Growth-uncoupled isoprenoid synthesis in Rhodobacter sphaeroides

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Abstract

Background Microbial cell factories are usually engineered and employed for cultivations that combine product synthesis with growth. Such a strategy inevitably invests part of the substrate pool towards the generation of biomass and cellular maintenance. Hence, engineering strains for the formation of a specific product under non-growth conditions would allow to reach higher product yields. In this respect, isoprenoid biosynthesis represents an extensively studied example of growth-coupled synthesis with rather unexplored potential for growth-independent production. Rhodobacter sphaeroides is a model bacterium for isoprenoid biosynthesis, either via the native 2-methyl-D-erythritol 4-phosphate (MEP) pathway or the heterologous mevalonate (MVA) pathway, and for poly-β-hydroxybutyrate (PHB) biosynthesis.

Results This study investigates the use of this bacterium for growth-independent production of isoprenoids, with amorpha-4,11-diene as reporter molecule. For this purpose, we employed the recently developed Cas9-based genome editing tool for R. sphaeroides to rapidly construct single and double deletion mutant strains of the MEP and PHB pathways, and we subsequently transformed the strains with the amorphadiene producing plasmid. Furthermore, we employed 13 C-metabolic flux ratio analysis to monitor the changes in the isoprenoid metabolic fluxes under different cultivation conditions. We demonstrated that active flux via both isoprenoid pathways while inactivating PHB synthesis maximizes growth-coupled isoprenoid synthesis. On the other hand, the strain that showed the highest growth-independent isoprenoid yield and productivity, combined the plasmid-based heterologous expression of the orthogonal MVA pathway with the inactivation of the native MEP and PHB production pathways.

Conclusions Apart from proposing a microbial cell factory for growth-independent isoprenoid synthesis, this work provides novel insights about the interaction of MEP and
MVA pathways under different growth-conditions.

Background

Isoprenoids (also known as terpenoids) have great industrial value as ingredients of pharmaceuticals, perfumes, food flavourings and most recently biofuels [1–6]. They are formed by the condensation of the five-carbon monomers isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). The two naturally existing IPP/DMAPP production pathways are the 2-C-methylerythritol 4-phosphate (MEP) pathway and the mevalonate (MVA) pathway [7]. While the former branches in the central metabolism from glyceraldehyde 3-phosphate and pyruvate, the latter uses acetoacetyl-CoA (AA-CoA) as precursor (Fig. 1). These two pathways are, with few exceptions, phylogenetically distinct: the MEP pathway is present in prokaryotes, while the MVA pathway is found in archaea and eukaryotes [8]. Plants express both metabolic routes, with the MEP pathway compartmentalized in the chloroplasts and the MVA pathway expressed in the cytosol [9]. Since the first implementation of a heterologous MVA pathway in Escherichia coli [10], the number of studies focusing on the synthesis of isoprenoids via microbial cell factories has increased a lot. Efforts for improving bioproduction have been focusing either on engineering of the endogenous MEP pathway [11–13], or by co-expressing the heterologous MVA counterpart [10, 14, 15]. Moreover, to limit the effect of unfavorable regulatory control of the endogenous pathway, substitution by an orthogonal isoprenoid pathway has been reported [16].

In microorganisms, isoprenoids are often membrane-bound molecules - like carotenoids, ubiquinones, chlorophylls and sterols - which are indispensable for growth [17, 18]. Therefore, during a batch cultivation, their volumetric concentration is expected to increase as consequence of microbial growth inside the reactor. Such type of metabolism for isoprenoid biosynthesis is defined as growth-coupled. For strain improvement
purposes, growth-coupled production has been largely employed. In such a scenario, the product of interest becomes a mandatory by-product of growth, and therefore microbial growth becomes the driving force of production [19]. This production-growth association has already been exploited for enhancing isoprenoid biosynthesis by laboratory evolution [20].

Nevertheless, growth-coupled production contains an inherent trade-off between substrate use for i) biomass production and maintenance, and ii) product formation [21]. Thus, if biomass formation is prevented, in principle more substrate is available for product synthesis.

Microbial metabolism has been engineered to produce non-native isoprenoid molecules as pharmaceuticals, perfumes, food flavourings and biofuels [1–6]. These compounds are not required for growth, and often excreted. Coupling of product formation to microbial growth is therefore not a necessity, and growth-uncoupled production would be an advantageous option.

Apart from C, H and O, isoprenoids do not contain other biomass-specific elements like P, S or N. Therefore, under P, S or N limited incubation conditions, glucose can in principle be converted into isoprenoid but not into microbial biomass. Improved isoprenoid/biomass ratios have already been obtained by using nutrient-limited culturing conditions [22–25]. Nonetheless, isoprenoid synthesis, as primary metabolite, is often strictly regulated to occur only under growing conditions, and this demotivated further studies on decoupling isoprenoid production from microbial growth as a production strategy.

In a recent study, the concept of redesigning biosynthetic networks based on orthogonality principles was introduced [26]. This idea entails that a non-native metabolic route that minimizes the interaction with the endogenous biomass-producing pathways can be exploited for bioproduction. Intrinsic independence exists between the two
isoprenoid pathways MEP and MVA. Therefore, regulatory control affecting the MEP pathway should not affect the MVA pathway, and vice versa. Moreover, in a recent study, functional replacement of the native MEP with the heterologous MVA pathway was described in the bacterium Rhodobacter sphaeroides [16], a microbial platform organism that is gaining interest for isoprenoid biosynthesis. This organism is able to synthesize intracellular membranes which can accommodate isoprenoids such as as carotenoids and bacteriochlorophylls. Moreover, it is also a natural producer of coenzyme Q$_{10}$. Apart from these native isoprenoid molecules, heterologous production of lycopene [27] and sesquiterpenes [28] has also been reported in this species.

Growth and isoprenoid synthesis in R. sphaeroides has been studied using defined medium, where the introduction of the heterologous MVA pathway revealed potential for growth-independent isoprenoid biosynthesis [29]. Under these conditions, the storage compound poly-β-hydroxybutyrate (PHB) was also accumulated [29]. Additionally, a mutual stimulating effect between the MEP and MVA pathways has been observed [30].

In this study, we investigate the behaviour of the two isoprenoid pathways for amorphadiene production in R. sphaeroides during different growth modes, as well as their interaction with the pathway for the carbon- and energy reserve material poly-β-hydroxybutyrate, PHB. By means of $^{13}$C metabolic flux ratio analysis, we assess the effect of genetic modifications (i.e. for elimination of PHB accumulation) and environmental changes (nitrogen limitation) on isoprenoid pathways capacities. Ultimately, we demonstrate that exclusive use of the orthogonal MVA pathway in combination with elimination of PHB synthesis is a promising strategy for attaining growth-independent production of isoprenoids.

Results
Prevention of PHB formation and its effect on amorphadiene biosynthesis

Culturing *R. sphaeroides* under nitrogen-limited conditions could theoretically result in growth-independent isoprenoid synthesis via the native MEP and the heterologous MVA pathways. However, upon consumption of the limited available nitrogen, *R. sphaeroides* stores excess carbon intracellularly as PHB, a nitrogen-free carbon and energy storage compound [29]. Aiming to increase isoprenoid production, we reasoned that deletion of one of the PHB synthesis genes would block PHB production under nitrogen-limited conditions and could therefore increase the flux through the MVA pathway. Deletion of the phaC1 and phaC2 genes, that code for the PHB polymerase, is the established approach for eliminating PHB biosynthesis in *R. sphaeroides* [31–34]. Nonetheless, this does not prevent activity of the NADPH-dependent acetoacetyl-CoA reductase PhaB, which could result in the undesired accumulation of 3-hydroxybutyryl-CoA or excretion of 3-hydroxybutyrate (Fig. 1). In a recent study, we demonstrated that deletion of the phaB gene prevents PHB biosynthesis [35]. Here, we confirmed that the deletion of either the phaB gene (Rs265_ΔphaB strain) or the combined deletion of the phaC1 and phaC2 genes (Rs265_ΔphaC1ΔphaC2 strain) prevents PHB formation both under nitrogen excess and nitrogen-limited conditions (Fig. 2A). As observed before [29], the wild type (Rs265) strain produced substantial amounts of PHB, especially under nitrogen-limiting conditions.

The pBBR-ads plasmid harbouring the heterologous amorphadiene synthase gene was transferred to the various *R. sphaeroides* strains by conjugation. The resulting strains were cultured under both nitrogen excess and nitrogen-limited conditions. At glucose depletion, we determined the amorphadiene concentration in the spent medium (Additional file 1: Table S1), the active biomass concentration reached (Additional file 1: Table S2) and, based on these two measurements, we calculated the amount of amorphadiene produced per biomass (Additional file 1: Table S3). We observed that, for
the Rs265_ΔphaB:pBBR-ads and Rs265_ΔphaC1ΔphaC2:pBBR-ads strains that use only the endogenous MEP pathway (MEP-only strains), elimination of PHB synthesis does not result in higher amorphadiene/biomass ratios compared to the Rs265 wt strain (Fig. 2B, Additional file 1: Table S3). In fact, these ratios remained unaffected when moving from nitrogen excess to nitrogen-limited conditions (Additional file 1: Table S3) at a value of 2.9 ± 0.2 mg • g of biomass⁻¹. Interestingly, the Rs265_ΔphaC1ΔphaC2:pBBR-ads strain showed an even lower amorphadiene/biomass ratio compared to the Rs265:pBBR-ads and the Rs265_ΔphaB:pBBR-ads strains (Additional file 1: Table S3). In summary, when only the endogenous MEP pathway was active, isoprenoid biosynthesis appeared to be strictly growth-coupled. Moreover, the MEP flux was insensitive or negatively affected by the impaired PHB synthesis.

We subsequently transformed the available Rs265, Rs265_ΔphaC1ΔphaC2 and Rs265_ΔphaB strains with the orthogonal MVA pathway, cloned on the pBBR-MVA-ads plasmid. The amorphadiene/biomass ratio increased 10- to 20-fold for all the strains tested (Fig. 2C, Additional file 1: Table S3). The highest increase was observed for the ΔphaB strain (Rs265_ΔphaB:pBBR-MVA-ads), reaching a ratio of 63.7 ± 4.0 mg • g of biomass⁻¹ (Fig. 2B, Additional file 1: Table S3). This value was significantly higher than the ratio reached by the strain with a functional PHB synthesis (Rs265:pBBR-MVA-ads), which was 35.9 ± 3.6 mg • g of biomass⁻¹ (Fig. 2B, Additional file 1: Table S3).

Increase of the MVA pathway flux, as consequence of the phaB deletion, was confirmed also for the Rs265-MVA_Δdxr strain, for which the MEP pathway is inactivated via the deletion of the 1-deoxy-D-xylulose 5-phosphate reductoisomerase (dxr) gene, after genomic integration of the MVA pathway (Fig. 1). This strain relies exclusively on the non-native isoprenoid route (MVA-only) [16]. Also here, a substantial increase in the
amorphadiene/biomass ratio was observed during both nitrogen excess and nitrogen-limited conditions (Fig. 2D, Additional file 1: Table S3). The highest value observed was for the Rs265-MVA_ΔdxrΔphaB:pBBR-MVA-ads strain, with 36.9 ± 1.6 mg • g of biomass−1 during nitrogen limitation.

In summary, although both the ΔphaC1ΔphaC2 and ΔphaB knockouts were equally effective in reducing PHB synthesis, the ΔphaB knockout strain produced more amorphadiene, both volumetrically and per biomass unit (Additional file 1: Tables S1, S3).

Organic acids secretion as consequence of PHB deletion

We reasoned that comparing the secretion profiles between ΔphaB and ΔphaC1ΔphaC2 could provide additional insights on the beneficial effect of ΔphaB on isoprenoid synthesis. Therefore, we quantified by HLPC analysis the organic acids in the spent medium of Rs265, Rs265_ΔphaC1ΔphaC2 and Rs265_ΔphaB harbouring either pBBR-ads or pBBR-MVA-ads plasmids (Fig. 2E-H). For both ΔphaB and ΔphaC1ΔphaC2 strains, 2-oxoglutarate (80 to 340 mg • g of biomass−1, Fig. 2F) and pyruvate (150 to 500 mg • g of biomass−1, Fig. 2G) were the main by-products. Both compounds require coenzyme A (CoA) for proceeding further in the metabolism via oxidative decarboxylation (Fig. 1). Excretion of these compounds suggests that free CoA is limiting when PHB biosynthesis is prevented.

The ΔphaC1ΔphaC2 strains secreted an additional unknown compound, which was identified as 3-hydroxybutyrate (3HB) by NMR (Additional file 1: Fig. S2). The spent medium for the Rs265_ΔphaC1ΔphaC2:pBBR-ads strain showed a value of 0.002 ± 0.001 mg 3HB • g of biomass−1 (Fig. 2H). In contrast, a value of 0.004 ± 0.001 mg 3HB • g of biomass−1 was observed for the Rs265_ΔphaC1ΔphaC2:pBBR-MVA-ads strain, which expressed the heterologous MVA pathway (Fig. 2H). Therefore, under nitrogen limitation and upon expression of the MVA pathway, the amount of 3HB secreted increased.
significantly compared to when only the MEP pathway was active.

\[ ^{13}C \] metabolic flux ratio analysis of isoprenoid biosynthesis under different growth conditions

The Rs265_\Delta phaB:pBBR-MVA-ads strain, that overexpresses the MVA pathway and still has an active MEP pathway, showed the highest amorphadiene/biomass ratio under nitrogen-limited conditions (Fig. 2C). Additionally, we observed that, independent from the cultivation conditions and the presence of an active PHB synthesis pathway, the dual-pathway (co-expressing MEP and MVA pathways) strains largely outperformed the single-pathway strains (Fig. 2B-D, Table S3). We therefore decided to further investigate the separate and combined contribution of the isoprenoid pathways to the amorphadiene production by \[ ^{13}C \] flux ratio analysis. MEP and MVA pathways are known to exert a reciprocal stimulation [30, 36]. To better understand their mode of interaction under the conditions tested, we determined their contribution via \[ ^{13}C \] metabolic flux ratio analysis of the Rs265:pBBR-MVA-ads and Rs265_\Delta phaB:pBBR-MVA-ads strains. We therefore compared the resulting amorphadiene/biomass ratios for each pathway with the ones determined for i) the Rs265:pBBR-ads and Rs265_\Delta phaB:pBBR-ads (MEP-only) strains, and ii) the Rs265-MVA_\Delta dxr:pBBR-MVA-ads and Rs265-MVA_\Delta dxr\Delta phaB:pBBR-MVA-ads (MVA-only) strains. Previously, this \[ ^{13}C \]-method provided important insights on the flux ratios upon co-expression of the MEP and MVA pathways [30]. Under nitrogen excess condition, we observed that the dual-pathway strains with active MEP and MVA pathways showed a higher amorphadiene/biomass ratio for each isoprenoid route compared to when these were active individually (Fig. 3A). We therefore confirmed that, during nitrogen excess conditions, co-expression of the two isoprenoid pathways resulted in enhancement of their
capacities in the Rs265 and Rs265\_AphaB strains harbouring the pBBR-MVA-ads plasmid (Fig. 3A, Table 1). Moreover, the capacity of the MVA pathway in the Rs265\_AphaB and Rs265-MVA\_AdxrAphaB strains was even further enhanced by the phaB deletion, as made obvious by comparison to strains that still contain the phaB gene (Fig. 3A, Table 1). In contrast, the flux through the native MEP pathway remained unaffected by the phaB deletion. Thus, under nitrogen excess conditions, deletion of the phaB gene results in an increase of the isoprenoid flux exclusively via the MVA pathway (Table 1).

| Table 1 | isoprenoid flux ratio calculated for the Rs265 and Rs265\_AphaB strains harbouring the pBBR-MVA-ads plasmid after growth on different initial nitrogen concentrations. |  |
|---------|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Genomic background | Isoprenoid flux ratios | Pathway capacities (mg amorphadiene \cdot g biomass\(^{-1}\)) |  |  |  |  |
|  | Nitrogen excess | Nitrogen limitation | Nitrogen excess | Nitrogen limitation |  |  |
| MEP | MVA | MEP | MVA | MEP | MVA | MEP | MVA |
| Rs265 | 0.29 ± 0.01 | 0.71 ± 0.01 | 0.46 ± 0.01 | 0.54 ± 0.1 | 9.4 ± 0.3 | 23.0 ± 0.3 | 16.9 ± 0.4 | 19.9 ± 0.5 |
| Rs265\_AphaB | 0.24 ± 0.03 | 0.76 ± 0.03 | 0.45 ± 0.02 | 0.55 ± 0.03 | 9.2 ± 1.3 | 29.3 ± 1.3 | 28.7 ± 1.3 | 35.0 ± 1.9 |

We further studied the isoprenoid flux ratio under nitrogen-limited conditions (Fig. 3B).

The Rs265:pBBR-ads and Rs265\_AphaB:pBBR-ads strains, which rely exclusively on the MEP pathway for isoprenoid production did not show any increase in the amorphadiene/biomass ratio when compared to nitrogen excess conditions (Fig. 3B). In contrast, the amorphadiene/biomass ratio for the strains that express only the MVA pathway was increased 3-fold when compared to this value under nitrogen excess conditions (Rs265-MVA\_Adxr and Rs265-MVA\_AdxrAphaB strains harbouring pBBR-MVA-ads plasmid, Fig. 3). Interestingly, for the strains that express both pathways (Rs265:pBBR-MVA-ads and Rs265\_AphaB:pBBR-MVA-ads strains, Fig. 3B), there was only a minor increase of the MVA pathway capacity in the Rs265\_AphaB strain when compared to nitrogen excess conditions (Table 1). On the other hand, the MEP pathway capacity increased by 80% for the Rs265 strain (from 9.4 ± 0.3 to 16.9 ± 0.4 mg amorphadiene \cdot g biomass\(^{-1}\)).
of biomass\(^{-1}\)) and by 300% for the Rs265\(_{\Delta \text{phaB}}\) strain (from 9.2 ± 1.3 to 28.7 ± 1.3 mg amorphadiene \(\cdot\) g of biomass\(^{-1}\)). Thus, for the dual-pathway strain, the increase of the amorphadiene/biomass ratio under nitrogen limitation conditions is attributed to the endogenous MEP pathway (Table 1).

**Amorphadiene biosynthesis during resting cells conditions**

Under nitrogen-limited conditions a short exponential growth phase occurred, and therefore a short growth-associated amorphadiene production phase could not be avoided. This resulted in non-linear growth and production kinetics, making it difficult to assess yields (mg amorphadiene \(\cdot\) glucose\(^{-1}\)) and productivities (mg amorphadiene \(\cdot\) L\(^{-1}\) \(\cdot\) h\(^{-1}\)). In order to focus exclusively on growth-uncoupled production, and to obtain linear kinetics, we decided to assess amorphadiene production during resting cell conditions in nitrogen-free medium. This cultivation setup simulates the production phase of a two-stage fermentation setup where growth and production are separated.

Since deletion of phaB and expression of the MVA pathway increased production during nitrogen limitation, we reasoned to assess the amorphadiene production levels in the presence of also an active MEP pathway. Therefore, we further cultivated the strains Rs265, Rs265\(_{\Delta \text{phaB}}\), Rs265-MVA\(_{\Delta \text{dxr}}\) and Rs265-MVA\(_{\Delta \text{dxr} \Delta \text{phaB}}\) under resting cells condition. All these strains contained the pBBR-MVA-ads plasmid (Fig. 4A).

A linear increase was observed in the OD\(_{600}\) of the strains with a functional PHB biosynthetic pathway (Rs265 and Rs265-MVA\(_{\Delta \text{dxr}}\), Fig. 4B). This trend is known to be associated with the accumulation of this storage compound [29], and it is associated with cell expansion rather than cell division. Accordingly, the corresponding \(\Delta \text{phaB}\) strains (Rs265\(_{\Delta \text{phaB}}\) and Rs265-MVA\(_{\Delta \text{dxr} \Delta \text{phaB}}\)) did not show any increase in OD\(_{600}\). Glucose consumption (Fig. 4C, Additional file 1: Fig. S1), pH and amorphadiene concentrations
(Fig. 4D, E) were followed over time. A decrease in the pH of the Rs265_ΔphaB and Rs265-MVA_ΔdxrΔphaB strains was observed (Fig. 4D), which can be explained by the secretion of organic acids upon prevention of PHB accumulation, mainly of pyruvate and 2-oxoglutarate (Fig. 4F).

Amorphadiene samples were collected over time from all the cultures (Fig. 4E), and yields and productivities were calculated for the first 24 h (Fig. 4G-I). The corresponding values for the Rs265 strain were the lowest among the tested strains. Deletion of phaB (Rs265_ΔphaB) resulted in a 2-fold increase of the amorphadiene/biomass ratio (Fig. 4G), the volumetric productivity (Fig. 4H) and the yield on glucose (Fig. 4I) compared to the Rs265 strain. Also, inactivation of the endogenous MEP pathway in the Rs265-MVA_Δdxr strain resulted in an increase of those values compared to Rs265 strain (Fig. 4H, I). Hence, inactivation of either the PHB production pathway or the endogenous MEP pathway stimulates growth-independent production. Combined inactivation of the MEP and PHB production pathways (Rs265-MVA_ΔdxrΔphaB strain) allowed to reach the highest amorphadiene/biomass ratio, volumetric amorphadiene productivity (Fig. 4H) and yield on glucose (Fig. 4I). All these values were 2,5-fold higher in the Rs265-MVA_ΔdxrΔphaB strain, compared to the Rs265 control strain. Hence, deletion of the endogenous MEP and PHB biosynthetic pathways resulted in the best metabolic setup for exploiting non-growing conditions for amorphadiene production.

Discussion

Strain optimization for improved bioproduction often relies on strategies that couple production to microbial growth [19, 37]. Nevertheless, an emerging approach for metabolic engineering strategies is the one of dissociating production and growth [26]. Following this view, in this work we engineered the isoprenoid and the PHB biosynthetic pathways in R. sphaeroides. Therefore, we could demonstrate that isoprenoid
biosynthesis, a typically growth-coupled type of metabolism in microorganisms, can be uncoupled from biomass production by means of rational metabolic engineering.

A previous work indicated that isoprenoid synthesis is strictly growth-coupled via the endogenous MEP pathway [29]. We applied nitrogen-limited conditions to a strain relying only on this isoprenoid pathway, but this did not result in increased amorphadiene/biomass ratio (Fig. 2B). We reasoned that targeting the storage compound (PHB) synthesis could increase the flux via the MEP pathway during this condition. Nevertheless, inactivation of the PHB synthetic pathway did not result in any improvement (Fig. 2B), thereby indicating that the endogenous MEP pathway is inhibited during non-growing conditions.

Introduction of a heterologous MVA pathway was described to allow isoprenoid synthesis also during non-growing conditions [29]. Here, we confirmed that nitrogen limited conditions increased the amorphadiene/biomass ratio in a strain relying exclusively on the MVA pathway (Fig. 2D). Moreover, inactivation of PHB synthesis by targeting phaB increased amorphadiene production when the MVA pathway was present (Fig. 2C, D). In order to calculate titers, rates and yields (TRY) of growth-independent amorphadiene synthesis, we performed cultivation under resting cells condition (Fig. 4). The experimental data confirmed that exclusive isoprenoid flux via the MVA pathway combined with inactivation of PHB synthesis result in maximal TRY values (Fig. 4E, G-I).

$^{13}$C metabolic flux ratio analysis was performed to understand the interaction between the two isoprenoid pathways during nitrogen limitation. The analysis indicated that in the dual-pathway strain the endogenous MEP pathway capacity is substantially enhanced during nitrogen limitation (Fig. 3B). Therefore, presence of the MVA pathway helps in deregulating the endogenous MEP pathway during nitrogen limitation.

Despite the increase in flux, only a small part of the carbon that originally went to PHB
production could be redirected to amorphadiene. Organic acids - especially pyruvate and 2-oxoglutarate - were excreted instead. The accumulation of these two organic acids indicates that their downstream reactions, catalysed by the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes respectively, were inhibited by the inability to produce PHB. These reactions require input of free CoA (Fig. 1), the co-enzyme released by the conversion of AA-CoA into PHB. We therefore speculate that knocking out eitherphaB orphaC1 and phaC2 decreased the availability of free CoA, that the capacity of the mevalonate pathway was insufficient to remedy this, and that this low CoA availability resulted in the accumulation of both pyruvate and 2-oxoglutarate. Possibly, secretion of 3HB after deletion of phaC1 and phaC2 allowed to release free CoA from the intermediate 3HB-CoA.

PHB synthesis results in [38]: i) carbon storage, ii) regeneration of NADP⁺ and iii) regeneration of free CoA for cellular homeostasis. Knocking out the ability to produce PHB should therefore increase the availability of precursors (AA-CoA) and NADPH for the MVA pathway. The relatively small increase in flux through the MVA pathway indicated that this pathway benefited from the increased amounts of AA-CoA and NADPH, but its capacity is limiting isoprenoid production. One of the rate-determining enzymes of the heterologous pathway is HMG-CoA reductase, already described as crucial enzyme for enhancing isoprenoid flux [15, 39]. We speculate that improving the catalytic efficiency of this enzyme might allow the MVA pathway to benefit from the higher availability of NADPH and AA-CoA. Such an improvement could allow to increase carbon flux towards isoprenoids, while reducing by-products secretion. Therefore, rational engineering strategies can build upon the findings of this work for further improving growth-independent isoprenoid biosynthesis in R. sphaeroides.
Conclusions

In this work, we assessed the contribution of the MEP and MVA pathway to amorphadiene biosynthesis under different culturing conditions. We confirmed that application of the heterologous MVA pathway holds potential for growth-independent production. In a dual-pathway strain, enhancement of the endogenous MEP pathway capacity was confirmed during nitrogen-limited conditions via $^{13}$C metabolic flux ratio analysis.

Nevertheless, isoprenoid synthesis during resting cells condition was limited by the presence of an active endogenous MEP pathway. On the other hand, exclusive isoprenoid flux via the MVA pathway increased amorphadiene synthesis during this condition.

Additionally, prevention of PHB synthesis via phaB resulted in the highest TRY values for growth-independent amorphadiene production.

Ultimately, this work proposed a metabolic engineering design for increasing growth-independent isoprenoid biosynthesis in *R. sphaeroides*, while providing novel insights about the interaction occurring between the two isoprenoid pathways.

Materials And Methods

Strains and standard cultivation conditions

The strains and plasmids used in this study are listed in Table 2 and Table 3, respectively. The Rs265_ΔphaC1ΔphaC2 strain was kindly donated by Isobionics BV. Preculturing of *R. sphaeroides* was performed in 250-mL Erlenmeyer flasks containing 25 mL of modified Sistrom’s minimal medium (SMM). As previously described [30], the medium contained glucose (3.0 g/L) as carbon source, and NH$_4$Cl (1.0 g/L) as nitrogen source. Moreover, the SMM contained (per liter): 3.48 g KH$_2$PO$_4$, 0.5 g/L NH$_4$Cl, 0.1 g glutamic acid, 0.04 g L-aspartic acid, 0.5 g NaCl, 0.02 g nitrilotriacetic acid, 0.3 g MgSO$_4$$\cdot$7H$_2$O, 0.0334 g CaCl$_2$$\cdot$2H$_2$O, 0.002 g FeSO$_4$$\cdot$7H$_2$O, and 0.0002 g (NH$_4$)$_6$Mo$_7$O$_{24}$. Trace elements were added
(0.01% v/v) from a stock solution containing: 17.65 g/L disodium EDTA, 109.5 g/L 
ZnSO$_4$·7H$_2$O, 50 g/L FeSO$_4$·7H$_2$O, 15.4 g/L MnSO$_4$·7H$_2$O, 3.92 g/L CuSO$_4$·5H$_2$O, 2.48 g/L 
Co(NO$_3$)$_2$·6H$_2$O, and 0.0114 g/L H$_3$BO$_3$. Vitamins were added (0.01% v/v) from a stock 
containing: 10 g/L nicotinic acid, 5 g/L thiamine HCl, and 0.1 g/L biotin.

Table 2
Strains used in this study.

| Strain          | Description                                                                 | Source of reference               |
|-----------------|-----------------------------------------------------------------------------|-----------------------------------|
| E. coli S17-1   | Host strain for conjugation, thi pro recA hsdR [RP42Tc::MuKm::Tn7] Tp$^r$ Sm$^r$ | Laboratory stock                  |
| R. sphaeroides  | R265 wild-type                                                             | Derivative of ATCC35035, Isobionics BV |
| Rs265ΔphaC1ΔphaC2| Rs265 with deletion of phaC1 and phaC2 for PHB biosynthesis                 | Isobionics BV                     |
| Rs265ΔphaB      | Rs265 with deletion of phaB for PHB biosynthesis                            | [35]                              |
| Rs265-MVA_∆dxr  | Rs265 with chromosomally integrated MVA pathway operon and deletion of the endogenous MEP pathway (dxr) | [16]                              |
| Rs265-MVA_∆dxrΔphaB | Rs265 with chromosomally integrated MVA pathway operon and deletion of the endogenous MEP pathway (dxr) and PHB biosynthetic pathway (phaB) | This study                        |
Table 3
Plasmids used in this study.

| Plasmid          | Description                                           | Source of reference |
|------------------|-------------------------------------------------------|---------------------|
| pBBR-ads         | pBBR1MCS-2 + crtE promoter and ads (amorphadiene synthase) | [29]                |
| pBBR-MVA-ads     | pBBR1MCS-2 + crtE promoter controlling MVA enzymes and ads | [28]                |
| pBBR_Cas9_ΔphaB_HR| pBBR1MCS-2 + codon harmonized cas9 sequence, sgRNA targeting phaB and 1 kb homologous-recombination flanks for recombination with phaB | [35]                |

Figures legends

Figure 1. Network investigated in this work. The metabolic map shows the steps involved in the biochemical conversion of glucose to isoprenoids. Isoprenoid biosynthesis can occur via the two orthogonal 2-C-methyl-D-erythritol-4-phosphate (MEP, blue arrows) and mevalonate (MVA, orange arrows) pathways. For the MEP pathway, the 1-deoxy-D-xylulose 5-phosphate reductoisomerase (dxr) gene is shown. This gene is targeted for inactivating the endogenous isoprenoid pathway. Both modules branch from the central metabolism and converge to isopentenyl-diphosphate (IPP) and dimethylallyl-diphosphate (DMAPP), which are the precursors of all isoprenoids. Moreover, the biosynthetic pathway of the storage compound PHB is included (box with red outline). This consists of two enzymatic reactions encoded by the genes phaB and phaC1 and phaC2 (both in green). The network includes also the schematic representation of the Krebs cycle, which includes conversion of 2-oxoglutarate (2-OXO) to succinyl-CoA. Eventually, accumulation of PYR or 2-OXO can result in their secretion in the medium (boxes with yellow and light-blue outline, respectively). Additionally, conversion of (R)-3-hydroxybutyril-CoA ([(R)-3HB-CoA] to (R)-3HB can result in the secretion of the latter compound (box with purple outline). Other abbreviations: reduced flavodoxin (Fld), ferredoxin (Fd) red: reduced, ox: oxidized. GAP (glyceraldehyde-3-phosphate), PYR (pyruvate), CDP-ME (4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol), CDP-MEP (2-phospho-4-(cytidine 5'S-diphospho)-2-C-methyl-D-erythritol), MEP (2-C-methyl-D-erythritol 2,4-cyclodiphosphate), pyrF (uridylate kinase, gene), frr (ribosome recycling factor, gene), uppS (undecaprenyl-diphosphate synthase, gene), cdsA (phosphatidate cytidylyltransferase, gene), rseP (Regulator of sigma E protease, gene) Ac-CoA (acetyl-CoA), AA-CoA (acetoacetyl-CoA), HMG-CoA (S)-3-hydroxy-3-methylglutaryl-CoA, MVA-P (S)-5-phosphomevalonate), MVA-PP (S)-5-diphosphomevalonate).

Figure 2. Prevention of PHB pathway and effect on amorphadiene yield on biomass. A) effect of initial medium C/N ratio on active biomass and PHB concentrations. B-D) mg amorphadiene • g of biomass−1 for wt, ΔphaC1ΔphaC2 and ΔphaB background in strains relying on B) only MEP pathway, C) MEP and MVA pathway, D) only MVA pathway. E-F) mg • g of biomass−1 calculated for E) PHB, F) 2-oxoglutarate, G) pyruvate and H) 3-hydroxybutyrate during nitrogen excess and nitrogen limited conditions.

Figure 3: 13C metabolic flux ratio analysis of R. sphaeroides with different initial C/N in the medium. The values are obtained from parallel labeling cultivation experiment. The isoprenoid flux ratios obtained were multiplied for the yield on biomass obtained for some of the strains of Fig. 2. A) Nitrogen excess conditions: enough nitrogen is provided to support cell division until depletion of glucose. B) Nitrogen limitation: nitrogen will be depleted from the medium before glucose, and cell division is expected to stop and allow PHB accumulation.

Figure 4. Growth-independent amorphadiene production. A) schematic overview of the strain tested. In blue is shown the endogenous MEP pathway, while in orange the orthogonal MVA pathway. In green the PHB biosynthetic pathway is depicted. Red cross represent genes deletion in either dxr (MEP pathway) or in phaB (PHB pathway). C15H24 is the brute formula of the reporter molecule amorphadiene. B-E) Monitoring of B) OD600, C) glucose concentration, D) pH, E) amorphadiene concentration. F) volumetric productivities of PHB and organic acids (PYR: pyruvate; 2-OXO: 2-oxoglutarate). Determination of G) amorphadiene/biomass ratios, H) amorphadiene volumetric productivity and i) amorphadiene yield on glucose.

Generation of double-KO strain via CRISPR-Cas9 counter-selection

Deletion of phaB was performed as previously described [35], using the pBBR_Cas9_ΔphaB_HR plasmid. Such plasmid was transferred from E. coli S17 cells to Rs265-MVA_Δdxr via diparental conjugation, resulting in the double mutant strain Rs265-MVA_ΔdxrΔphaB. By employing the pBBR_Cas9_ΔphaB_HR plasmid, phaB could be removed by homologous-recombination, and Cas9-based counter-selection of cells with intact genomic copies of phaB.
Diparental conjugation of R. sphaeroides

Diparental conjugation for transferring amorphadiene producing plasmids was performed as previously described [30] using RÄ medium. Such medium contained, per liter: 3 g malic acid, 0.2 g MgSO$_4$$\cdot$7H$_2$O, 1.2 g (NH$_4$)$_2$SO$_4$, 0.07 g CaCl$_2$$\cdot$2H$_2$O, 1.5 mL of microelements stock solution, 2 mL of vitamin stock solution and 5 mL of phosphate buffer. In case of RÄ agar medium, 15 g/L agar was added. The microelements solution contained: 0.5 g/L Fe(II)-Citrate, 0.02 g/L MnCl$_2$$\cdot$4H$_2$O, 0.005 g/L ZnCl$_2$, 0.0025 g/L KBr, 0.0025 g/L KI, 0.0023 g/L CuSO$_4$$\cdot$5H$_2$O, 0.041 g/L Na$_2$MoO$_4$, 0.005 g/L CoCl$_2$$\cdot$6H$_2$O, 0.0005 g/L SnCl$_2$$\cdot$2H$_2$O, 0.0006 g/L BaCl$_2$$\cdot$2H$_2$O, 0.031 g/L AlCl$_3$, 0.41 g/L H$_3$BO$_3$, 0.02 g/L EDTA. The vitamin solution contained: 0.2 g/L nicotinic acid, 0.4 g/L thiamine HCl, 0.008 g/L biotin, 0.2 g/L nicotinamide. The phosphate buffer contained 0.6 g/L KH$_2$PO$_4$ and 0.9 g/L K$_2$HPO$_4$.

Cultivation in nitrogen excess and nitrogen limited conditions

After overnight preculturing on SMM, R. sphaeroides cultures were transferred to fresh SMM with a starting OD$_{600}$ of 0.1, and incubated at 30 °C with 250 rpm. Cultivations were performed for biological triplicates in 250-mL Erlenmeyer flasks, each filled with 45-mL of SMM medium and 5-mL of filter-sterilized dodecane. SMM composition differed between nitrogen excess condition and nitrogen limited conditions only in the initial NH$_4$Cl concentration: 1.0 g/L and 0.25 g/L, respectively. Initial glucose concentration remained 3.0 g/L in both cases. Amorphadiene titers were measured after glucose depletion. This occurred after 24 h (for nitrogen excess condition) or after 48 h (for nitrogen limited condition). At the same time, the content of the flasks was harvested by centrifugation and further processed for analytical measurements.

$^{13}$C-metabolic flux ratio analysis of isoprenoid biosynthesis
Isoprenoid flux ratios analyses were performed as previously described [30] in 10-mL Erlenmeyer flasks containing 1.8-mL of labeled SMM medium and 0.2-mL of filter-sterilized dodecane. [1-\(^{13}\)C]- and [4-\(^{13}\)C]-glucose tracers had an initial concentration of 3.0 g/L. For nitrogen excess and limited conditions, the initial NH\(_4\)Cl concentration used was the same as for the cultivations in 250-mL flasks: 1.0 and 0.25 g/L, respectively. Samples for GC-MS measurement were taken at glucose depletion. MEP and MVA pathways capacities were obtained by multiplying the flux ratios determined via GC-MS with the amorphadiene titers measured via GC-FID for the 250-mL cultivations.

Cultivation under resting cell conditions

For resting cell cultivations, exponentially growing cells on SMM with nitrogen excess conditions were incubated until mid-exponential phase. Then, cells were pelleted by centrifugation at 4255 g for 10 min at room temperature. Subsequently, pellets were washed twice with sterile physiologic solution (NaCl 9 g/L) and centrifuged at 4255 g for 5 min. Washed pellets were inoculated with a starting OD\(_{600}\) of 1.0 on ‘nitrogen free SMM’ containing 5.0 g/L glucose and 0.0 g/L NH\(_4\)Cl. Initial biomass concentration was determined at the moment of the inoculum with a sample of 5-mL for TOC-L analysis. Then, incubation proceeded at 30 °C with 250 rpm. Samples for OD\(_{600}\), pH and amorphadiene measurements were taken at a regular interval until the pH dropped below 5.5. Determination of productivity and yields were performed for samples taken within the first 24 h.

Analytics

Cell density was monitored by measuring the optical density at 600 nm (OD\(_{600}\)).

Amorphadiene concentration was measured via GC-FID as previously described [29].

Glucose, PHB and organic acid concentrations were determined via (U)HPLC as previously
described (REF). For pyruvate and crotonic acid (resulting from PHB hydrolysis) identification, DAD detector was used, while for glucose, 2-oxoglutarate and 3-hydroxybutyrate determination, the RID detector was used. Determination of biomass concentration via TOC-L was performed by measuring the nitrogen content of the pellet, which was then used to calculate the active biomass concentration using the elemental composition of R. sphaeroides of CH$_{1.99}$O$_{0.5}$N$_{0.19}$ [29]. Identification of unknown compounds in the spent medium was obtained via $^1$H-nuclear magnetic resonance spectroscopy ($^1$H-NMR) measurements performed in D$_2$O on a Bruker Avance III 400 MHz NMR spectrometer.

**Abbreviations**

PHB
poly-β-hydroxybutyrate;

MEP
2-methyl-D-erythritol 4-phosphate;

MVA
mevalonate;

CRISPR-Cas
Clustered Regularly Inter-spaced Short Palindromic Repeats-CRISPR associated proteins;

PYR
pyruvate;

CoA
coenzyme-A;

NADPH
dihydronicotinamide-adenine dinucleotide phosphate;

Ac-CoA
acetyl-CoA;

AA-CoA
acetoacetyl-CoA;

HMG-CoA
hydroxymethylglutaryl-CoA;
3-HB-CoA
3-hydroxybutyryl-CoA;
3HB
3-hydroxybutyrate;
2-OXO
2-oxoglutarate;
phaB
acetoacetylreductase (gene);
dxr
1-deoxy-D-xylulose 5-phosphate reductoisomerase (gene);
ads
amorphadiene synthase (gene);

Declarations

Availability of data and material
The dataset supporting the conclusions of this article are included within the article and in the additional file.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Competing interests
Not applicable

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Authors' contributions

EO, IM, SWMK, GE, JvO and RAW designed the work. IM, EO, WP JB and MD conducted, analyzed, and interpreted the experiments. IM and EO drafted and wrote the manuscript. All authors read and approved the final manuscript.

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Figures
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