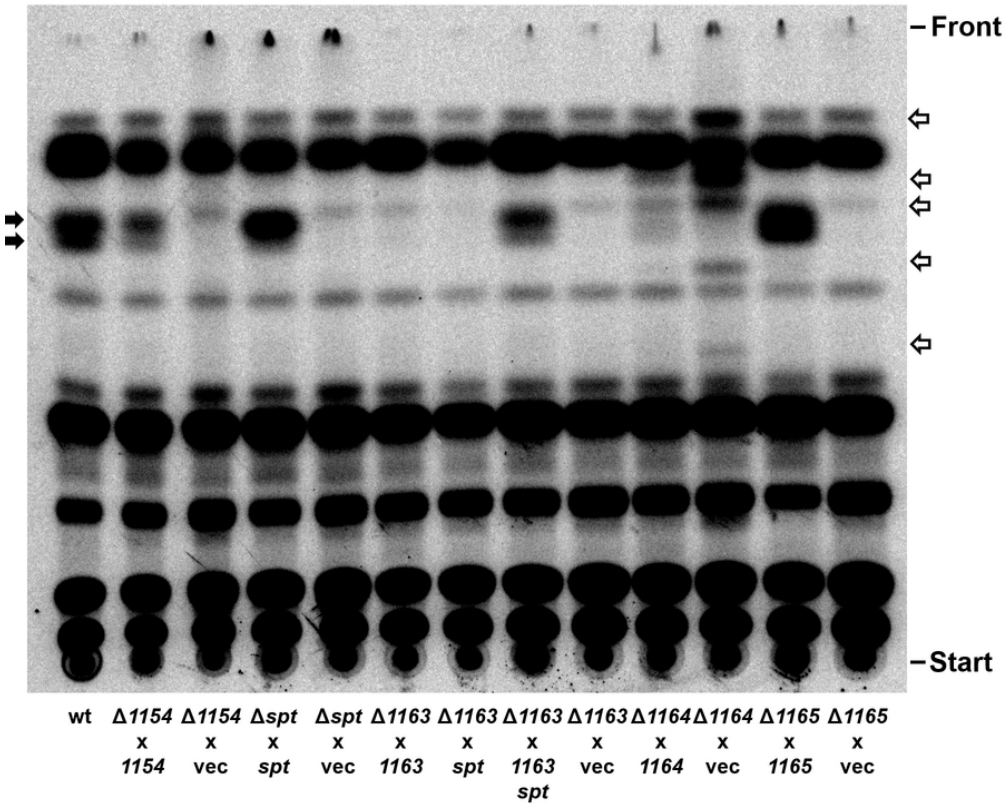

Fig. 9. Complementation of dihydroceramide-deficient *C. crescentus* mutants by their native genes in trans. Mutants of *C. crescentus* deficient in CC_1154, *spt (CC_1162), CC_1163, CC_1164, or CC_1165 carrying the respective intact gene in the xylose-inducible plasmid pRXMCS-2 (Δ1154 x 1154, Δspt x spt, Δ1163 x 1163, Δ1164 x 1164, Δ1165 x 1165) or carrying the empty pRXMCS-2 vector (vec) in trans as well as the mutant deficient in CC_1163 expressing spt (Δ1163 x spt) or 1163 together with spt (Δ1163 x 1163 spt) were radiolabeled with 14C-acetate for 16 h. At the end of the labeling period, cells were harvested, lipids were extracted, separated by TLC and developed chromatograms were subjected to autoradiography. Filled arrows indicate potential dihydroceramides formed by C. crescentus whereas empty arrows indicate compounds accumulated in the mutant deficient in CC_1164 (Δ1164) harbouring the empty vector pRXMCS-2 (vec).*

76x62mm (300 x 300 DPI)

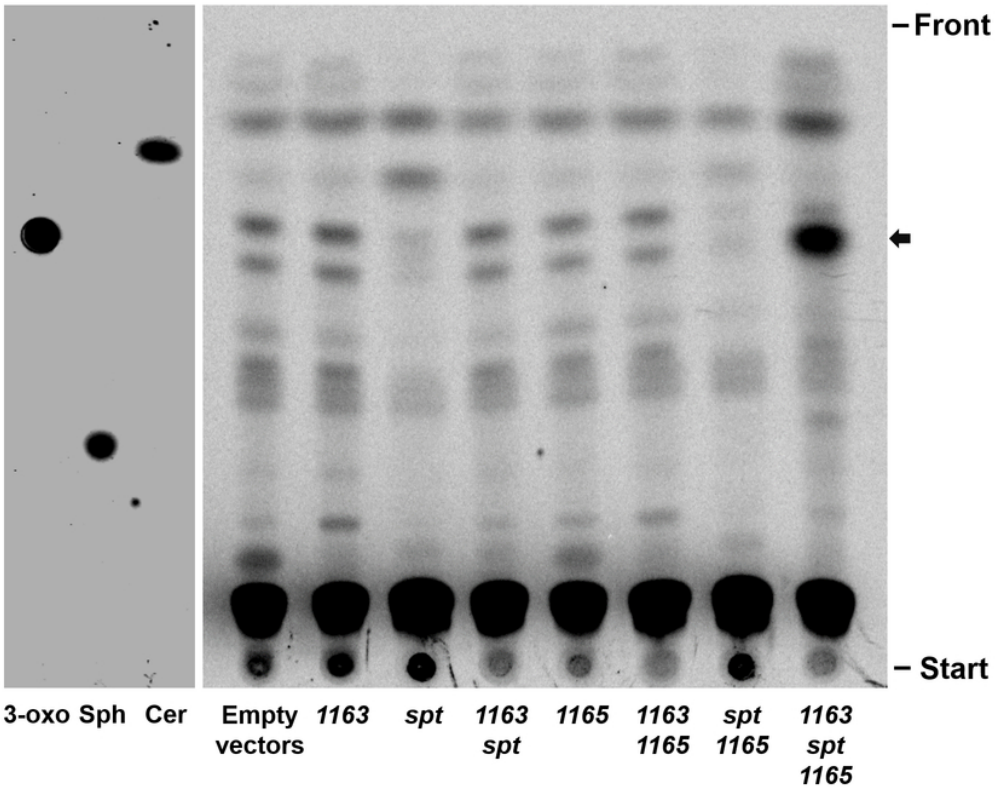

Fig. 10. Reconstitution of caulobacterial 3-oxo-sphinganine synthesis in *E. coli*. Combined expression of three structural genes from *C. crescentus* in *E. coli* BL21(DE3) leads to the formation of a compound that migrates like 3-oxosphinganine in TLC. Radiolabeling with 14C-acetate was performed on complex medium at 30ºC for 4 h (transition of exponential to stationary phase of growth) after induction with IPTG at an OD_{600} = 0.3 with *E. coli* BL21(DE3) pLysS expressing different caulobacterial SphL biosynthesis genes. Strains of *E. coli* BL21(DE3) pLysS employed harboured the empty vectors pET9a and pCDFDuet-1 (Empty vectors), vectors pDG01 and pCDFDuet-1 (1163), vectors pJPG08 and pCDFDuet-1 (*spt*), pRJ02 and pCDFDuet-1 (1163 *spt*), vectors pET9a and pDG06 (1165), vectors pDG01 and pDG06 (1163 1165), vectors pJPG08 and pDG06 (spt 1165), or vectors pRJ02 and pDG06 (1163 *spt* 1165). At the end of the labeling period, cells were harvested, lipids were extracted, separated by TLC and developed chromatograms were subjected to autoradiography. The arrow indicates a compound migrating like 3-oxo-sphinganine. Reference compounds 3-oxo-sphinganine (3-oxo), sphinganine (Sph), and *N*-palmitoyl-D-sphingosine (Cer) were developed in the same TLC and visualized by iodine staining.

70x55mm (300 x 300 DPI)

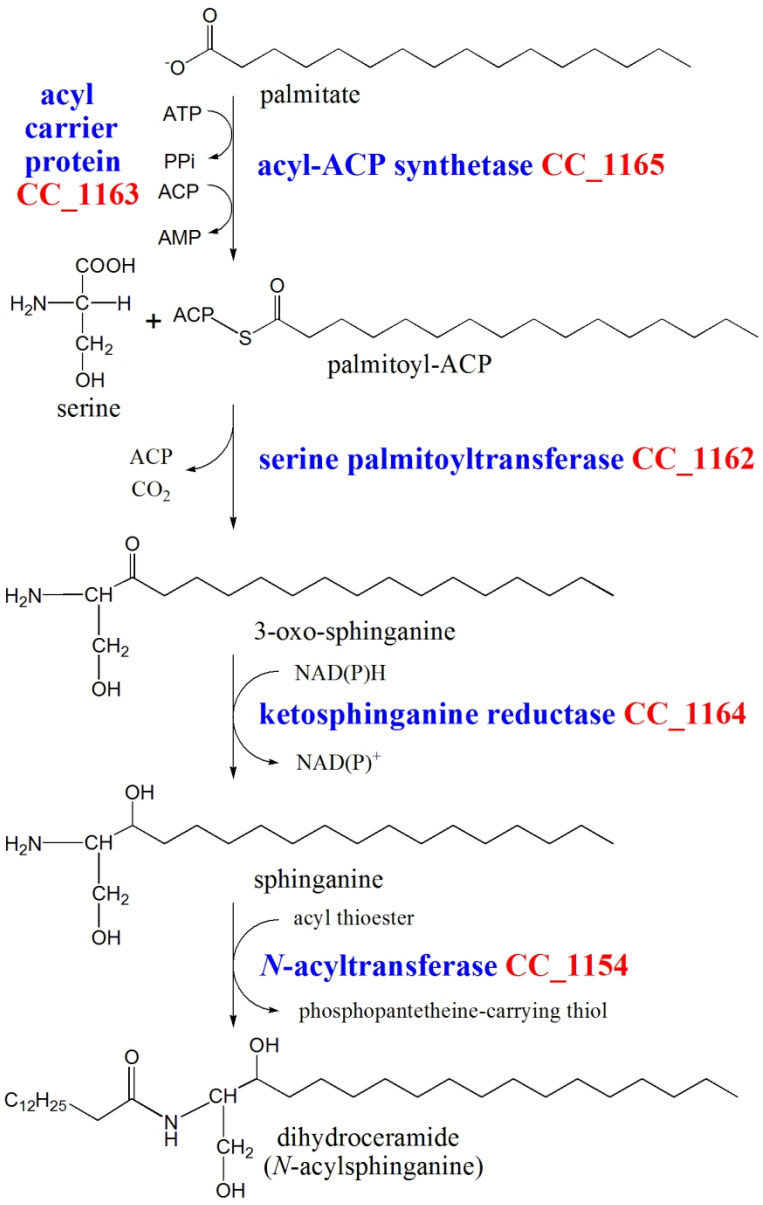

Fig. 11. Model for dihydroceramide biosynthesis in *C. crescentus*. A special acyl-ACP synthetase (CC_1165) links a free fatty acid, i.e. palmitate, in a thioester bond to a special acyl carrier protein (ACP) CC_1163 thereby activating it. The special acyl-ACP is used by serine palmitoyltransferase (Spt, CC_1162) to condense palmitate to serine thereby decarboxylating the latter resulting in the formation of 3-oxo-sphinganine. The ketosphinganine reductase step is probably catalyzed the NAD(P)H-dependent dehydrogenase CC_1164 converting 3-oxo-sphinganine to sphinganine. Finally, CC_1154 is thought to be the *N*-acyltransferase which attaches a shorter-chain fatty acyl residue, i. e. myristate, to sphinganine forming dihydroceramide (*N*-acylsphinganine).

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