Investigating the Phylogenetic Range of Gibberellin Biosynthesis in Bacteria

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Certain plant-associated microbes can produce gibberellin (GA) phytohormones, as first described for the rice fungal pathogen *Gibberella fujikuroi* and, more recently, for bacteria, including several rhizobia and the rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzicola*. The relevant enzymes are encoded by a biosynthetic operon that exhibits both a greater phylogenetic range and scattered distribution among plant-associated bacteria. Here, the phylogenetic distribution of this operon was investigated. To demonstrate conserved functionality, the enzymes encoded by the disparate operon from *X. translucens* pv. *translucens*, along with those from the most divergent example, found in *Erwinia tracheiphila*, were biochemically characterized. In both of these phytopathogens, the operon leads to production of the bioactive GA₄. Based on these results, it seems that this operon is widely dedicated to GA biosynthesis. However, there is intriguing variation in the exact product. In particular, although all plant pathogens seem to produce bioactive GA₄, rhizobia generally only produce the penultimate hormonal precursor GA₉. This is suggested to reflect their distinct interactions with plants, because production of GA₄ counteracts the jasmonic-acid-mediated defense response, reflecting the importance of wounds as the entry point for these phytopathogens, whereas such suppression presumably is detrimental in the rhizobial symbiotic relationship.

The development of multicellular organisms is regulated by a multitude of hormones. In plants, gibberellins (GA) control stem elongation, germination, dormancy, flowering, leaf senescence, and fruit development (Hedden and Thomas 2012). Notably, biosynthesis of GA is not limited to plants. Indeed, GA were first isolated from the fungus *Gibberella fujikuroi*, causal agent of the bakanae or foolish seedling disease in rice (*Oryza sativa*) (Hedden and Sponsel 2015). This hemibiotrophic fungal phytopathogen seems to produce GA as a virulence factor (Wiemann et al. 2013). The bioactive GA presumably causes hormonal imbalances in the plant, which negatively affects the defense response, particularly that dependent on jasmonic acid (JA) (Robert-Seilantantz et al. 2007).

Certain bacteria also produce GA (Bottini et al. 2004). However, although plant and fungal GA biosynthesis has been extensively characterized (Hedden and Thomas 2012), the

relevant pathway in bacteria has only recently been fully elucidated (Nagel et al. in press; Nett et al. 2017; Tatsukami and Ueda 2016). Bacterial GA biosynthesis is associated with a cytochrome P450 (CYP)-rich operon (Fig. 1), as demonstrated by functional characterization of the encoded enzymes. This includes the relevant synthases that together produce *ent*-kaurene—that is, an isoprenyl diphosphate synthase (IDS) that produces (*E,E,E*)-geranylgeranyl diphosphate (GGPP), subsequently acting *ent*-copalyl diphosphate (*ent*-CPP) synthase (CPS), and *ent*-kaurene synthase (KS)—which were first characterized from symbiotic rhizobia that fall within the *Rhizobiales* order in the α -*proteobacteria* class (Hershey et al. 2014; Morrone et al. 2009). In addition, the homologous CPS and KS from a more distantly related operon found in *Xanthomonas oryzae* pv. *oryzicola* from the separate γ -*proteobacteria* class were found to be functionally analogous, and the ultimate product of the operon also was demonstrated to act as a virulence factor for this rice pathogen (Lu et al. 2015). Even more recently, the three CYP (CYP112, CYP114, and CYP117), along with ferredoxin (Fd_{GA}) and short-chain alcohol dehydrogenase/reductase (SDR_{GA}), found in all copies of the operon, were shown to transform *ent*-kaurene to GA₉, although this is not thought to exert hormonal activity (Nagel et al. in press; Nett et al. 2017; Tatsukami and Ueda 2016). Notably, the additional CYP (CYP115) found associated with the operon in the phytopathogen *X. oryzae* pv. *oryzicola* carries out the final 3 β -hydroxylation reaction necessary to produce the bioactive GA₄ (Nagel et al. in press). Intriguingly, although almost all copies of the operon characterized from rhizobia have only a fragment of CYP115 remaining, it has been shown that some (approximately 16%) also contain a functional copy of CYP115 elsewhere in their genomes, which seems to have been acquired via a separate horizontal gene transfer event from the core GA biosynthetic operon (Nett et al. in press).

Perhaps not surprisingly, although the GA biosynthetic operon exhibits a highly scattered distribution, it seems to be exclusively found in plant-associated bacteria (A. Levy, US-DoE Joint Genome Institute, *personal communication*). In particular, the operon is found in subsets of both symbiotic nitrogen-fixing rhizobia and phytopathogens. It has been suggested that the production of GA₉ by rhizobia is followed by conversion to bioactive GA₄ by the host plant, and further proposed that this acts to block any further nodulation (Tatsukami and Ueda 2016), although the investigated *Mesorhizobium loti* MAFF303099 is capable of producing GA₄ directly (Nett et al. in press). On the other hand, the direct production of GA₄ has been hypothesized to reflect the importance of entry via wounded leaf tissue, with associated JA-dependent defense response, for the relevant phytopathogens (Nagel et al. in press).

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Here, bacterial GA biosynthesis is further investigated by functional characterization of all the encoded enzymes from two copies of the relevant operon whose sequences are only distantly related to those already elucidated and each other, covering the full phylogenetic range of this sequence element. These both lead to production of the bioactive GA₄, and speculation about the relevance of bacterial production of bioactive GA or the penultimate hormonal precursor is discussed.

RESULTS

It has been previously reported that the GA biosynthetic operon also is found in various pathovars of *X. translucens*, where it also includes CYP115, but is only distantly related to either those from the order *Rhizobiales* or *X. oryzae* pv. *oryzicola* (Lu et al. 2015). To determine whether the phylogenetically distinct operon from *X. translucens* also can impart the ability to produce GA₄ (i.e., is functionally analogous to that from *X. oryzae* pv. *oryzicola*), the encoded enzymes were investigated here, specifically those from *X. translucens* pv. *translucens* DSM18974. In addition, the phylogenetic range of the GA biosynthetic operon was further investigated by searching the publically available sequence databases at National Center for Biotechnology Information (NCBI) and the Joint Genome Institute, which revealed the presence of a homologous sequence element in a variety of other plant-associated bacteria. In every case, these contained at least the eight genes that compose the core GA biosynthetic operon (Fig. 1). Indeed, although homologs for these genes can be found in other settings (i.e., in operons with different compositions, containing no more than three of these genes), those exhibit much lower homology, <65% amino acid (aa) sequence identity (Supplementary Table S1), relative to those found in the putative GA operons, which have >75% aa sequence identity. Beyond the copies of the operon already noted above, even more

divergent examples were found here, as demonstrated by phylogenetic analysis (Fig. 2; Supplementary Fig. S1). These include examples from not only additional genera in the *γ-proteobacteria* class but also rhizobia that fall within the distinct *β-proteobacteria* class. To determine whether the range of operons observed here is involved in GA biosynthesis as expected, the encoded enzymes from the most divergent example (those from *Erwinia tracheiphila*, which share <77% aa sequence identity with any homolog) also were investigated here. Specifically, each of the genes from these two copies of the GA biosynthetic operon were expressed in *Escherichia coli* and the activity of the recombinant enzymes was functionally characterized.

Because bacteria do not typically produce diterpenes such as GA, such biosynthesis requires production of the general diterpene precursor GGPP from the universal isoprenoid 5-carbon precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The IDS from the GA biosynthetic operon has only been indirectly characterized thus far, by demonstrating that coexpression of the IDS with the CPS and KS is required for recombinant production of *ent*-kaurene, specifically using these genes from the *α*-rhizobia *Sinorhizobium fredii* (Hershey et al. 2014). Here, the activity of the IDS from *X. translucens* pv. *translucens* (*Xt*IDS) and *Erwinia tracheiphila* (*Et*IDS) were more directly investigated. In particular, using in vitro assays with purified enzymes and direct detection of the resulting enzymatic products by liquid chromatography with tandem mass spectral detection (LC-MS/MS) demonstrated that both *Xt*IDS and *Et*IDS react with IPP and DMAPP to specifically produce the 20-carbon GGPP, with only very minor amounts of the initial 10-carbon elongation intermediate geranyl diphosphate, and none of the intermediary 15-carbon intermediate (*E,E*)-farnesyl diphosphate observed (Fig. 3). Thus, these IDS are specific GGPP synthases.

Previous characterization of the CPS from the GA biosynthetic operon relied upon indirect detection of the *ent*-CPP

Fig. 1. Schematic representation of the gibberellin (GA) biosynthetic operon. Arrows indicate the direction of translation. Abbreviations for the genes: CYP = cytochrome P450, Fd = ferredoxin, SDR = short-chain alcohol dehydrogenase/reductase, IDS = isoprenyl diphosphate synthase, CPS = copalyl diphosphate synthase, KS = *ent*-kaurene synthase, and IDI = isopentenyl diphosphate isomerase.

Fig. 2. Phylogeny of the gibberellin (GA) biosynthetic operon. Unrooted maximum-likelihood phylogenetic tree of the concatenated amino acid sequence of the enzymes from the operon spanning the completely conserved cytochrome P450 (CYP)112 to kaurene synthase. Bootstrap values were determined with MEGA 7 and the scale bar represents substitutions per site. Also indicated are the relevant classes of proteobacteria and the presence of CYP115.

product, with observation by gas chromatography with mass spectral detection (GC-MS) of the dephosphorylated *ent*-copalol, or subsequently produced *ent*-kaurene upon coexpression of a KS, from bacteria also engineered to produce GGPP (Hershey et al. 2014; Lu et al. 2015; Morrone et al. 2009). Although analogous studies were carried out with the CPS from *X. translucens* pv. *translucens* (*Xtt*CPS) and *E. tracheiphila* (*Et*CPS) (Supplementary Fig. S2), a more direct approach was taken here to characterize these CPS, again using purified enzymes in in vitro enzyme assays with GGPP as substrate and direct detection of the enzymatic products by LC-MS/MS. This approach was first validated by analysis of the known CPS from *Arabidopsis thaliana* (*At*CPS), which further provided an authentic sample of *ent*-CPP for comparison with the products of *Xtt*CPS and *Et*CPS, both of which exhibited the predicted activity (Fig. 3).

Characterization of the KS from *X. translucens* pv. *translucens* (*Xtt*KS) and *E. tracheiphila* (*Et*KS) was carried out as previously described (Hershey et al. 2014; Lu et al. 2015; Morrone et al. 2009); in particular, using a metabolic engineering approach in which the KS were expressed in *Escherichia coli* also engineered to produce *ent*-CPP. Such cultures expressing either *Xtt*KS or *Et*KS exhibited the predicted ability to produce *ent*-kaurene, as detected by extraction and GC-MS analysis (Fig. 4).

The oxidative enzymes catalyzing the remaining steps in GA biosynthesis were functionally characterized by recombinant feeding studies, much as previously described (Nagel et al. in press). Briefly, each of the CYP and SDR_{GA} were individually

expressed in *E. coli*, which cannot otherwise catalyze these transformations. The CYP114 also were coexpressed with the accompanying Fd_{GA}, because this has been shown to be required for full activity of this CYP—the other CYP are presumably efficiently reduced by endogenous ferredoxins (Nett et al. 2017). The resulting recombinant strains were each fed the relevant substrate, and the cultures then extracted for GC-MS analysis to determine whether the predicted enzymatic function was observed.

E. coli expressing CYP117 from either *X. translucens* pv. *translucens* (*Xtt*CYP117) or *Erwinia tracheiphila* (*Et*CYP117) efficiently converted *ent*-kaurene to *ent*-kauren-19-oic acid (Fig. 5; Supplementary Fig. S3). Indeed, this multistep transformation proceeded to essentially completion, because the intermediates *ent*-kauren-19-ol and *ent*-kauren-19-al were not detected.

Escherichia coli expressing CYP114 and Fd_{GA} from either *X. translucens* pv. *translucens* (*Xtt*CYP114 + *Xtt*Fd_{GA}) or *Erwinia tracheiphila* (*Et*CYP114 + *Et*Fd_{GA}) efficiently converted *ent*-kaurenoic acid to GA₁₂-aldehyde and, less efficiently, to the further oxidized GA₁₂ (Fig. 5). Only trace amounts of the intermediate *ent*-7 α -hydroxykaurenoic acid were observed with these cultures but it was the sole product when *Escherichia coli* expressing CYP114 only (either *Xtt*CYP114 or *Et*CYP114) was fed *ent*-kaurenoic acid. Notably, unlike recombinantly expressed CYP114 + Fd_{GA} from other bacteria, including those from *X. translucens* pv. *translucens*, *E. coli* expressing *Et*CYP114 + *Et*Fd_{GA} was able to convert *ent*-7 α -hydroxykaurenoic acid to a similar mixture of

Fig. 3. Isoprenyl diphosphate synthase (IDS) and copalyl diphosphate synthase (CPS) activity. Left: Liquid chromatography with tandem mass spectral detection chromatograms from in vitro assays with purified IDS from either *Xanthomonas translucens* pv. *translucens* (*Xtt*IDS) or *Erwinia tracheiphila* (*Et*IDS) with isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) as substrates, with comparison with a GGPP standard (also shown are standards for geranyl diphosphate [GPP] and (E,E)-farnesyl diphosphate [FPP]), or with purified CPS from either *Arabidopsis thaliana* (*At*CPS), *X. translucens* pv. *translucens* (*Xtt*CPS), or *E. tracheiphila* (*Et*CPS), with GGPP as substrate. Right: Condensation of DMAPP with three molecules of IPP to form GGPP catalyzed by IDS, and cyclization of GGPP to *ent*-CPP catalyzed by CPS.

GA₁₂-aldehyde and lesser amounts of GA₁₂ (Supplementary Fig. S4B). Consistent with previous work on the CYP114 + Fd_{GA} from the α -rhizobia *S. fredii* (Nett et al. 2016), trace amounts of *ent*-6 α ,7 α -dihydroxykaurenoic acid was observed here but neither this (Supplementary Fig. S4C and B) nor the closely related kaurenolide or 7 β -hydroxykaurenolide (neither of which were observed here) appeared to be further transformed by *Et*CYP114 + *Et*Fd_{GA} (data not shown).

Although CYP114 + Fd_{GA} is able to further oxidize GA₁₂-aldehyde to GA₁₂, this transformation does not seem to be efficiently catalyzed. By contrast, this reaction is efficiently catalyzed by the SDR_{GA} from either *X. translucens* pv. *translucens* (*Xt*SDR_{GA}) or *Erwinia tracheiphila* (*Et*SDR_{GA}) as *Escherichia coli* expressing either completely converted GA₁₂-aldehyde to GA₁₂ (Fig. 5).

E. coli expressing CYP112 from either *X. translucens* pv. *translucens* (*Xt*CYP112) or *Erwinia tracheiphila* (*Et*CYP112) efficiently converted GA₁₂ to GA₉ (Fig. 5). This multistep transformation was catalyzed almost to completion, because GA₁₅ (formed by initial hydroxylation) is not observed, and only small amounts of the intermediary GA₂₄ are observed (Supplementary Fig. S5).

Finally, just as in *X. oryzae* pv. *oryzae*, the GA biosynthetic operons in both *X. translucens* pv. *translucens* and *E. tracheiphila* contain a full-length CYP115. *Escherichia coli* expressing CYP115 from either *X. translucens* pv. *translucens* (*Xt*CYP115) or *Erwinia tracheiphila* (*Et*CYP115) catalyzed 3 β -hydroxylation of GA₉ to form the bioactive GA₄ (Fig. 5).

DISCUSSION

The results reported here strengthen the association of this operon with GA biosynthesis. For example, whereas previous

characterization of the enzymes producing isoprenyl diphosphates (i.e., IDS and CPS) has relied on indirect observations, using GC-MS to analyze the dephosphorylated products, these have been directly characterized here via LC-MS/MS (Fig. 3). This enabled demonstration of the specific production of GGPP by the associated IDS, rather than a mixture of different chain-length products as seen with other such enzymes (Nagel et al. 2015; Schmidt et al. 2010), which is consistent with dedicated production of the derived GA. In addition, the ability of *Et*CYP114 + *Et*Fd_{GA} to convert *ent*-7 α -hydroxykaurenoic acid to GA₁₂-aldehyde further supports the relevance of this intermediate, consistent with recent labeling experiments (Nett et al. 2016).

More importantly, the results presented here demonstrate the conserved function for production of GA of this operon across the full phylogenetic range that is currently available (Fig. 2). Accordingly, bacteria containing this operon are then predicted to produce GA. This includes species not only from additional α -rhizobia genera within the order *Rhizobiales* (i.e., *Microvirga* and *Azorhizobium*) but also from the *Pseudomonas* genus in the γ -*proteobacteria* class, as well as β -rhizobia from the *Burkholderia* genus in the distinct β -*proteobacteria* class (albeit only in three strains). Hence, plant-associated bacteria spanning three orders of *Proteobacteria* have acquired the GA biosynthetic operon, almost certainly via horizontal gene transfer (Fig. 2). However, the scattered distribution of the operon within these plant-associated bacteria indicates that production of GA only provides a selective advantage under certain circumstances.

Intriguingly, there seems to be a correlation between the plant–microbe relationship, specifically pathogenic versus symbiotic, and presence or absence of CYP115, with associated production of bioactive GA₄ or the penultimate precursor GA₉,

Fig. 4. Kaurene synthase (KS) activity. Left: Gas chromatography with mass spectral detection chromatograms of hexane extract from *Escherichia coli* metabolically engineered to produce *ent*-CPP and expressing the KS from either *Xanthomonas translucens* pv. *translucens* (*Xt*KS) or *Erwinia tracheiphila* (*Et*KS), and of an authentic *ent*-kaurene standard. Right: Cyclization of *ent*-CPP to *ent*-kaurene catalyzed by KS.

respectively. In particular, phytopathogens seem to invariably contain the full GA biosynthetic operon (i.e., including CYP115) and, thus, directly produce the bioactive GA₄, whereas rhizobia generally (although not always) contain only the core GA biosynthetic operon (i.e., without CYP115) and only produce the penultimate hormonal precursor GA₉. Indeed, beyond the previously noted loss of CYP115 in more than 80% of α -rhizobia (Nett et al. in press), its absence in all the β -rhizobia containing the GA biosynthetic operon (i.e., the core operon)

further supports the hypothesis that production of bioactive GA₄ may be deleterious to the symbiotic relationship between these rhizobia and their leguminous host plants. In particular, given that the bioactive GA₄ produced by *X. oryzae* pv. *oryzicola* acts as a virulence factor by suppressing the JA-dependent defense response (Lu et al. 2015), it has been hypothesized that the rhizobia may leave the final step in bioactive GA production to their hosts in order to not compromise the ability of the plant to respond to infection by

Fig. 5. Activity of the oxidative enzymes. Left: Gas chromatography with mass spectral detection chromatograms of organic extracts from *Escherichia coli* cultures expressing the indicated enzymes and fed the indicated substrate, with chromatograms of standard compounds as a reference. Standards and extracts were methylated and, in case of gibberellin 4 (GA₄), also silylated. Enzymes from *Xanthomonas translucens* pv. *translucens* are indicated by a Xtt prefix and those from *Erwinia tracheiphila* by an Et prefix. Right: Reactions catalyzed by the indicated enzymes.

pathogenic microbes (Nett et al. in press). However, although it has been proposed that rhizobial production of GA affects nodulation by limiting nodule numbers (Tatsukami and Ueda 2016), it remains unclear why only certain rhizobia contain the GA biosynthetic operon, particularly given the much wider phylogenetic distribution of this sequence element shown here.

The presence of the (full) GA biosynthetic operon in phytopathogenic bacteria has been hypothesized to reflect the importance of entry via wounded leaf tissue for that bacterial species (Nagel et al. in press). The basis for this hypothesis arose from the similar lifestyles of *X. oryzae* pv. *oryzicola* and *X. translucens*. Although not otherwise closely related (Naushad et al. 2015), these nominally enter via stomata and propagate throughout the mesophyll parenchyma, causing leaf streak disease in small grain cereals and forage grasses from a subset of the *Poaceae* or grass plant family, specifically the BEP clade (Grass Phylogeny Working Group II 2012). However, these plants have highly water-repellent leaves (Neinhuis and Barthlott 1997), a function of their vertical or upright nature, and a hydrophobic surface (Koch et al. 2008), which limits bacterial colonization and stomatal access (Beattie 2011). Hence, it seems likely that these phytopathogens are largely dependent on entry via wounded tissue, such that the GA biosynthetic operon provides a means for suppressing the JA response associated with wounding (Nagel et al. in press), consistent with the known antagonistic interactions between GA and JA (Bari and Jones 2009; Robert-Seilanian et al. 2007; Wasternack and Hause 2013). The appearance of the full GA biosynthetic operon in *X. bromi* noted here (Fig. 2) falls neatly within this paradigm, because *X. bromi* also nominally enters via stomata and causes leaf streak disease in grasses from the *Poaceae* BEP clade (Hersemann et al. 2016). Although little is known about the lifestyle of the *Pseudomonas psychrotolerans* strain that was also found here to contain the full GA biosynthetic operon, this genus contains many phytopathogens, and this strain was isolated from rice (Midha et al. 2016), consistent with the overall scenario presented above.

By contrast, the very different lifestyle of the cucurbit pathogen *E. tracheiphila* might seem more difficult to reconcile. Nevertheless, this phytopathogen also seems to depend on entry via wounded tissue. In particular, *E. tracheiphila* is transmitted by herbivorous beetles via deposition of bacteria-infested frass on fresh feeding wounds (Rojas et al. 2015). Thus, the production of bioactive GA₄ imparted by the operon, as shown here, presumably provides *E. tracheiphila* a means of suppressing the JA response induced by feeding.

In conclusion, the results reported here demonstrate functional conservation of the GA biosynthetic operon across a wide phylogenetic range of plant-associated bacteria, both rhizobia and plant pathogens. Intriguingly, based on the current distribution of this operon, as well as the CYP115 that catalyzes the final step in biosynthesis of the bioactive GA₄, such hormone production seems to increase the virulence of plant pathogens that must attenuate a preexisting JA-mediated defense response, while leaving the final step to the host plant may be beneficial in the rhizobia-legume symbiotic relationship. Thus, it will be interesting to see where else the operon will be found in the future, along with more direct investigation of the physiological relevance of GA production, either the bioactive GA₄ or penultimate hormonal precursor GA₉, in phytopathogens and rhizobial symbionts.

MATERIALS AND METHODS

Chemicals were purchased from Sigma and Fisher Scientific if not otherwise specified. Authentic GA standards

(GA₁₂-aldehyde, GA₁₂, GA₉ and GA₄) were purchased from OlChemIm Ltd., whereas authentic *ent-7α*-hydroxykaurenoic acid, GA₁₅, and GA₂₄ were kindly provided by P. Hedden (Rothamsted Research).

To investigate the currently available phylogenetic range of the GA biosynthetic operon, the protein sequence of CYP114 from *X. oryzae* pv. *oryzicola* BLS256 was used in a BLASTP search against the NCBI nonredundant protein sequences database. Homologs in other bacteria with known genome sequences provided an entry point for examination of the presence of the other relevant genes in the vicinity (Fig. 1). To investigate homologs of each gene from the operon, those from *E. tracheiphila* were used for a BLAST search against the protein database at NCBI, with the default number of target sequences increased from 100 to 1,000. The closest homolog that is not part of a GA operon is noted in Supplementary Table S1.

For phylogenetic analysis, the protein sequences for CYP112, CYP114, Fd_{GA}, SDR_{GA}, CYP117, IDS, CPS, and KS from a representative subset of species were downloaded from GenBank for *S. fredii* NGR234, *Bradyrhizobium japonicum* USDA110, *M. loti* MAFF303099, *X. oryzae* pv. *oryzicola* BLS256, *X. oryzae* pv. *oryzicola* BOXR1, *X. oryzae* X11-5A, *X. bromi* LMG 947, *X. translucens* pv. *translucens* DSM18974, *X. translucens* pv. *cerealis* CFBP2541, *X. translucens* pv. *graminis* ART-Xtg29, *Burkholderia* sp. JPY251, *P. psychrotolerans* NS274, and *E. tracheiphila* PSU-1. CYP115 and the isopentenyl diphosphate isomerase were not included due to their absence in certain rhizobia. Sequences were aligned in MEGA 7 using the Muscle algorithm, and the phylogenetic tree was constructed and tested with the bootstrap method in the same program. For the maximum-likelihood, neighbor-joining, and minimum-evolution algorithms, the JTT model was used with a γ distribution. With the maximum-likelihood algorithm, all sites were used whereas, for the other algorithms, gaps were deleted.

The targeted enzymatic genes were cloned by amplification from genomic DNA of *X. translucens* pv. *translucens* strain DSM18974 or *E. tracheiphila* strain PSU-1 with Q5 Hot Start High-Fidelity DNA polymerase (NEB), according to the product manual, using 5 μ l of the high-GC-content enhancer for genes from *X. translucens* pv. *translucens* and gene-specific primers (Supplementary Table S2). The forward primer included a CACC sequence before the start codon to allow cloning into pET100 (Invitrogen) according to the instructions supplied by the manufacturer. Clones were sequenced to verify the correct DNA sequence. CYP114 was cloned either alone or in tandem with the neighboring Fd_{GA}, using the forward primer of CYP114 and the reverse primer of Fd_{GA}.

For recombinant expression, the pET100 constructs containing either IDS or CPS were transformed into *Escherichia coli* strain BL21 Star (Invitrogen) for expression. Starter cultures were grown in 10 ml of NZY media (NaCl at 10 g/liter, casein at 10 g/liter, yeast extract at 5 g/liter, and MgSO₄ [anhydrous] at 1 g/liter [pH 7.0]) with carbenicillin at 50 μ g/ml, 18°C, and 200 rpm for 3 days. The starter culture (5 ml) was used to inoculate 100 ml of fresh NZY media, with carbenicillin at 50 μ g/ml, after the cultures reached an optical density (OD) of 0.6 they were induced with 1 mM isopropylthio-galactopyranoside (IPTG) and grown under continuous shaking at 200 rpm at 18°C for 1 day. Cells were harvested by centrifugation at 3,000 $\times$ g for 20 min. The cell pellet was resuspended in 5 ml of buffer (25 mM 3-[N-morpholino]-2-hydroxypropanesulfonic acid [pH 7.2], 10 mM MgCl₂, and 10% [vol/vol] glycerol) with 20 mM imidazole, and homogenized using an EmulsiFlex C-5 (Avestin). The homogenized suspensions were centrifuged at 14,000 $\times$ g for 30 min. The supernatant was mixed with 1 ml of Ni-NTA agarose

(Qiagen), incubated under gentle shaking for 1 h at 4°C, and washed with 5 ml of buffer containing 20 mM imidazole and an additional 5 ml with 60 mM imidazole. Proteins were eluted with 2 ml of buffer containing 250 mM imidazole.

Enzyme assays were carried out with 10 µg of purified heterologously expressed protein in 300 µl of buffer. IDS assays were run with 50 µM IPP and 50 µM DMAPP as substrates, while CPS assays were run with 20 µM GGPP as substrates. Assays were incubated for 1 h at 30°C, then flash frozen in liquid nitrogen and kept at –80°C until their analysis by LC-MS/MS, which was carried out as previously described (Nagel et al. 2015).

Metabolic engineering to test the activity of the CPS and KS from *X. translucens* pv. *translucens* was carried out using a modular expression system in *E. coli* (Cyr et al. 2007), with analysis by GC-MS, as previously described (Hershey et al. 2014; Lu et al. 2015; Morrone et al. 2009).

For recombinant feeding studies, the pPET100 constructs with CYP117, CYP112, CYP114, CYP114 + Fd_{GA}, CYP115, or SDR_{GA} were transformed into *E. coli* strain BL21 Star (Invitrogen). For each of the resulting recombinant strains, three colonies were inoculated into 10 ml of terrific broth (TB) broth (casein at 12 g/liter, yeast extract at 24 g/liter, and 0.4% glycerol), including carbenicillin at 50 µg/ml, and grown at 18°C for 2 days with constant shaking at 200 rpm. From the starter culture, 2 ml was transferred into 25 ml of fresh TB broth, including carbenicillin at 50 µg/ml. Cultures were induced with 1 mM IPTG. Those expressing CYP were induced upon reaching an OD of 0.8, whereas those expressing SDR_{GA} were induced at an OD of 0.6. Upon induction, 5 ml of 1 M phosphate buffer (pH 7.5) with 10 µM specific substrates was added to all cultures whereas, for cultures expressing CYP, this further contained 1 mM aminolevulinic acid, 1 mM riboflavin, and 0.1 mM FeCl₃. The induced and fed cultures were then grown for 3 days at 18°C with constant shaking at 200 rpm. Products were then extracted and analyzed by GC-MS, as previously described (Nagel et al. in press). For detection of *ent*-6 α ,7 α -dihydroxykaurenoic acid, the relevant hexane extracts were silylated with N-methyl-N-(trimethylsilyl) trifluoroacetamide after methylation.

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