Biosynthesis

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The Molecular Basis of Conjugated Polyyne Biosynthesis in Phytopathogenic Bacteria**

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Abstract: *Polyynes (polyacetylenes), which are produced by a variety of organisms, play important roles in ecology. Whereas alkyne biosynthesis in plants, fungi, and insects has been studied, the biogenetic origin of highly unstable bacterial polyynes has remained a riddle. Transposon mutagenesis and genome sequencing unveiled the caryoynencin (cay) biosynthesis gene cluster in the plant pathogen B. caryophylli, and homologous gene clusters were found in various other bacteria by comparative genomics. Gene inactivation and phylogenetic analyses revealed that novel desaturase/acetylenase genes mediate bacterial polyyne assembly. A cytochrome P450 monooxygenase is involved in the formation of the allylic alcohol moiety, as evidenced by analysis of a fragile intermediate, which was stabilized by an in situ click reaction. This work not only grants first insight into bacterial polyyne biosynthesis but also demonstrates that the click reaction can be employed to trap fragile polyynes from crude mixtures.*

Polyynes are structurally intriguing molecules with alternating triple and single carbon–carbon bonds and they have attracted considerable interest in organic chemistry because of their unique rod-like architectures and high reactivity.[1] Interestingly, various polyynes have also been found in nature. Often imprecisely referred to as polyacetylenes, these compounds constitute a family of natural products from a variety of organisms, including plants, algae, insects, lichen, fungi, and bacteria.[2] Naturally occurring polyynes often have potent biological activities and are of ecological relevance to the producing organism.[2,3] The unique molecular scaffold of multiple conjugated triple bonds is usually essential for bioactivity, yet these molecules are highly unstable and their characterization poses a major challenge.[4] In fact, numerous synthetic and natural polyynes undergo exothermic crosslinking reactions and many polyyne natural products are even considered to be explosives that need to be handled with caution.

Whereas many polyacetylenes have been characterized, especially from plants, basidiomycetes, and insects,[2,3c,5] polyynes are a rarity among bacterial metabolites. The very few partially characterized bacterial polyynes, such as Sch 31828 (**1**) from *Microbispora* sp.,[4c] the cepacins (**2**) from the human pathogen *Burkholderia cepacia*,[4a] and the antibacterial compound caryoynencin (**3**) from the carnation pathogen *Burkholderia caryophylli*,[4b] possess a terminal alkyne function (Figure 1 A). Because of their high instability, synthetic approaches to derivatives of **3** were developed to gain more insight into their structure and function, and the results indicate that the intact polyyne, in particular the terminal triple bond, seems to be crucial for bioactivity.[6] Notably, caryoynencin (**3**) is the only known bacterial polyyne to possess four conjugated triple bonds; it thus represents an intriguing molecule for studying the biogenetic origin of this rare pharmacophore. In natural products, alkynes are typically formed by stepwise O_2-dependent dehydrogenation of aliphatic chains in a process catalyzed by di-iron enzymes named desaturases (or acetylenases for triple bond formation; dual functions have been observed).[7] Compared to the fairly high number of known polyynes, there have been only few reports to date on plant-derived desaturases that mediate the progressive desaturation of fatty acids into polyacety-

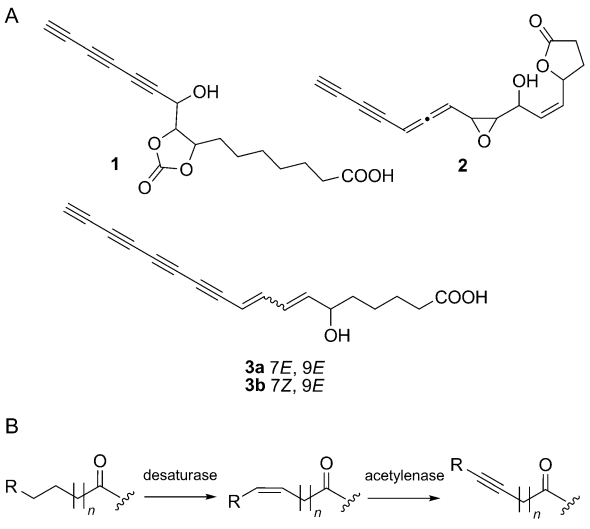

Figure 1. A) Structures of selected bacterial polyynes; Sch 31828 (**1**) from *Microbispora* sp., cepacin (**2**) from *Burkholderia cepacia*, and caryoynencin (**3**) from *Burkholderia caryophylli*. B) Sequential desaturation of fatty acids by plant-derived desaturases (acetylenases for triple bonds).

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lenes[2,8] (Figure 1B). A set of desaturases that evolved independently of their plant congeners was shown to be involved in alkyne formation in insects.[5b] In the fungus *Cantharellus formosus*, a bifunctional desaturase as well as an acetylenase, which is phylogenetically distinct from known plant and fungal desaturases, were reported to catalyze alkyne formation.[9] By contrast, the biogenetic origin of polyacetylenes in prokaryotes has remained completely enigmatic. Herein, we report the discovery of the first bacterial gene cluster coding for the biosynthesis of a polyyne, namely caryoynencin in *Burkholderia* spp., and this discovery led to the identification of numerous related clusters in other bacteria. We also show that specialized desaturases and a cytochrome P450 monooxygenase are involved in the formation of the diene-tetrayne compound. Moreover, we describe the application of a chemical trapping approach as a strategy for isolating and characterizing these highly unstable molecules directly from the fermentation broth extract.

To identify the genes involved in the biosynthesis of the unusual polyyne metabolite, we performed a random transposon mutagenesis of the caryoynencin producer *Burkholderia caryophylli* (DSM50341). We hypothesized that disruption of caryoynencin biosynthesis genes would affect the phenotype. First, a null mutant could be detected by using an antibacterial plate assay with *B. subtilis* as a sensitive test strain (Figure 2A, trace I). Second, we reasoned that a visual mutant screening would be viable since *B. caryophylli* produces high amounts of polyynes even on agar plates and these compounds tend to polymerize to form dark brown polymerization products,[4b,6] thus resulting in brownish colonies. In the mutant library, we noticed one clone that displayed white colonies instead of the usual brownish phenotype (Figure 2B). Analysis of secondary metabolite formation and a negative *B. subtilis* plate assay revealed that caryoynencin production was fully abolished in the mutant strain (Figure 2A, trace II), thus implying that a gene putatively involved in polyyne biosynthesis had been disrupted by the transposon. To locate its insertion site, we performed transposon rescue. Sequence mapping of the transposon insertion site revealed integration within a gene that shows homology to Δ^9 desaturases (Figure 3, clade II), which catalyze the oxidation of stearic acid to oleic acid, for example, and are involved in the formation of polyunsaturated fatty acids.[2,10] A Blast homology search pointed to orthologous genes in the genomes of the plant pathogens *Burkholderia gladioli* BSR3[11] and *Burkholderia gladioli* pv. *cocovenenans*.[12] To investigate whether *B. gladioli* is also able to produce polyacetylenes, we analyzed its secondary metabolome by LC–MS. Indeed, after optimization of the media and culture conditions, we could show for the first time that *B. gladioli* produces a polyyne derivative as indicated by the characteristic UV spectrum. LC–HRESI-MS, MS/MS, and co-elution experiments with an authentic standard unequivocally identified the polyacetylene as caryoynencin (see the Supporting Information). With two caryoynencin producers in hand, we aimed at comparative genomics analyses. Therefore, we submitted genomic DNA from *Burkholderia caryophylli* for 454 shotgun sequencing, and by matching the genome

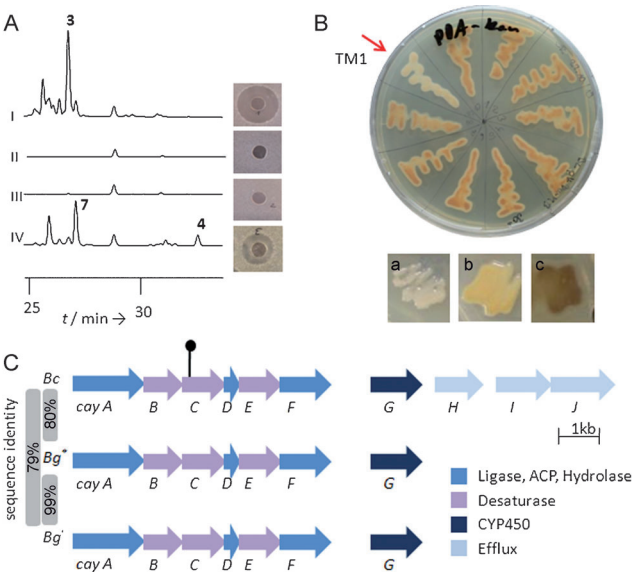

Figure 2. A) HPLC analyses (PDA 200–800 nm) of microbial cultures and bioactivity of the culture extracts against *Bacillus subtilis*: I) *B. caryophylli* wild type, II) *B. caryophylli* TM1, III) *B. caryophylli ΔcayB/C*, IV) *B. caryophylli ΔcayG*. B) Transposon mutagenesis and targeted gene knockouts. The plate shows the results of in vivo mutagenesis of *B. caryophylli*, including a white phenotype mutant (TM1). Shown below are the phenotypes of targeted knockouts of *cayB/C* (a, white phenotype) and *cayG* (b, yellow phenotype) compared to the wild type (c). C) The organization of the caryoynencin biosynthesis gene cluster in *B. caryophylli* (*Bc*) and comparative genomics among *Burkholderia* spp. (amino acid sequence similarities computed by BlastP). Bg = *Burkholderia gladioli* pv. *cocovenenans*, Bg′ = *Burkholderia gladioli* BSR3. The black pin indicates the site of transposon integration.

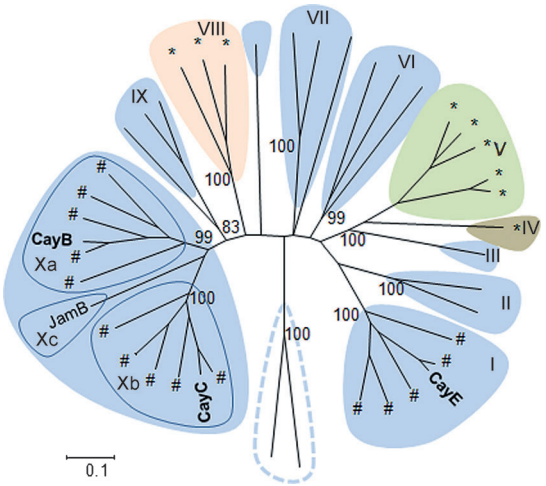

Figure 3. A phylogenetic tree (neighbor joining algorithm) of desaturases/acetylenases from plants (green), fungi (brown), insects (orange), and bacteria (blue). Caryoynencin gene products are highlighted in bold, triple-bond introducing enzymes from studied polyyne pathways are marked with an asterisk (*), and desaturases from cryptic bacterial polyyne gene clusters are indicated with a hash (#). The outgroup is marked with a dashed line (for a detailed tree, see the Supporting Information).

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data, we found that the genes that putatively code for the desaturases are located within a conserved gene cluster. Besides three desaturase genes (*cayB, cayC,* and *cayE*), we identified genes encoding a fatty acyl-AMP ligase (*cayA*), a hydrolase (*cayF*), and a cytochrome P450 monooxygenase (*cayG*). Additional genes for transport and efflux (*cayHIJ*) were found downstream of the biosynthesis genes (Figure 2C, and Table S1 and Figure S3 in the Supporting Information).

A phylogenetic analysis including the deduced *cay* gene products and characterized and tentative desaturases (acetylenases) from plant, insect, and microbial pathways revealed that the bacterial enzymes are clearly distinct from their eukaryotic counterparts, thus suggesting an independent evolution (Figure 3). Notably, CayB and CayC form a separate clade with the closest homologue JamB, which has been suggested to mediate alkyne formation in the jamaicamide pathway of *Lyngbya majuscula*.[13] CayB and CayC are thus the best candidates for enzymes involved in triple-bond formation. To confirm their involvement in caryoynencin biosynthesis, we created a targeted knockout of the predicted desaturase (acetylenase) genes *cayB* and *cayC* through insertion of a kanamycin resistance cassette. Metabolic profiling of the resultant mutant strain revealed that polyyne formation was fully abolished (Figure 2A, trace III). Furthermore, the mutant completely lost its antibacterial potency, another indication for loss of the polyyne residue. These findings indicate that the disrupted genes are crucial for alkyne formation. Given that no enrichment of unusual unsaturated fatty acid precursors could be detected, it appears that the endogenous fatty acid pool is tapped for the formation of the polyynes. Furthermore, since a ligase (CayA), an acyl carrier protein (ACP, CayD) and a hydrolase (CayF) are encoded in the *cay* gene cluster, the fatty acids are likely ACP-bound during desaturation, thus preventing the detection of intermediates in the broth.

In order to provide additional functional evidence for the identity of the genes belonging to the *cay* biosynthesis gene cluster, we inactivated *cayG*, the putative cytochrome P450 monooxygenase gene, through insertional disruption. HPLC-DAD–HRESI-MS analysis of the *ΔcayG* mutant extract showed the accumulation of two metabolites, with $m/z = 265.1238$ $[M-H]^-$ (calcd. for $C_{18}H_{17}O_2$: 265.1234; **4**) and $m/z = 285.1499$ $[M-H]^-$ (calcd. for $C_{18}H_{21}O_3$: 285.1496; **7**) as shown in Figure 2A, trace IV. Both compounds display a shifted UV spectrum, which indicates a change in the degree of unsaturation of the fatty acid backbone.

To fully elucidate their structures, we attempted the challenging isolation of the fragile polyyne intermediates. Typically, the inherent instability of polyynes with a terminal alkyne moiety prevents their isolation and purification. To overcome this hurdle, we intended to chemically trap the polyynes. Derivatization of the terminal alkyne by Cu^I-catalyzed azide–alkyne cycloaddition (CuAAC, "click reaction")[14] appeared to be most promising. As a proof of principle, we selectively trapped caryoynencin from the crude fermentation extract of the wild type *Burkholderia caryophylli*. Using a protocol that selectively targets terminal alkynes,[15] we treated the crude mixture with benzyl azide and the Cu^I catalyst. LC–MS analysis of the mixture revealed that caryoynencin (**3**) disappeared with formation of a new compound (**5b**) that matches the expected mass of the proposed triazole (Figure 4). By performing preparative HPLC, we were able to purify **5b**, which was sufficiently stable to be fully characterized. 1D and 2D NMR spectra were in full agreement with the proposed structure. Moreover, we succeeded in elucidating the absolute configuration of the hydroxymethine

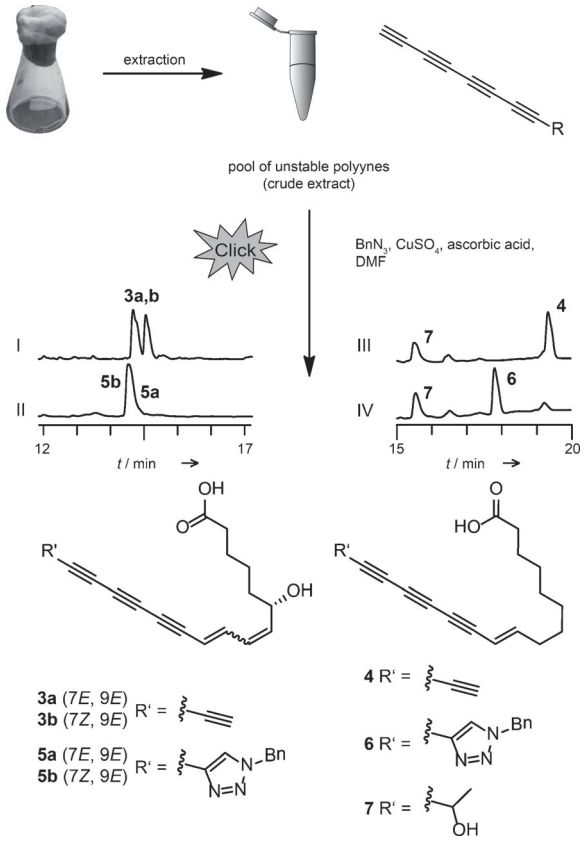

Figure 4. Chemical trapping of the tetraynes by an in situ click reaction with benzyl azide. Shown are a schematic representation of the general approach and HPLC profiles of the culture extracts (PDA 200–800 nm): I) *B. caryophylli* wild type, II) *B. caryophylli* wild type subsequent to click reaction, III) *B. caryophylli ΔcayG*, and IV) *B. caryophylli ΔcayG* subsequent to click reaction. Shown below are the structures of **7** and triazoles **5a**,**b** and **6**, and the inferred structures of **3a**,**b** and **4**. DMF = *N*,*N*-dimethylformamide.

group (6*S*) by using the Mosher method (see the Supporting Information).

Since the polyyne click method proved to be viable for stabilizing the polyyne residue, we used the same approach for the *ΔcayG* mutant. The intermediate (**4**) was derivatized in situ, which led to the formation of a new compound (**6**) at the expense of **4**. The triazole was isolated, purified, and fully characterized by NMR spectroscopy. Compared to **5b**, the lack of two olefinic methine signals pointed to the reduction of a double bond. Furthermore, the ^1H NMR data supported the idea that compound **6** is not hydroxylated. In agreement with the HRESI-MS and 2D NMR data for **6**, metabolite **4** features an alkyl chain instead of the allylic alcohol motif, while the tetraalkyne-ene residue remained congruent

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(Figure 4). By contrast, compound **7** was not susceptible to derivatization by benzyl azide, which indicates the absence of a terminal alkyne moiety. By using preparative HPLC, we achieved the isolation of trace amounts of this compound, which finally allowed its structural elucidation. ^{13}C NMR analysis clearly revealed that the terminal triple bond was missing. Instead, the presence of a methyl carbon atom and an additional methine carbon atom was noticed; a chemical shift of $\delta = 56.7$ ppm for C-17 pointed to proximity to an oxygen atom. H,H-COSY coupling of the methyl protons H-18 and H-17 and HMBC coupling of H-18 and H-17 with C-16 and C-15, respectively, finally established the structure of **7** (Figure 4). The discovery of an alcohol residue in lieu of a triple bond can be rationalized by the mechanistic link between hydroxylation and desaturation.[16] Alcohol **7** thus likely represents a shunt product of terminal alkyne formation.

It is remarkable that the deletion of the cytochrome P450 enzyme (CayG) results in the accumulation of compounds lacking not only the hydroxy group but also one double bond. To test whether CayG could belong to the family of CYP450 desaturases, we performed a phylogenetic analysis including representatives from secondary metabolite pathways (see the Supporting Information). CayG is most closely related to polyketide tailoring enzymes, in particular to the multifunctional CYP450 monooxygenase AurH[17] from the aureothin pathway, and it is only remotely related to studied CYP450 desaturases from plants and yeasts. However, in geldanamycin biosynthesis, deletion of a CYP450 monooxygenase gene (*gdmP*) leads to the accumulation of a dihydro variant of geldanamycin, which suggests that GdmP acts as a desaturase.[18] By analogy, CayG could introduce the double bond by desaturation or through a hydroxylation–dehydration sequence (potentially leading to the observed *E*/*Z*-isomers), followed by hydroxylation at the allylic position (Scheme S1). This model is in agreement with reported cases of competing desaturation and hydroxylation by CYP450 enzymes.[19]

In terms of bioactivity, it is surprising that **5b**, in which the terminal alkyne was converted into a triazole moiety, is still active in antibacterial assays (minimum inhibitory concentration (MIC): *B. subtilis* 3.12 $\mu g\,mL^{-1}$, 7.6 μM; MRSA 12.5 $\mu g\,mL^{-1}$, 30.3 μM). This finding contrasts with earlier studies on structure–activity relationships, carried out with synthetic model compounds, which suggested that the terminal alkyne is indispensable for the antibacterial activity.[6]

The discovery of the novel polyyne biosynthesis gene cluster in three plant-pathogenic *Burkholderia* spp. raises the question of how widespread related gene clusters are amongst other bacteria. In a broader genome-mining analysis, we found six orthologous gene clusters in other bacterial species from the genera *Burkholderia*, *Pseudomonas*, *Collimonas*, and *Mycobacterium* (Figure S3). This result is particularly interesting since many Burkholderiales and Pseudomonales live in close association with eukaryotes and their metabolic capabilities are often essential for the eukaryotic partner.[20] The potent antibacterial activity of **3** and the specific occurrence of polyyne gene clusters among these genera may suggest an ecological function for the producing organisms. In plants and insects, polyacetylenes have been proposed as defensive chemicals, since most polyynes possess strong antimicrobial properties.[2,5b] The discovery of the molecular basis of polyyne biosynthesis in bacteria will now help in the identification of further bacterial polyacetylenes by genome mining and may thus lead to a better understanding of their ecological function.

In conclusion, we have identified the first biosynthesis gene cluster coding for a polyyne biosynthetic pathway in bacteria. A crucial role for novel desaturase genes in bacterial polyyne formation was revealed, and these enzymes seem to have evolved independent of the equivalent genes known from plant metabolism. Bacterial polyyne pathways appear to be rare but comparative genome analyses unveiled numerous orthologues in diverse bacteria, and through metabolic profiling, we verified that the important phytopathogen *B. gladioli* produces caryoynencin too. Knowledge of the molecular basis of bacterial polyyne biosynthesis and the discovery of several polyyne biosynthesis gene clusters will greatly aid functional studies and set the basis for the genomics-based discovery of new bacterial polyynes. To tackle the challenging handling of fragile polyynes, we successfully employed an in situ click reaction. Although Huisgen azide-alkyne cycloadditions have been used for the chemical derivatization of biomolecules and their chemical derivatives,[21] this is the first report of the successful trapping of highly reactive polyynes from the crude metabolite mixture of a microbial culture. This generally applicable approach enabled the isolation and full characterization of the polyyne natural products, thus providing spectral data that is otherwise difficult or impossible to retrieve. As a proof of concept, we could rigorously determine the structures of a tetrayne pathway intermediate from a targeted knockout mutant (*ΔcayG*) and elucidate the absolute configuration of caryoynencin. Furthermore, analysis of the metabolites of the mutant revealed that a cytochrome P450 monooxygenase plays a key role in the formation of the allylic alcohol residue of caryoynencin and that the presence of a terminal alkyne residue is not crucial for bioactivity. These results thus not only shed light on bacterial polyyne biosynthesis but also demonstrate the power of combining synthetic methods and genetics to analyze the biosynthesis of highly reactive pathway intermediates.

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