INTRODUCTION

Microorganisms can withstand a diversity of environmental stresses ranging from extreme temperatures to the immune defenses of multicellular organisms. The cell surface membrane serves as a first line of defense against environmental perturbations and the membrane’s lipid composition is critical for stress resistance. On the one hand, the membrane must be robust enough to withstand chemical and physical challenges. On the other hand, the membrane must be fluid enough to support bioactivity. In eukaryotic organisms such as yeast, sterols play a crucial role in achieving a fluid yet mechanically robust cell surface membrane (Mouritsen & Zuckermann, 2004).
However, bacteria generally do not synthesize sterols with very few exceptions (Ourisson et al., 1987; Rivas-Marin et al., 2019).

The absence of sterols from most prokaryotes suggests that alternate lipids may serve analogous roles in surface membranes. All three domains of life possess isoprenoid synthesis pathways derived from a common C\textsubscript{5} isoprene building block which give rise to a broad suite of diverse lipid classes including sterols, but also carotenoids and hopanoids, and the majority of archaeal lipids. Because of their structural similarities that are derived from a common C\textsubscript{5} isoprene building block, resulting in rigid and often semi-planar structures, isoprenoid-derived lipids may share certain biophysical features in membranes (Ourisson et al., 1987). However, the mechanism and exact influence of isoprenoid lipids on prokaryotic membrane properties and cellular fitness remains relatively unexplored.

There is increasing evidence pointing to the role of bacterial isoprenoid-derived lipids such as hopanoids and carotenoids in membrane stabilization in bacteria (Belin et al., 2018; Bramkamp & Lopez, 2015; Tookmanian et al., 2021). Hopanoids are predominately found in Gram-negative bacteria where they have been shown to order outer membrane lipids by interacting with lipid A in a similar manner to that exhibited by cholesterol and sphingolipids in eukaryotes (Saenz et al., 2012, 2015; Silipo et al., 2014). In contrast, carotenoids (ji-carotene and zeaxanthin) have been shown using molecular dynamics (MD) simulations to have a condensing effect similar to that of cholesterol on phospholipids (Mostofian et al., 2020). Physiologically, there is evidence that hopanoids are important for growth at higher temperatures (Belin et al., 2018; Doughty et al., 2011; Kulkarni et al., 2013a; Poralla et al., 1984; Schmidt et al., 1986), whereas carotenoids have been linked to cold acclimation in some bacteria (Chattopadhyay & Jagannadham, 2001; Fong et al., 2001; Seel et al., 2020). These contrasting phenotypes for temperature acclimation suggest that hopanoids and carotenoids may serve opposing roles in modulating membrane properties. Taken together, these observations suggest functional similarities between sterols and bacterial isoprenoid lipids. However, the extent to which carotenoids and hopanoids have analogous or diverging biophysical properties and functions in biomembranes is not known and has not been systematically explored in a living model system. How do hopanoids and carotenoids contribute to the role of the outer membrane in adaptation to varying temperatures?

Methylobacterium extorquens is a Gram-negative bacterium with a well-characterized genome and a simple lipidome (Chwastek et al., 2020) that produces both hopanoids and carotenoids. This makes it an attractive model organism for studying the global phenotypes of disrupting the two pathways. In this study we have genetically disrupted the biosynthetic pathways of the two main isoprenoid lipid precursors; squalene (precursor for hopanoids) and phytoene (precursor for carotenoids), thus confirming the function of the gene hydroxyisqualene oxidoreductase (hpnE) in M. extorquens PA1. Additionally, we show that even though the genome of M. extorquens has the genes for the canonical C\textsubscript{40} carotenoid biosynthetic pathway, the pigmentation has a C\textsubscript{30}-based backbone that is squalene derived.

We propose that the genes for the C\textsubscript{30} squalene derived pathway were acquired through lateral gene transfer (LGT). We demonstrate the importance of hopanoids, but not carotenoids for growth at high temperature, and low divalent cation concentration, as well as maintaining low membrane permeability. In contrast, the carotenoid pathway may play a role in protection against oxidative stress.

## 2 | RESULTS

### 2.1 | Characterizing the function of the genes hpnE and crtB in M. extorquens PA1

To compare the effects of hopanoids and carotenoids on growth, adaptation, and outer membrane mechanics, we first aimed to create strains deficient in either hopanoid or carotenoid synthesis. Hopanoids are derived from squalene (Kannenberg & Poralla, 1999) and carotenoids were previously predicted to be derived from phytoene in M. extorquens (Dien et al., 2003). Therefore, we first disrupted the biosynthesis of the isoprenoid precursors squalene and phytoene by deleting the putative squalene synthase gene hydroxyisqualene oxidoreductase (hpnE) and the phytoene synthase gene (crtB). We next aimed to evaluate the effect of knocking out hpnE and crtB on hopanoid and carotenoid biosynthesis. First, we ran total lipid extracts of the mutant strains on thin layer chromatography (TLC) and, as expected, diplopterol (hopanoids) and hydroxyisqualene (carotenoids) were present in the WT strain but not in the ΔhpnE mutant strain. LC-MS analysis also revealed that the ΔhpnE strain no longer produced detectable amounts of diplopterol (hopanoids; Table 1), and that it accumulated hydroxyisqualene which is the precursor of squalene biosynthesis (Table 1 and Figure S2; Pan et al., 2015). Next, we measured the absorbance spectra of lipids extracted from the strains WT, ΔcrtB and ΔhpnE as a readout for carotenoid pigmentation. Surprisingly, the ΔcrtB mutant strain showed no loss in pigmentation compared to the WT (Figure 1a), whereas the ΔhpnE mutant strain was non-pigmented (Figure 1b). These unexpected observations led us to investigate whether carotenoid biosynthesis in M. extorquens was derived from squalene (Furubayashi et al., 2014) rather than phytoene.

| Compound         | WT     | Δhsc   | ΔhpnE   |
|------------------|--------|--------|---------|
| Hydroxyisqualene | n.d    | n.d    | 84.8 ± 10.1 |
| Diplopterol      | 19 ± 1.4 | n.d    | n.d     |
| Squalene         | n.d    | 318.4 ± 2.8 | n.d     |

### 2.2 | Phylogeny reveals co-occurrence of C\textsubscript{30} and C\textsubscript{40} pathways in M. extorquens PA1 and suggests that the C\textsubscript{30} pathway was acquired through horizontal gene exchange

Carotenoid biosynthesis in M. extorquens has been previously hypothesized to be derived from phytoene (Dien et al., 2003).
Nonetheless, the loss of pigmentation observed in the ΔhpnE mutant strain suggested that carotenoids are squalene derived (C\textsubscript{30}-based carotenoid backbone). Most bacterial carotenoids that have been characterized have C\textsubscript{40} backbones and are derived from phytoene (Goodwin, 1980; Umeno et al., 2002). However, recently a squalene-derived C\textsubscript{30} carotenoid pathway was discovered and has been identified in a few species, including Planctomycetes, which also produce hopanoids (Santana-Molina et al., 2020; Steiger et al., 2012; Takaichi et al., 1997; Taylor, 1984). Hence, we analyzed the distribution of both pathways in Proteobacteria (Figure 2a). Additionally, we performed the phylogeny of the FAD-dependent desaturases which are involved in the initial steps of C\textsubscript{40} and C\textsubscript{30} carotenoid biosynthesis (Figure 2b). We found in the M. extorquens genome the genes coding for the enzymes CrtB-CrtD-CrtI (for C\textsubscript{40} carotenoids), CrtN-CrtP (for C\textsubscript{30} carotenoids), and HpnCDE (for C\textsubscript{30} squalene) (Figure 2a). The phylogeny of the respective CrtD-CrtI enzymes located M. extorquens sequences branching within the Alpha- and Gammaproteobacteria group (Figure 2b). The phylogeny of HpnCDE enzymes showed monophyly of Alpha- and Gammaproteobacteria (Santana-Molina et al., 2020), but HpnCDE appeared more conserved than CrtB-CrtD-CrtI (Santana-Molina et al., 2020) (Figure 2a). This monophyly of Alpha- and Gammaproteobacteria suggested that both squalene and C\textsubscript{40} carotenoid biosynthesis were ancestral in Proteobacteria. By contrast, the C\textsubscript{30} FAD-dependent desaturase enzymes CrtN and CrtP, displayed a more limited distribution in Alphaproteobacteria, particularly in Rhodospirillales, Rhizobiales, Acetobacterales, Azospirillales orders (taxonomic orders according to GTDB; Figure 2a). In addition, these sequences, including those from M. extorquens, did not branch close to, nor monophyletically with, the Gammaproteobacteria. Instead, the respective alphaproteobacterial groups of CrtN and CrtP branched within the Planctomycetes (Figure 2b), suggesting LGT from this group (Figure 2b). Planctomycetes are a distant bacterial phylum that had recently been proposed to produce C\textsubscript{30} carotenoids via squalene synthesis enzymes HpnCDE (Santana-Molina et al., 2020). The similar topology between CrtN and CrtP branches (Figure 2b) suggested that these genes were transferred together, that is, in the same DNA fragment/ locus. Therefore, unlike CrtI-CrtD or HpnCDE enzymes which indicated an ancestral feature of Proteobacteria, the C\textsubscript{30} carotenoid pathway in some Alphaproteobacteria orders suggest that they originated later by LGT from Planctomycetes.

### 2.3 Carotenoids are derived from the C\textsubscript{30} pathway in M. extorquens PA1

In order to confirm that carotenoid biosynthesis uses squalene as a precursor in M. extorquens, we knocked out the genes in the C\textsubscript{30} pathway (crtN, crtP; Furubayashi et al., 2014), analyzed the absorbance spectra of the lipids extracted from different mutant strains, and performed LC-MS on the pigments to determine the chemical composition of their carbon backbone. We observed that knocking out crtN and crtP resulted in loss of pigmentation (Figure 3a,b), whereas, knocking out crtB did not (Figure 1a), hence, we amended the carotenoid biosynthetic pathway in M. extorquens PA1 (Figure 3c). Moreover, LC-MS analysis confirmed that carotenoids detected in M. extorquens all have a C\textsubscript{30} backbone, and no C\textsubscript{40} backbone-based carotenoids were detected confirming its squalene origin (Figure S3).

Our observations demonstrated that the deletion of genes in the proposed squalene-derived C\textsubscript{30} carotenoid pathway (crtN, crtP) produced non-pigmented mutant strains, where the phenotype was eliminated by gene complementation on an inducible plasmid (Figure S4a,b). Whereas knocking out genes in the C\textsubscript{40} carotenoid biosynthesis pathway had no effect on pigmentation, LC-MS analysis confirmed the presence of a C\textsubscript{30} backbone of the carotenoid pigment extracted from the WT, Δshc and ΔcrtB strains (Figure S3). These results suggest that C\textsubscript{40} biosynthetic pathway was not used to synthesize carotenoids, at optimal growth conditions in M. extorquens PA1.

When characterizing phenotypes of mutant strains, it is important to keep in mind that in addition to depleting hopanoids and carotenoids, other intermediates or related products may accumulate and complicate interpretations. The deletion of the genes crtN and crtP did not yield any detectable accumulation of carotenoid precursors.
at the tested conditions (Figure S3). However, we observed an accumulation of squalene in the ∆shc mutant strain as detected by LC-MS and TLC (Table 1 and Figure S5), which could generate artifactual phenotypes not linked to the absence of hopanoids. The accumulation of squalene could also explain an increase in abundance of carotenoids in the ∆shc strain observed here (Figure 3d) and previously by Bradley et al. (2017). The ∆hpnE mutant strain, in contrast, exhibited an accumulation of the squalene precursor hydroxysqualene (Table 1).

2.4 Isoprenoid-deficient mutant strains are more sensitive to physicochemical stresses

In order to explore how hopanoids and carotenoids contribute to cellular growth and acclimation to environmental physical stresses, we investigated how disrupting the biosynthesis of hopanoids and carotenoids affected cellular growth at different temperatures. Temperature change is a key environmental stress that M. extorquens must withstand in its native habitat on plant leaves, where it can experience wide diurnal variations. Temperature variations can perturb the physical properties of the membrane (e.g., lipid packing and permeability) and sterol-like lipids can play an important role in buffering the effects of such perturbations. For instance, diplopterol and cholesterol both reduce variation in lipid packing of Lipid A caused by changing pH (Saenz et al., 2012). We previously showed that temperature has one of the largest effects on lipidomic remodeling and growth rate, relative to other experimental parameters such as detergent and salt concentrations in M. extorquens (Chwastek et al., 2020). Here, we observed that interrupting hopanoid biosynthesis (∆hpnE, ∆shc) caused a growth impairment especially at temperatures higher than the optimum (30°C) (Figure 4a). Moreover, growth of the ∆shc mutant strain was impaired even more than for the ∆hpnE mutant strain, which could potentially be linked...
to the observed accumulation of squalene and carotenoids in the \( \Delta \text{shc} \) strain (Figure 4a and Table 1). On the other hand, knocking out the C\(_{30}\) pathway desaturases (\( \text{crtN}, \text{crtP} \)) did not have any effect on growth at different temperatures (Figure 4b). These results confirm the importance of hopanoids for acclimation to higher temperatures and suggest that carotenoids are not essential for temperature acclimation in \( M. \text{extorquens} \).

The outer membrane serves as a first barrier to environmental chemical stress. To examine the importance of the hopanoid and carotenoid pathways for resistance to chemical stressors, we performed a disk diffusion assay of the hopanoid and carotenoid deficient mutant strains compared against the WT with a panel of antibiotics and hydrogen peroxide (Figure 4c,d). Carotenoid-deficient strains \( \Delta \text{crtN} \) and \( \Delta \text{crtP} \) did not show any significant increase in susceptibility to antibiotics, except for chloramphenicol (Figure 4d).

\( \text{H}_2\text{O}_2 \), however, resulted in a significantly larger zone of inhibition for \( \Delta \text{crtN} \) and to a lesser extent \( \Delta \text{crtP} \) mutant strains (Figure 4d), which is consistent with an antioxidant role for carotenoids against oxidative damage from reactive oxygen species in the membrane (Kim et al., 2019; Kumar et al., 2019). In contrast, both hopanoid-deficient strains \( \Delta \text{shc} \) and \( \Delta \text{hpnE} \) exhibited higher sensitivity to all antibiotics tested. Increased antibiotic sensitivity is consistent with our previous work showing the impairment of multi-drug efflux transporters (Saenz et al., 2015) in hopanoid-deficient \( \Delta \text{shc} \), as well as the proposed role of hopanoids in maintaining low membrane permeability (Kannenberg et al., 1980; Mangiarotti et al., 2019; Schmerk et al., 2011). These observations show that while absence of carotenoid biosynthesis renders cells more sensitive to oxidative stress, the hopanoid pathway is important for antibiotic resistance in \( M. \text{extorquens} \) PA1.

**Figure 3** Identification of the genes involved in the squalene-derived C\(_{30}\) carotenoid biosynthetic pathway in \( M. \text{extorquens} \) PA1. (a) Absorbance spectra normalized to cell mass of lipids extracted from mutant strains in the C\(_{30}\) pathway \( \Delta \text{crtN} \), and (b) \( \Delta \text{crtP} \) which resulted in loss of pigmentation. (c) Amended \( M. \text{extorquens} \) carotenoid biosynthetic pathway confirmed by loss of pigmentation resulting from knocking out genes in the C\(_{30}\) pathway. (d) Absorbance spectra of lipids extracted from \( \Delta \text{shc} \) mutant strain demonstrated an increase in pigmentation relative to WT (normalized to cell mass).
Disruption of isoprenoid synthesis influences lipid packing, membrane permeability, and sensitivity to divalent cation concentration

The high temperature growth impairment observed for mutant strains that cannot produce hopanoids implicated a membrane-induced defect. Therefore, we next asked how hopanoid and carotenoid synthesis influenced outer membrane properties. Such an approach is complicated by the fact that membranes have complex lipidomes that can compensate to varying degrees for the loss of individual lipid species. While some lipids are crucial for viability (e.g., sterols in mammalian cells), others may have little or no effect at all when they are removed from the system. In this sense, the uniqueness of a lipids’ property or function is implicated by how much its absence affects the membrane. In this regard, rather than asking what particular biophysical effect different isoprenoids have on membranes, we instead asked how much does the absence of a particular isoprenoid pathway impact the ability for the membrane to maintain its physical properties, using the WT as a reference.

To investigate the membrane properties of different hopanoid knockout strains, we used the lipophilic dye Di-4 ANEPPDHQ (Di-4) which reports on lipid packing density. The emission spectrum of Di-4 is sensitive to changes in lipid packing density and can be semiquantitatively measured through the calculation of the general polarization (GP) index, whereby higher GP values indicate more densely packed lipids (Amaro et al., 2017). Lipid packing is correlated with a number of key membrane properties including viscosity and bending rigidity, thereby providing a robust and sensitive readout of variations in the physical state (Ma et al., 2018; Steinkühler et al., 2019). It has been previously shown that Di-4 selectively labels the surface membrane,
most likely due to its bulky polar headgroup which prevents flipping to the inner leaflet (Lorent et al., 2020). We therefore used Di-4 to measure in vivo outer membrane lipid packing of the WT and isoprenoid-deficient mutant strains ΔcrtN, ΔcrtP, Δshc, and ΔhpnE (Figure 5).

Our first observation was that the GP values obtained for WT in vivo were comparable to the GP values reported by Sáenz et al. for the in vitro measurement of purified outer membranes (Saenz et al., 2015). This confirmed that under normal conditions, Di-4 is selectively reporting outer membrane lipid packing. ΔcrtN, and ΔcrtP mutant strains had slightly increased lipid packing compared to the WT strain (Figure 5). Since precursors for carotenoid synthesis were not detectable in ΔcrtN, and ΔcrtP mutant strains (Figure S3), this suggested that increased lipid packing was due to the absence of carotenoids, and not the accumulation of precursors. In contrast we observed dramatically lower Di-4 GP values in Δshc, and ΔhpnE mutant strains. On the one hand, this could indicate that the absence of hopanoids resulted in lower lipid packing, which we previously demonstrated in purified outer membranes from WT and Δshc mutant strains (Saenz et al., 2015). However, GP values that we observed in vivo for Δshc and ΔhpnE mutant strains were considerably lower than observed for Δshc purified outer membranes (Saenz et al., 2015), raising the possibility that Di-4 was not selectively reporting outer membrane lipid packing in the hopanoid-deficient mutants.

Since hopanoids are crucial for modulating lipid order and membrane permeability (Kannenberg et al., 1980; Mangiarotti et al., 2019; Poralla et al., 1980; Saenz et al., 2012, 2015; Schmerk et al., 2011), it is reasonable model for inner membrane lipids in M. extorquens. A phospholipid with two double bonds per lipid, which is a reasonable model for inner membrane lipids in M. extorquens. In contrast, the GP value of the WT and carotenoid-deficient strains is similar to purified outer membranes (Saenz et al., 2015), and closer to the values of liposomes composed of SM (Sphingomyelin) and cholesterol, which are in a liquid ordered state (Figure S6). Thus, the large negative shift in GP values for the hopanoid-deficient mutants indicates that Di-4 is reporting the less ordered phospholipid inner membrane rather than exclusively the more ordered outer leaflet of the outer membrane. Taken together, these observations point toward dramatically increased outer membrane permeability in the hopanoid-deficient mutants.

To measure changes in membrane permeability we used an assay that determined relative changes in fluorescein diacetate (FDA) diffusivity (Levental et al., 2020). Fluorescein diacetate is non-fluorescent, and rapidly diffuses through the membrane where it is hydrolysed by cellular esterases. The hydrolysis of FDA yields fluorescein, which is fluorescent. Furthermore, fluorescein is charged thereby preventing it from diffusing rapidly out of the cell (Levental et al., 2020). Thus, the rate of increase in fluorescein fluorescence can be used to estimate the rate of diffusion of FDA across the membrane. Relative differences in diffusivity can be estimated from the slope of fluorescein fluorescence intensity versus time. We observed that the hopanoid deficient mutant strains Δshc and ΔhpnE had much higher relative permeabilities to FDA compared with the WT, and carotenoid deficient mutant strains ΔcrtN or ΔcrtP (Figures 6 and S7).

Finally, we aimed to test whether increased membrane permeability in the hopanoid-deficient mutant strains was linked to the absence of hopanoid ordering of Lipid A in the outer membrane (Saenz et al., 2015). The accumulation of squalene and carotenoids in Δshc and hydroxyqualene in ΔhpnE could also introduce changes in membrane properties and generate growth phenotypes. For example, it has been shown that squalene has a lipotoxic effect when it accumulates in the membranes of yeast (Csáky et al., 2020). Since hopanoid synthesis is carried out in the inner membrane and hopanoids are subsequently transported to the outer membrane (Doughty et al., 2011), hopanoid precursors would likely accumulate in the inner membrane. In such a case, outer membrane properties should not be influenced, but the effects of accumulation of hopanoid precursors in the inner membrane could still influence growth rates. One way to test whether the phenotypes we observed are resulting from changes in outer membrane properties due to the absence of hopanoids, or rather due

![Figure 5](image-url) Effect of disrupted isoprenoid synthesis on lipid packing. (a) Lipid packing measured by Di-4-ANEPPDHQ general polarization (GP) index for hopanoid-deficient strains (Δshc, ΔhpnE), and carotenoid-deficient (ΔcrtN, ΔcrtP) at 27°C. (b) Lipid packing of hopanoid-deficient strains (Δshc, ΔhpnE) upon complementation with genes shc and hpnE, respectively.
to the accumulation of hopanoid precursors would be to introduce a chemical that rescues outer membrane lipid packing.

Divalent cations are crucial for the stability of Gram-negative bacterial outer membranes because of their ordering effect on lipids in the outer leaflet (Clifton et al., 2015). We therefore examined whether supplementing the growth medium with Ca\(^{2+}\) could rescue growth and membrane permeability of the hopanoid-deficient strains. First, we evaluated the effect of increasing Ca\(^{2+}\) concentration on WT, Δshc and ΔhpnE. We observed a negligible effect for WT, but saw a rescue of both Δshc and ΔhpnE growth with an optimal concentration around 1.67 mM Ca\(^{2+}\) (Figure S8). We next examined growth with amended (1.67 mM) and unamended Ca\(^{2+}\) with varying temperature and observed a rescue of growth of both Δshc and ΔhpnE mutant strains at higher temperature (34°C; Figure 7). Finally, we measured relative changes in membrane permeability by FDA hydrolysis (Figure 6), and observed that permeability was rescued

**FIGURE 6** Membrane permeability is increased in hopanoid-deficient strains and rescued by Ca\(^{2+}\). Relative changes in membrane permeability are estimated by the rate of fluorescein diacetate (FDA) hydrolysis upon diffusing into the cell. Relative permeability is presented as the slope of increase in fluorescence with time upon the hydrolysis of FDA to fluorescein (a) Hopanoid-deficient strains; Δshc, ΔhpnE and the rescue effect of the divalent cation Ca\(^{2+}\), and gene complementation. (b) Carotenoid-deficient mutant strains; ΔcrtN, and ΔcrtP and the rescue effect by Ca\(^{2+}\), and gene complementation
in the presence of additional Ca\(^{2+}\). The rescue of both permeability and growth at higher temperatures for both hopanoid-deficient mutants provides strong evidence that the role of hopanoids in ordering outer membrane lipids is critical to outer membrane function. Furthermore, this suggested that the hopanoid pathway provides a means for maintaining outer membrane stability for Gram-negative bacteria that occupy environments with low or fluctuating divalent cation concentrations.

3 | DISCUSSION

In order to examine the comparative roles of carotenoids and hopanoids in *M. extorquens* PA1, we first determined how to perturb biosynthesis of the two pathways. Hopanoid biosynthesis in *M. extorquens* has been relatively well-described (Bradley et al., 2010), however, the squalene synthase was never formally identified or confirmed by a knockout strain. We identified and confirmed the function of *hpnE* as a key gene that would disrupt squalene synthesis (Pan et al., 2015), thereby disrupting hopanoid synthesis and preventing squalene accumulation (Bradley et al., 2017; Csáky et al., 2020; Valachovic et al., 2016). Under the assumption that carotenoids were derived from phytoene in *M. extorquens* (Dien et al., 2003), we targeted a phytoene synthase gene *crtB*. Surprisingly, deletion of *crtB* showed no phenotype in pigmentation, whilst deletion of *hpnE* yielded non-pigmented mutant strains that also lacked hopanoids. These unexpected results revealed that carotenoid biosynthesis was derived from squalene rather than phytoene through a non-canonical pathway that has recently been shown to produce C\(_{30}\) carotenoids (Furubayashi et al., 2014).

The presence of genes associated with both C\(_{30}\) and C\(_{40}\) carotenoid pathways, combined with evidence for the synthesis of only C\(_{30}\) carotenoids prodded us to examine the phylogeny of the two pathways for insights on their origins. An interesting clue emerged from the recent observation that squalene-derived carotenoids were also present in the hopanoid-producing Planctomycete, *Planctopirus limnophilus* (Santana-Molina et al., 2020). Why would such distantly related organisms possess both squalene-derived lipid synthesis pathways for carotenoids and hopanoids? Our phylogenetic analyses revealed that the genes involved in the C\(_{40}\) and C\(_{30}\) carotenoid biosynthetic pathways co-occur in the genomes of specific Alphaproteobacteria orders like Acetobacterales, Azospirillales and Rhodospirillales, and the latter including *M. extorquens* (Figure 2). While the phylogeny of HpnCDE and C\(_{40}\) carotenoid enzymes suggest an ancestral feature of Alpha- and Gammaproteobacteria, the C\(_{30}\) carotenoid pathway in Alphaproteobacteria orders most likely represents a secondary acquisition by LGT from Planctomycetes. By confirming that *M. extorquens* produces only C\(_{30}\) carotenoids under laboratory conditions (Figure 3 and S3), and given that the enzyme CrtM is absent in Alphaproteobacteria we propose that the C\(_{40}\) carotenoid pathway via CrtB-CrtI-CrtD does not produce C\(_{40}\) carotenoids in *M. extorquens*. Indeed, no pigmentation was observed in mutants with disrupted C\(_{30}\) carotenoid synthesis at any of the temperatures considered in this study. Together, these observations suggest a replacement of C\(_{40}\) carotenoid biosynthesis in *M. extorquens* and possibly other related species.

The discovery that hopanoids and carotenoids are both derived from squalene allowed us to identify the genes required to disrupt carotenoid and hopanoid synthesis in *M. extorquens*. Deletion of *hpnE*, and thus squalene synthesis, yielded a strain deficient in both hopanoids and carotenoids that also lacked carotenoids. These unexpected results revealed that carotenoid biosynthesis was derived from squalene rather than phytoene through a non-canonical pathway that has recently been shown to produce C\(_{30}\) carotenoids (Furubayashi et al., 2014).

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The discovery that hopanoids and carotenoids are both derived from squalene allowed us to identify the genes required to disrupt carotenoid and hopanoid synthesis in *M. extorquens*. Deletion of *hpnE*, and thus squalene synthesis, yielded a strain deficient in both hopanoids and carotenoids that also lacked carotenoids. These unexpected results revealed that carotenoid biosynthesis was derived from squalene rather than phytoene through a non-canonical pathway that has recently been shown to produce C\(_{30}\) carotenoids (Furubayashi et al., 2014).

The presence of genes associated with both C\(_{30}\) and C\(_{40}\) carotenoid pathways, combined with evidence for the synthesis of only C\(_{30}\) carotenoids prodded us to examine the phylogeny of the two pathways for insights on their origins. An interesting clue emerged from the recent observation that squalene-derived carotenoids were also present in the hopanoid-producing Planctomycete, *Planctopirus limnophilus* (Santana-Molina et al., 2020). Why would such distantly related organisms possess both squalene-derived lipid synthesis pathways for carotenoids and hopanoids? Our phylogenetic analyses revealed that the genes involved in the C\(_{40}\) and C\(_{30}\) carotenoid biosynthetic pathways co-occur in the genomes of specific Alphaproteobacteria orders like Acetobacterales, Azospirillales and Rhodospirillales, and the latter including *M. extorquens* (Figure 2). While the phylogeny of HpnCDE and C\(_{40}\) carotenoid enzymes suggest an ancestral feature of Alpha- and Gammaproteobacteria, the C\(_{30}\) carotenoid pathway in Alphaproteobacteria orders most likely represents a secondary acquisition by LGT from Planctomycetes. By confirming that *M. extorquens* produces only C\(_{30}\) carotenoids under laboratory conditions (Figure 3 and S3), and given that the enzyme CrtM is absent in Alphaproteobacteria we propose that the C\(_{40}\) carotenoid pathway via CrtB-CrtI-CrtD does not produce C\(_{40}\) carotenoids in *M. extorquens*. Indeed, no pigmentation was observed in mutants with disrupted C\(_{30}\) carotenoid synthesis at any of the temperatures considered in this study. Together, these observations suggest a replacement of C\(_{40}\) carotenoid biosynthesis in *M. extorquens* and possibly other related species.
absence of any significant phenotype associated with disrupted carotenoid synthesis was surprising, since it has been shown in model membranes that carotenoids could share some of the lipid order- ing properties of sterols (Gabrielska & Gruszecki, 1996; Kostecka-Gugała et al., 2003; Mostofian et al., 2020; Socaciu et al., 2000; Subczynski et al., 1992). We did observe a small increase in lipid packing in the carotenoid-deficient mutants, opposite to what is predicted from measurements in model membranes but similar to observations in Pantoea sp. (Kumar et al., 2019). In the case of Pantoea sp., increased membrane rigidity (qualitatively comparable to increased lipid packing) was attributed to a compensatory increase in the abundance of saturated acyl chains, rather than the depletion of carotenoids (Kumar et al., 2019). However, it has been shown that, in some bacteria, carotenoid production is increased at lower temperatures (Fong et al., 2001; Seel et al., 2020), pointing toward a role in fluidizing membranes. Carotenoids have a plethora of diverse structures that could alter their physicochemical effects on the membrane (Milon et al., 1986; Wisniewska et al., 2006), which could account for diverse biophysical effects. Alternatively, carotenoids may serve in a different capacity unrelated to the physical properties of the membrane in M. extorquens. For example, carotenoids play an important role in light scavenging in photosynthetic organisms (Polivka & Frank, 2010), and protecting cells from oxidative stress (Kim et al., 2019; Kumar et al., 2019). Indeed, a role for carotenoids in oxidative stress is suggested by our observation of increased sensitivity of ∆phmE (Saenz et al., 2012, 2015). In M. extorquens, disruption of hopanoid synthesis by deleting the gene shc resulted in a large growth deterioration at higher temperatures as well as increased membrane permeability comparable to what we observed for deleting the gene hpmE. It has previously been shown in other organisms that hopanoids are associated with sensitivity to high temperatures (Belin et al., 2018; Doughty et al., 2011; Kulkarni et al., 2013b; Poralla et al., 1984; Schmidt et al., 1986), and MD simulations also suggest that hopanoids could reinforce membranes at higher temperatures (Caron et al., 2014). The ordering effect of hopanoids, like sterols, has also been shown to reduce membrane permeability (Kannenberg et al., 1980; Mangiarotti et al., 2019; Poralla et al., 1980; Schmerk et al., 2011). We further demonstrated that the growth deficiency and increased membrane permeability could be partially rescued by increasing the Ca$^{2+}$ concentration of the media. Since Ca$^{2+}$ and other divalent cations stabilize the outer membrane by ordering lipopolysaccharide, this result has two important implications. First, it provides evidence that the phenotypes associated with disrupted hopanoid synthesis are linked to a reduction in lipopolysaccharide packing density from the loss of hopanoids. Second, the sensitivity of hopanoid-deficient mutants to stress at lower Ca$^{2+}$ concentrations suggested that hopanoids may be especially important for Gram-negative bacteria that occupy environments with low divalent cation concentrations.

*Methylobacterium extorquens* is on its way toward becoming a well-characterized and robust model system for studying the role of lipid structure in membrane function and organismal fitness. It was recently shown that *M. extorquens* has the simplest lipidome so far observed in any organism (Chwastek et al., 2020), making it an ideal system for exploring the principles of lipidome adaptation. While the phospholipidome is relatively well-explored by comparison, the role of isoprenoid lipids is still relatively undefined. Our observations show that carotenoids and hopanoids serve divergent roles in the outer membrane. By revealing that carotenoids are squalene-derived and identifying genes in the carotenoid pathway, this study now provides a new tool to explore the property-function relationship of carotenoids and their relationship with hopanoids in *Methylobacterium*.

### 4 | MATERIALS AND METHODS

#### 4.1 | Media, growth conditions

*Methylobacterium* strains were grown at 30°C in minimal medium described by Delaney et al. (2013) referred to as hypho medium, with 9.9 mM disodium succinate (Sigma Aldrich, W327700) as the carbon source at 160 rpm shaking (ISFI-X Kuhner shaker). *Escherichia coli* strains were grown at 37°C in LB medium (Carl Roth, X968). Triparental conjugation was performed on Nutrient broth medium (Carl Roth, X929.1). All solid media plates were prepared with 1.5% Agar-Agar (Carl Roth, X929.1). Antibiotics for selection were at the following concentrations for *Methylobacterium*: Trimethoprim (Tmp) 10 µg/ml (Cayman chemicals, 16,473), Tetracycline (Tc) 10 µg/ml (Carl Roth, HP63), Kanamycin 25 µg/ml (Carl Roth, T832), for *E. coli*: Kanamycin (Km): 50 µg/ml, Chloramphenicol (Cm) 25 µg/ml (Sigma Aldrich, C1919). Plasmid pLC291 (Chubiz et al., 2013) was induced using Anhydrotetracycline hydrochloride 25 ng/ml (Alfa Aesar, J66688).

#### 4.2 | Evolutionary analyses for C$_{30}$ and C$_{40}$ carotenoid pathway

We performed protein searches of CrtI (P54980), CrtD (Q01671), CrtN (O07855), and CrtP (Q2FV57) against NCBI database using phmmer (Finn et al., 2011) and with e-value threshold of 1e-5. We combined all the sequences obtained and using GTDB taxonomy (Parks et al., 2018), we removed redundant sequences by taxonomic orders (from 90 up to 50% of identity threshold for the less and more represented groups respectively). We then aligned this set of non-redundant sequences using MAFFT (Katoh & Standley, 2013) and
performed a fast phylogenetic tree using FastTree (Price et al., 2009) to exclude spurious sequences. Once we obtained the final set of sequences, we re-aligned with MAFFT and removed those enriched gap positions using trimAl (Capella-Gutierrez et al., 2009). For the final phylogenetic reconstruction, we used IQ-TREE (Nguyen et al., 2015). We obtained branch supports with the ultrafast bootstrap (Hoang et al., 2017) and the evolutionary models were automatically selected using ModelFinder (Kalyaanamoorthy et al., 2017) implemented in IQ-TREE and chosen according to BIC criterion.

For the phylogenetic profile, the distribution of HpnCDE, Sqs and CrtB enzymes were obtained from data previously generated (Santana-Molina et al., 2020). The distribution of FAD-dependent desaturases was obtained from the phylogenetic reconstruction performed in this study. The taxonomic tree was obtained from GTDB repository (https://gtdb.ecogenomic.org/) pruning those sequences of interest. Phylogenetic trees were visualized and annotated in iTOL (Letunic & Bork, 2019).

4.3 Growth rate at different temperatures

Fresh cells were passaged at least once in Erlenmeyer flasks at 30°C, cells were then diluted to OD600 of 0.02, and cultured in 96-half-deepwell microplate (enzyscreen, CR1469c). Cells were then grown at 650 rpm shaking on orbital thermoshaker (inheco, Vancomycin (30 µg 1,461.2), Tetracycline (30 µg 1,498.2), Penicillin (10 U 1,494.2), and 500 µl of 1 M H2O2 was added to the disk diffusion stress assays

The protocol was adapted after the Kirby Bauer method (Hudzicki, 2009) with a few modifications. M. extorquens bacterial strains were grown to mid-exponential OD600 of ~0.5 then 200 µl of cell suspensions at OD600 were spread on pre-dried Hyphoagar plates, then antibiotic disks (Carl Roth) were tested individually on each plate; Ampicillin (10 µg 1,456.2), Penicillin (10 U 1,494.2), Chloramphenicol (30 µg 1,461.2), Tetracycline (30 µg 1,498.2), Vancomycin (30µg 1501.1). For testing the sensitivity of H2O2, cells were spread on the plate as mentioned then disk was loaded in the middle of the plate and 10 µl of 1 M H2O2 was added to the disk (Merck 216,763). Agar plates were incubated at 30°C for 72 hr and zone of inhibition was measured. Experiments were repeated at least three times.

4.5 Carotenoid extraction for absorbance scan

Bligh and Dyer extraction (Bligh & Dyer, 1959) was used to extract carotenoids for cells grown to late exponential. 10 ml of cells of different Methyllobacterium mutants were collected at 5,000 rcf, for 10 min, washed once with 1× D-PBS. Wet weight of cell pellet was weighed. Cells were resuspended in water to 200 µl (taking weight into account), adding 250 µl chloroform (Carl Roth, Y015), and 500 µl of methanol (VWR chemicals, 20,903.368). The homogenous mixture was then sonicated in ultrasonic bath (Bandelin, Ultrasonic bath SONOREX DIGITEC DT 510 F) for 30 min. Samples were centrifuged at 12,000 rcf for 1 min (Thermo Scientific, Microcentrifuge Pico™ 21), supernatant was collected and extracted by adding 250 µl water, and 500 µl chloroform, vigorously mixing the cells, and collecting the lower organic phase into a new tube. Extraction was repeated three times, then the collected extract was dried using vacuum concentrator (Christ, RVC 2-25 CD). Finally, the dried extract was dissolved in ethyl acetate (EtOAc) (Merck, 1.06923.2511) to a final concentration 0.1 mg/µl (of pellet wet weight). Absorbance Scan was performed on (Tecan, plate reader Spark M20) the pigments in ethyl acetate, using cuvette (Hellma, 105-202-15-40).

4.6 Isolation and saponification of carotenoids

Carotenoids extraction was adapted from Kim and Lee (2012). Briefly, 10 mg wet weight of each sample was extracted using 1 ml methanol containing 6% KOH and incubated for at least 14 hr at 4°C in the dark. Supernatant was collected after centrifugation (1,500xg, 5 min) and reduced in a speedvac concentrator (Savant SPD111V; Thermo Fisher Scientific, Massachusetts, USA). EtOAc and saturated NaCl were added in equal volumes while thoroughly mixing after each addition. Upper organic phase was collected after centrifugation (10,000xg at 4°C for 5 min), washed twice with distilled water, and completely dried.

4.7 LC-MS analysis

Dried extracts, squalene (Sigma Aldrich) and diplopterol (Chiron AS) were dissolved in acetonitrile and filtered with Minisart RC 4 (Sartorius, Stonehouse, UK) before applying 5 µl aliquots to a Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 150 mm; Waters, Milford, Massachusetts) using an Agilent 1,290 Infinity II HPLC system equipped with a diode array detector. Extracts were eluted with a gradient solvent system consisting of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient selected was: 0 min: 80% B, 5 min: 80% B, 15 min: 95% B, 35 min: 95% B, 40 min: 80% B at a constant flow rate of 0.4 ml/min. Carotenoids were identified using a combination of absorption spectra, retention time, and mass spectra. For the differentiation between pigments with C30 and C40 carbon backbones Zeaxanthin and β-carotene (DHI Lab Products, Denmark) were run as examples for C40 pigments, and extracts from Staphylococcus aureus 533 R4 (DSM 20,231) and Methylobacterium rhodinum (DSM 2,163) were prepared as controls for C30 pigments. Mass spectra were monitored in positive electron spray ionization (ESI) mode in a mass range of m/z 300–1,000 on the Agilent 6,545 Q-TOF system (Agilent, Waldbronn, Germany).
Germany) using the following conditions: drying gas temperature 300°C, drying gas flow rate 8 L/min, sheat gas temperature 350°C, sheat gas flow rate 12 L/min, capillary voltage 3000V.

4.8 In vivo DI-4 spectroscopy

Three biological triplicates of cells were grown at either 27 or 32°C until the cultures reached mid exponential growth at around OD600 ~0.5. Cells were then diluted to OD600 0.2, washed and resuspended in succinate-free media. Cells were then incubated with 80 nM Di-4 ANEPPDHQ (ThermoFisher, D36802) for 10 min at 950 rpm shaking on a thermomixer (Eppendorf, Thermomixer C). Subsequently, cells were plated onto a black 96-well plate in analytical triplicates per sample, and measured in a plate reader (Tecan Spark M20). Excitation was set to: 485 nm, and emission was recorded at Channel 1 (Ch1) 540 nm and Channel 2 (Ch2) 670 nm with a bandwidth of 20 nm. The GP value was calculated from emission at 540 nm and 670 nm with the following equation:

\[
GP = \frac{I_{540 \text{nm}} - I_{670 \text{nm}}}{I_{540 \text{nm}} + I_{670 \text{nm}}}
\]

where \(I\) is the intensity measured at the specified channels.

4.9 Liposome preparation and in vitro DI-4 spectroscopy

Liposomes were prepared from two mixtures of lipids: DOPC to achieve a liquid disordered membrane and SM:Chol 1:1 (molar ratio) to achieve a liquid ordered membrane. 0.2 µmol of lipid in chloroform was mixed in a glass vial and dried under vacuum for 3 hr. The lipid film was hydrated with 1 ml HBS buffer (10 mM HEPES, 150 mM NaCl, at pH 7.0) at 45°C for 30 min, then sonicated for 5 min to create liposome that are more unilamellar and have more uniform size distribution. 120 nM Di-4-ANEPPDHQ was added and then the mixture was shaken at 45°C for 10 min. Liposomes of each mixture were independently mixed and prepared in triplicate. The spectra were recorded at 30°C using Spark® multimode microplate reader (TECAN). Samples were incubated at 30°C for 20 m. Excitation was set to: 485 nm, and emission was recorded at Channel 1 (Ch1) 540 nm and Channel 2 (Ch2) 670 nm with a bandwidth of 20 nm. The GP value was calculated from emission at 540 nm and 670 nm with the following equation:

\[
GP = \frac{I_{540 \text{nm}} - I_{670 \text{nm}}}{I_{540 \text{nm}} + I_{670 \text{nm}}}
\]

4.11 Thin layer chromatography

Lipids were extracted using the Bligh and Dyer extraction method as previously mentioned, and resuspended to final concentration of 0.1 mg/µl (wet weight of cell pellet), HPTLC Silica gel with concentrating zone were used (Merck, 1.13748.0001). Plates were pre-run in mobile phase prior to loading the samples. For diplopterol detection and quantification chloroform was used as the mobile phase. Diplopterol (Chiron, C1391.30-1MG) and squalene (Carl Roth, 3.185.1) were used as standards at concentrations (0.27 and 0.1 mg/ml respectively). For squalene detection, Hexane:Chloroform 3:1 (Sigma 1,070,232,511, and Carl Roth Y015.2) was used as mobile phase.

4.12 Strains, construction of plasmids, generation of mutants, and gene complementation

M. extorquens PA1 with cellulose synthase deletion was used in this study and referred to hereafter as WT (Chubiz et al., 2013; Delaney et al., 2013), Δshc was already available (Saenz et al., 2015). Genes for carotenoid biosynthesis were identified based on M. extorquens gene annotations for phytoene desaturase and phytoene synthases, and BLASTp was used to reconstruct the carotenoid biosynthesis pathway as shown.

Mutants were constructed by unmarked allelic exchange as described (Hmelo et al., 2015; Marx, 2008), for each gene primers were designed to include 500 bp upstream and 500 bp downstream overhangs of the gene. The produced PCR product was then used as a template for the construction of two plasmids one to delete the gene and one for the inducible expression of the gene in the knockout strain as explained in (Table 2). For gene deletion: plasmid pCM433 (Marx, 2008) was linearized via restriction digestion using enzymes NotI-HF, and SacI-HF (NEB, R3189, R3156 respectively), overhangs upstream and downstream of the gene of interest were amplified (primers sequences available in Table S1) and purified then cloned into linearized pCM433 using In-Fusion HD Cloning plus kit (Takara), primer design was done using primer design tool (Takara). For inducible expression of the gene: plasmid pLC291 was linearized...
TABLE 2 Names of targeted genes/proteins and plasmids used in this study

| Gene | Protein ID | Deletion vector | Inducible expression vector |
|------|------------|-----------------|-----------------------------|
| hpnE | WP_012253488.1 | pLMM013 | pLMM019 |
| crtN | WP_012254689.1 | pMG027 | pMG028 |
| crtP | WP_003603441.1 | pMG029 | pMG030 |
| crtB | WP_012254336.1 | pMG007 | - |

Note: Overhangs for gene deletion were introduced into pCM433 (Marx, 2008). Gene was cloned into pLC291 under the control of anhydrotetracycline inducible promoter (Chubiz et al., 2013).

using EcoRI-Hf, and KpnI-HF restriction enzymes (NEB R3101, R3142 respectively), gene was then PCR amplified and purified then cloned into plasmid pLC291 (Chubiz et al., 2013) using In-Fusion HD Cloning plus kit (Takara). All PCR products and linearized vector were purified (Macherey and Nagel, Nucleospin PCR clean-up Gel extraction).

Deletion vectors were introduced into WT via triparental conjugation. WT cells were mated with E. coli pRK2073 helper cells, and E. coli Stellar cells that carry the deletion/expression vector, and the mating was done using a ratio of 5:1:1 Acceptor-strain : helper-strain : donor-strain. The conjugation was done on NB-Agar plates at 30°C, overnight, then the cells were plated on hypho media-agar plates with Tmp and Tc. The clones were then grown for 9 hr in liquid media, then plated on 10% sucrose plates for selection of mutants. Colony PCR was then performed on clones from the sucrose plates, using Primers (Table S1) for gene template. PCR products of the correct size for gene deletion were then sequenced to confirm deletion of genes.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
James P. Saenz https://orcid.org/0000-0001-8901-4377

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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