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A Type III Polyketide Synthase Specific for Sporulating Negativicutes is Responsible for Alkylpyrone Biosynthesis

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Genomic analyses indicate that anaerobic bacteria represent a neglected source of natural products. Whereas a limited number of polyketides have been reported from anaerobes, products of type III polyketide synthases (PKSs) have remained unknown. We found a highly conserved biosynthetic gene cluster (BGC) comprising genes putatively encoding a type III PKS and a methyltransferase in genomes of the Negativicutes, strictly anaerobic, diderm bacteria. By *in vivo* and *in vitro* expression of a type III PKS gene, *dquA* from the oak-associated *Dendrosporobacter quercicolus* in *E. coli* we show production of long-chain alkylpyrones. Intriguingly, this BGC is specific for sporulating Sporomusaceae but absent in related Negativicutes that do not sporulate, thus suggesting a physiological role.

Introduction

A large number of ecologically important aromatic natural products are produced from type III PKSs. These monofunctional enzymes catalyze the condensation of coenzyme A (CoA) activated starter units with (methyl)malonyl-CoA (MMCoA/MCoA) extender units by decarboxylative Claisen condensations to produce a variety of cyclized secondary metabolites.[1] Plant-derived type III PKSs mainly produce stilbenes, chalcones and flavonoids, whereas pyrone- and resorcinol-producing type III PKSs are found in fungi.[2] Two decades ago, type III PKSs have been firstly described in bacteria.[3] A variety of bacterial type III PKSs have since been reported to produce phenolic lipids that contribute to key microbial membrane-associated processes like resistance to antibiotics,[4] electron transport within the respiratory chain,[5] or cyst formation.[6] The presence of type III PKSs was found to be distributed over a diverse set of bacteria, ranging from *Streptomyces* spp.,[4,7] *Mycobacterium* spp.,[5,8] *Bacillus subtilis*,[9] and *Azotobacter vinelandii*.[6,10] We have extensively scrutinized the existence of type III PKSs in anaerobic bacteria on a genomic level with a putative candidate gene found in *Heliobacterium modesticaldum*.[11] However, to this date, no type III PKS from anaerobes has been characterized. Here we report the first characterization of a type III PKS from anaerobic bacteria. The exclusive abundance of this BGC within the Sporomusaceae suggests a biological role of type III PKS derived alkylpyrones for these underexplored anaerobic bacteria.

Results and Discussion

A type III PKS locus is highly conserved in the Sporomusaceae

Negativicutes, strict anaerobic diderm bacteria within the otherwise monoderm Firmicutes have been underexplored regarding their potential of producing specialized metabolites.[12] By investigating the biosynthetic potential of the oak-associated *Dendrosporobacter quercicolus* using the Anti-SMASH platform,[13] we found a putative type III PKS gene called *dquA* (Figures 1A and S1). BLASTp (pdb) using the predicted gene product DquA as query, revealed many putative type III PKS gene products from strains of the Sporomusaceae family (Table S1). This rich abundance could not be related to any reported natural product. DquA was therefore used for BLASTp (Swiss-Prot) analysis of characterized enzymes, which revealed the *B. subtilis* type III PKS BpsA as closest result (44%, Tables S1 and S6). BpsA is reported to produce a mixture of long-chain alkylpyrones and -resorcinols.[9] A downstream gene product BpsB was furthermore found to methylate these phenolic lipids at their free hydroxyl groups.[9] Accordingly, a putative methyltransferase gene, *dquB*, could be observed downstream of *dquA* (Figures 1A and S1, Tables S1–2). Furthermore, all *dquA* orthologs found in strains of the Sporomusaceae are encoded upstream of a *dquB*-orthologous gene (Figures S2–3). A phylogenetic analysis of the BpsA/DquA orthologs suggested a close relationship between putative type III PKSs of the Sporomusaceae (Negativicutes) and BpsA orthologs from strains belonging to the Firmicutes (Figures 1B and S4). Branching in the phylogenetic tree reflects bacterial relationships between these groups of organisms rather than a close structural similarity to characterized bacterial, fungal, and plant type III PKSs (Figur-

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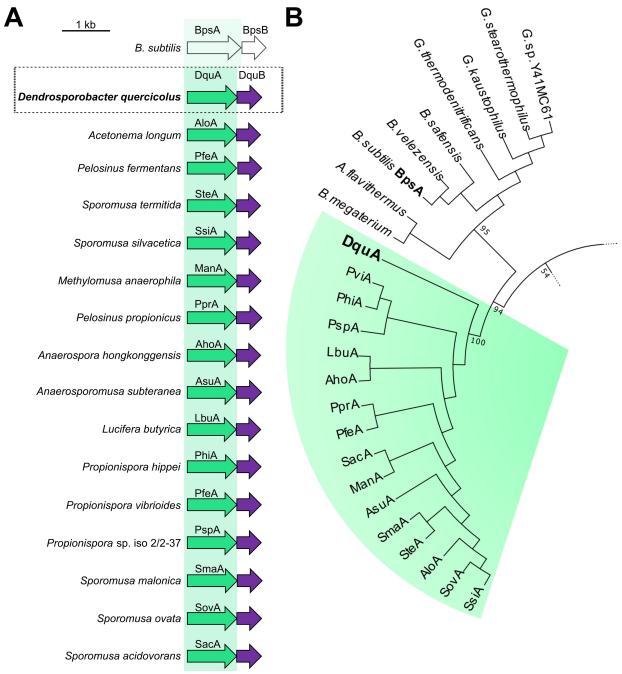

Figure 1. Bioinformatic analysis of the *dquA-B* gene locus. A) A two-gene locus encoding a BpsA/BpsB-like type III PKS/ICMT-like methyltransferase is highly conserved in members of the diderm family Sporomusaceae. B) Phylogenetic tree based on type III PKSs shows branching in accordance with strain-level phylogeny. Type III PKSs from Sporomusaceae branch together and share the BpsA containing Bacillaceae branch as closest group.

es 1B and S4). Having shown the high level of conservation of DquA-like proteins and their similarity to BpsA, we concluded that DquA might produce long-chain alkylpyrones and/or -resorcinols. To gain more insight into the potential function, we generated a protein sequence alignment using BpsA,[9] *Mycobacterium tuberculosis* (*Mt*) PKS11,[14] and the *"Candidatus* Entotheonella" CepA[15] (Figure S5).

Key residues reported to being responsible for catalysis (Cys141, His273, Asn306) as well as binding of long-chain fatty-acyl-CoA starter units are conserved in DquA (Figure S5). Bacterial type III PKSs producing phenolic lipids were shown to possess a long hydrophobic tunnel, which enables binding of the long lipid starter substrates.[14] A 3D model of DquA using the SWISS-MODEL tool[16] resulted in a best match with *Mt* PKS11.[17] Key catalytic residues and those reported to facilitate binding of activated long-chain fatty acids in *Mt* PKS11 aligned well with respective residues in DquA (Phe191, Trp226) (Figures S6-7). *Mt* PKS11 predominantly produces triketide pyrones by extending myristoyl- or palmitoyl-CoA and MCoA/MMCoA.[14] Based on the similarity to PKS11, we expected DquA to produce mainly triketide pyrones. DquA likely incorporates middle to long-chain starter units, such as activated palmitic or myristic acid, which have been detected in the long hydrophobic cavity of *Mt* PKS11 crystals.[14]

Heterologous expression of *dquA* in *E. coli* facilitates long-chain pyrone production

Guided by the bioinformatic analysis, we aimed at uncovering predicted products of the type III PKS. However, hydrophobic alkylpyrones or catechols could not be detected by HPLC-HRMS monitoring of various growing *D. quercicolus* cultures. Thus, we set out to heterologously express the native *dquA* gene (n-*dquA*) in *E. coli* BL21 (DE3) in a pET28a-based system. Compared to the vector control, *E. coli* expressing n-*dquA* produced new hydrophobic compounds with UV absorption maxima of 280–285 nm (Figure 2A), which were potentially metabolites derived from DquA. However, the production of compounds was too low to obtain sufficient amounts for further isolation and structure elucidation. We therefore cloned a codon-optimized synthetic variant of *dquA* and expressed it in analogous fashion (Table S8). The profile of *E. coli-dquA* showed increased production of the new hydrophobic compounds compared to the profile of *E. coli*-n-*dquA* (Figure 2A). A 5 L culture of *E. coli* expressing *dquA* was extracted, followed by purification with silica gel column chromatography and reverse-phase HPLC. During the course of preparative HPLC based purification, we recognized that the peak at 21.5 min in Figure 2A corresponds to two compounds, **1** and **2** (Figure S8). We applied a sequential set of preparative methods to separate and purify **1** and **2**. To this end, a pre-purification step using an open silica column, was followed by preparative reverse-phase HPLC using a NUCLEOSIL C18 column.

Finally, repeated preparative HPLC purification (NUCLEODUR C18 HTec column) enabled the separation of the two compounds, yielding pure **1** (5 mg) and **2** (6 mg), which were subjected to HRMS/MS and NMR spectroscopic analysis. Compound **1** (*m/z* of 295.2265 $[M+H]^+$) has a sum formula of $C_{18}H_{30}O_3$ (Figure S9). HRMS/MS analysis showed continuous losses of 14 atomic mass units (amu), suggesting a carbon chain (C13) attached to an aromatic system containing three oxygens (Figure S10). The chain of methylene groups could be observed with ^{1}H, ^{13}C, DEPT135, HSQC, and ^{1}H-^{1}H COSY NMR spectra (Figures S11-16, Table S10). HMBC analysis suggested a pyrone ring based on correlations between H2 (δ_H 5.61) to C1 (δ_C 168.2), C2 (δ_C 89.9) and C3 (δ_C 172.5), H4 (δ_H 6.00) to C2, C3, and C5 (δ_C 167.5), and H6 (δ_H 2.50) to C5 (Figures 2B and S17). Two sets of carbon connections C6-C8 and C16-C18 were determined by ^{1}H-^{1}H COSY correlations (Figures 2B, S11–12 and S15). Based on this analysis, **1** was elucidated as an alpha-pyrone decorated with a saturated C13-chain (Figures 2B and S11). This compound has been previously reported as product of several bacterial type III PKSs, including BpsA and CepA.[9,15] Compound **2** shows a similar UV spectrum and HRMS/MS fragmentation patterns to those of **1**. An observed ion of *m/z* 321.2424 $[M+H]^+$ detected by HRMS indicated a sum formula of $C_{20}H_{32}O_3$ (Figure S19). NMR spectral data analysis of **2** revealed equivalent correlations to **1**, confirming it to share the same pyrone structure, attached to a long carbon chain (Figures S18–26, Table S11). However, in contrast to the saturated chain in **1**, **2** possesses an additional double bond between C13 (δ_C 129.7) and C14 (δ_C 130.0), which are connected with C12 and C15,

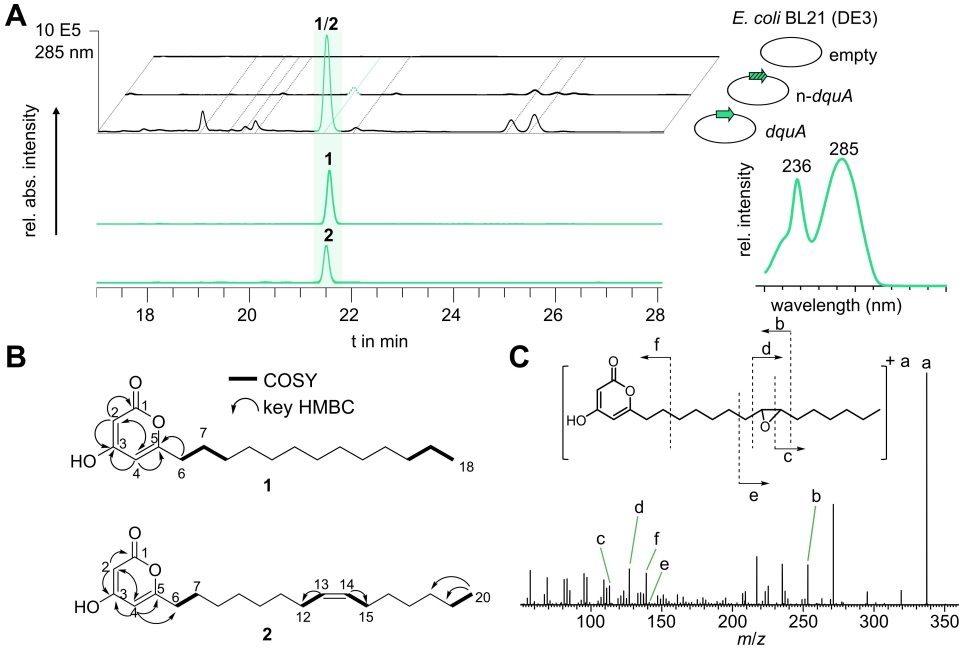

Figure 2. Heterologous production of DquA *in vivo* and purification of compounds **1** and **2**. A) New hydrophobic compounds are produced by *E. coli* expressing *dquA*. B) Compounds **1** and **2** were elucidated as alkylpyrones by NMR. C) Determination of the double-bond position in **2** by chemical modification and MS/MS analysis.

deduced from HMBC correlations, respectively (Figures S18–20 and S26). To unequivocally locate the double bond position, we derivatized **2** with *meta*-chloroperoxybenzoic acid (mCPBA) to give an epoxide, followed by HRMS/MS analysis. The double bond position was thus inferred to be located between C13 and C14 (Figures 2B–C, S20, and S27). Furthermore, judging from the differences between *cis* and *trans* olefinic proton signals in ^{1}H NMR, we concluded **2** is *cis*-configurated at position 9 (Figure S21).[18] The structures of minor congeners **3–6** were tentatively assigned by HPLC, HRMS and HRMS/MS analyses (Figures 3A and S28-31).

By analogy to BpsA and CepA, DquA would produce **1** using the C14 starter fatty acyl-CoA (myristoyl-CoA), which is extended with two MCoAs, followed by lactonization to yield the pyrone ring.[9,15] In addition to myristoyl-CoA, DquA is able to incorporate a range of other CoA-activated fatty acids. Palmitoleic acid (9*Z*, C16:1), which accounts for a major proportion of fatty acid abundance shown for *E. coli*,[19] would

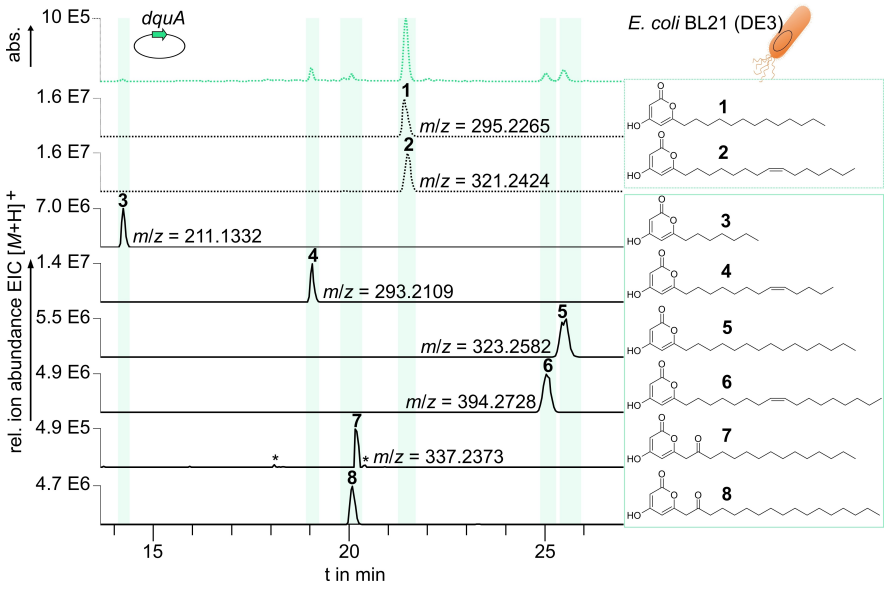

Figure 3. Characterization of further alkylpyrones produced by *E. coli* expressing *dquA*. Compounds **1–8** were produced by *E. coli-dquA*.

be the starter unit to produce **2**. Incorporation of octanoic (C8:0), myristoleic (C14:1, 9*Z*), palmitic (C16:0), and oleic acid (C18:1, 9*Z*) would give rise to **3**, **4**, **5**, and **6**, respectively (Scheme S1).

Two compounds, **7** and **8**, were detected for which HRMS analysis suggests sum formulas of $C_{20}H_{32}O_4$ (*m/z* 337.2373 $[M+H]^+$, 335.2228 $[M\text{-}H]^-$) and $C_{22}H_{36}O_4$ (*m/z* 365.2686 $[M+H]^+$, 363.2541 $[M\text{-}H]^-$), respectively (Figures S32-33). Compared to **1**–**6**, these two pyrones contain an additional oxygen, which could be explained by incorporation of an additional MCoA unit (Schemes S1-2). The HRMS/MS fragmentation pattern of a tetraketide pyrone produced by CepA[15] shows a highly similar pattern as that of **8** (Figure S33). We therefore propose the structure of **8** to be a tetraketide pyrone, which may be biosynthesized by extending palmitic acid (C16:0) with three units MCoA (Scheme S1). Accordingly, **7** represents a tetraketide, which may be biosynthesized from myristic acid (C14:0), extended with three MCoAs (Figure S32, Scheme S1).

Type III PKSs share a highly conserved catalytic Cys-His-Asn triad, facilitating decarboxylative extension of starter substrate with extender units.[1] Introducing amino acid exchanges of each of these residues to Val in DquA by point mutation of *dquA* completely abolished production of both triketide (**1**–**6**) and tetraketide (**7**, **8**) pyrones (Figure S34). A long hydrophobic tunnel shown to facilitate starter substrate binding in *Mt* PKS11 is assumed to be present in DquA as well.[14] Whereas DquA and *Mt* PKS11 produce pyrones, other examples are shown to produce exclusively either resorcinols (ArsB),[10] pyrones (ArsC)[10] or a mixture (BspA,[9] CepA[15]). Trp281 in ArsB (Gly284 in ArsC) has been linked to determine resorcinol- versus pyrone-formation.[10] DquA possesses a Trp at this position, assuming to produce resorcinols (Figure S5). However, our experimental data from *in vivo* and *in vitro* expression in *E. coli* show exclusive production of pyrones.

A putative methyltransferase is encoded by *dquB*. This locus is conserved among Sporomusaceae and always encoded downstream of *dquA* orthologs (Figures 1A and S1-3). Comparison to BpsB and characterized enzymes by sequence alignment assumed DquB to belong to the isoprenylcysteine carboxyl methyltransferase (ICMT) family (Figures S35-36). BpsB has been shown to *O*-methylate phenolic lipids produced from BpsA.[9] We assumed that DquB is a membrane protein responsible for methylation of pyrones produced by DquA. We co-expressed *dquA* together with the codon-optimized downstream gene *dquB* (Table S8) in *E. coli* BL21 (DE3) and analyzed crude extracts for the presence of methylated alkyl pyrones **1a**–**8a**. However, no methylated products could be observed (Figure S37). The production of pyrones **1**–**8** in *E. coli-dquA/dquB* was not impaired (Figure S37). The phylogenetic distance between *E. coli* and strains of the Sporomusaceae could account for the non-functional expression of *dquB*, which may have special requirements for membrane compositions necessary for correct protein folding. SDS-PAGE analysis of *E. coli-dquA/dquB* indeed revealed production of DquA but not DquB (Figure S38). Still, the high level of conservation of DquB orthologs suggests a similar role as BpsB in *B. subtilis*.[9]

DquA recognizes various fatty-acyl-CoA starter substrates

Type III PKSs can elongate either CoA-activated starter substrate or employ an additional acyl carrier protein (ACP). A key position within type III PKSs has been shown to indicate loading with either CoA- (Ala) or ACP-bound (Arg/Lys) substrates.[1,20] The corresponding residue Lys278 (Figure S5) suggests DquA to be dependent on a further ACP delivering activated substrates. As heterologous expression of *dquA* alone succeeded in the production of alkylpyrones, the substrates could, in theory, be provided as fatty-acyl-ACPs produced by *E. coli*. To test if DquA can accept CoA-activated substrates, we heterologously produced and purified His_6-tagged DquA. Due to poor yields of soluble DquA, we co-expressed with the GroES/EL chaperonin genes to assist DquA folding and successfully improved the solubility of His_6-DquA (Figure S39). As the most abundant pyrones were derived potentially from saturated fatty acids with 8, 14 and 16 carbon-chains, we focused on these starter substrates in *in vitro* experiments using octanoyl-, myristoyl-, and palmitoyl-CoA, respectively. Incubation of DquA with either of these substrates together with equimolar amounts of MCoA yielded expected products **3**, **1** and **5**, which were not detected in reactions without enzyme (Figure S40). These results show that DquA can produce alkylpyrones independent of additional ACPs.

Orthologs of DquA are functionally equivalent and specific for spore-forming Negativicutes

Since the *dquA*/*dquB* locus is highly conserved among the Sporomusaceae, we wondered whether the orthologs are functionally equivalent. A sequence alignment with all type III PKS sequences encoded by these loci showed a high similarity and conservation of catalytic residues as well as residues putatively involved in substrate specificity (Figure S41). A selection of putative type III PKS encoding genes from five strains of the Sporomusaceae, namely *Acetonema longum*, *Pelosinus fermentans*, *Sporomusa termitida*, *Sporomusa silvacetica*, and *Methylomusa anaerophila* were chosen, and their codon-optimized type III PKS genes were heterologously expressed using the pET28a vector in *E. coli* BL21 (DE3). Expression of each of the five genes led to production of triketide pyrones **1**–**6** (Figures 4 and S42). The tetraketides **7** (only trace amounts for *S. silvacetica* and *M. anaerophila*) and **8** were produced, too (Figure S43).

The high conservation suggests that alkylpyrones play an important role in Sporomusaceae. Related compounds have been implicated with changing membrane properties.[4,6,7b] For example, type III PKS derived phenolic lipids including long-chain alkylpyrones make up a majority in *A. vinelandii* cysts and mutants lacking the ability to produce these compounds failed in forming cysts.[6] A similar role has been suggested for BpsA/BpsB-derived phenolic lipids in *B. subtilis*.[9] In order to get more hints towards a potential role of these alkylpyrones, we scrutinized further Negativicutes genomes for the presence of the *dquA*/*dquB* locus. We found that orthologs of *dquA*/*dquB* are

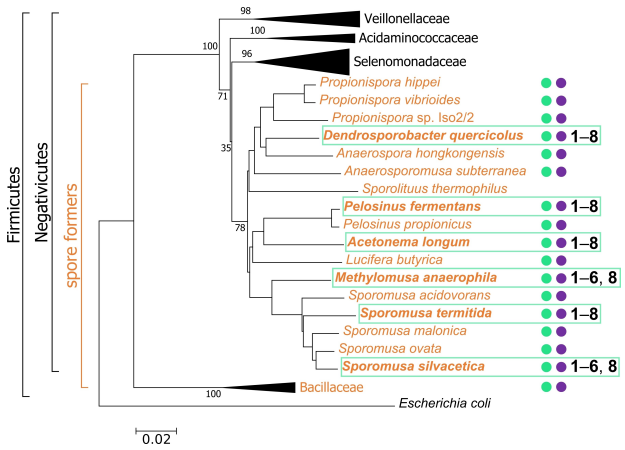

Figure 4. Phylogenetic analysis of selected Firmicutes. Spore-forming strains are depicted in orange. The *dquA-dquB*-like BGC is highly conserved in spore forming Gram-positive (monoderm) Bacillaceae and Gram-negative (diderm) Sporomusaceae but not in non-sporulating Gram-negative diderms. Phylogenetic tree based on 16S rDNA genes. Heterologous expression of *dquA* orthologs from highlighted strains in *E. coli* led to production of alkylpyrones. Strains to encode orthologs of DquA and DquB are labeled with green (type III PKS) and purple (putative ICMT) dots.

missing in non-sporulating families of the Negativicutes, namely Veillonellaceae, Acidaminococcaceae, and Selenomonadaceae, but are highly specific for the sporulating Sporomusaceae (Figures 4 and S44). Furthermore, the locus is conserved in the sporulating monoderm Bacillaceae, exemplified by *bpsA*/*bpsB* (Figures 4 and S44). Thus, the alkylpyrones described in this study may play a role that is specific for sporulating Negativicutes, which is dispensable for non-sporulating Negativicutes.

Conclusion

The Negativicutes are strict anaerobic diderm bacteria, which have been vastly neglected regarding their potential of producing secondary metabolites. Only two examples, naphthalecin from *Sporotalea colonica*,[12a] and the dendrubins from *D. quercicolus*[12b] have been reported to this date. In this study we uncovered the potential of these bacteria to produce long-chain alkylpyrones. Related compounds are implicated in electron transport in *Mycobacterium tuberculosis*,[5] aid in cyst formation in *A. vinelandii*,[6] and confer resistance to cell-wall targeting antibiotics in *Streptomyces griseus*,[4] implying a common trait of influencing membrane properties. BpsA/BpsB-derived lipids may play a role in membrane-modifying processes in *B. subtilis*.[9] It is remarkable that the potential for alkylpyrone production is highly conserved in Sporomusaceae, the only sporulating family of the Negativicutes, but completely absent in non-sporulating Negativicutes. Our findings suggest a specialized role of lipid pyrones in spore-forming Negativicutes, potentially through influencing membrane processes. Moreover, DquA was identified as a new member of the growing family of bacterial type III PKSs.[4–9,14–15,21] DquA is an alkylpyrone synthase, which elongates long-chain fatty acyl-CoA starters to produce tri- and tetraketide pyrones. We report the first functional characterization of a bacterial type III PKS from anaerobes, thereby expanding the natural product repertoire of this vastly untapped source of new secondary metabolites.

Experimental Section

For experimental details, see the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: heterologous expression · natural products · negativicutes · polyketides · type III PKS

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