

Antifungal activity improved by coproduction of cyclodextrins and anabaenolysins in Cyanobacteria

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Cyclodextrins are cyclic oligosaccharides widely used in the pharmaceutical industry to improve drug delivery and to increase the solubility of hydrophobic compounds. Anabaenolysins are lipopeptides produced by cyanobacteria with potent lytic activity in cholesterol-containing membranes. Here, we identified the 23- to 24-kb gene clusters responsible for the production of the lipopeptide anabaenolysin. The hybrid nonribosomal peptide synthetase and polyketide synthase biosynthetic gene cluster is encoded in the genomes of three anabaenolysin-producing strains of *Anabaena*. We detected previously unidentified strains producing known anabaenolysins A and B and discovered the production of new variants of anabaenolysins C and D. Bioassays demonstrated that anabaenolysins have weak antifungal activity against *Candida albicans*. Surprisingly, addition of the hydrophilic fraction of the whole-cell extracts increased the antifungal activity of the hydrophobic anabaenolysins. The fraction contained compounds identified by NMR as α -, β -, and γ -cyclodextrins, which undergo acetylation. Cyclodextrins have been used for decades to improve the solubility and bioavailability of many drugs including antifungal compounds. This study shows a natural example of cyclodextrins improving the solubility and efficacy of an antifungal compound in an ancient lineage of photosynthetic bacteria.

natural products | bioactive compounds | hydroxyamino fatty acid | NRPS | PKS

Cyclodextrins are small cyclic oligosaccharides composed of six (α), seven (β), or eight (γ) glucose units (1). Cyclodextrins were discovered in 1891 from *Bacillus amylobacter* and had their chemical structure determined by X-ray crystallography in the 1940s (2). Strains producing higher amounts of cyclodextrins were obtained in the 1970s, thereby enabling pharmaceutical applications (3). Cyclodextrins are capable of forming inclusion complexes with different compounds, thereby improving their solubility, reactivity, and stability, a property that has been widely exploited in the pharmaceutical industry (4–6). Cyclodextrins are used in the pharmaceutical industry to improve drug delivery and the encapsulation of antibiotics for the prevention and treatment of infections (1, 4, 7).

Cyanobacteria are photosynthetic bacteria capable of producing a broad range of bioactive compounds (8, 9). More than 600 peptides or peptidic compounds have been detected in different genera of cyanobacteria (8). Lipopeptides are commonly produced by cyanobacteria and present a broad range of bioactivity, including different types of cytotoxicity, growth, and enzyme inhibition of various organisms (fungi, bacteria, plants, protozoa, microalgae, and viruses) (9). A large number of lipopeptides have been described from cyanobacteria, such as barbamide (10), jamaicamide (11), curacin A (12), lyngbyatoxin (13) hassallidin (14), and puwainaphycins (15). Many lipopeptides are synthesized by large multifunctional enzymes known as polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) (10–13). The lipid structure of lipopeptides is synthesized in a variety of ways. PKS modules directly synthesize the lipophilic part of the curacin A (12), whereas the activation of the fatty acid chain incorporated in jamaicamide

involves an acyl-acyl carrier protein synthetase (JmA) (10). In the hassallidin gene cluster, produced by *Anabaena* sp., the involvement of an acyl-protein synthetase and ligase (HasG), an acyl carrier protein (HasH), and a 3-oxoacyl (acyl-carrier-protein) reductase (HasL) are predicted to be involved in the fatty acid synthesis and incorporation in this nonribosomal glycolipopeptide (14), whereas the synthesis of the hybrid puwainaphycins in *Cylindrospermum alatosporum* involves a fatty-acyl ligase (FAAL) (15).

Anabaenolysins are lipopeptides produced by species of *Anabaena* isolated from the Baltic Sea (16). Our previous study found them to consist of two glycines, a 2-(3-amino-5-oxotetrahydrofuran-2-yl)-2-hydroxyacetic acid and a long (C16–C19) unsaturated α -hydroxy- β -amino carboxylic acid (16). *Anabaena* sp. XPORK15F produces 10 variants of anabaenolysins, which differ in the length of the methylene units and the degree of unsaturation of the hydroxyamino fatty acid. Anabaenolysins may account for up to 0.1% of the dry weight of *Anabaena* strains (16). Anabaenolysins are able to permeabilize mammalian cells in a cholesterol-dependent manner and show more hemolytic potency than do the plant saponin digitonin and the bacterial cyclic peptide surfactin (17). In this study, we report the discovery of the anabaenolysin gene cluster and demonstrate that anabaenolysins exhibit antifungal activity. Furthermore, the anabaenolysins work in synergy with the cyclodextrins produced by the same *Anabaena* strains, resulting in an increased antifungal activity.

Results

Synergistic Antifungal Activity of Cyclodextrins and Anabaenolysins.

We screened 151 cyanobacterial isolates from diverse sources and various genera to detect new producers of the lipopeptide anabaenolysin (SI Appendix, Table S1). We used high-performance liquid

Significance

Cyclodextrins are cyclic oligosaccharides used in the pharmaceutical industry to improve drug delivery. Here, we show that cyclodextrins improve the antifungal activity of the anabaenolysins A and B lipopeptides and that are both produced by the same cyanobacteria. This study identifies the putative biosynthetic gene cluster involved in the synthesis of these unique cyanobacterial lipopeptide anabaenolysins.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. [KP715718](#) (*Anabaena* sp. XPORK1C), [KP715719](#) (*Anabaena* sp. XPORK1D), [KP715720](#) (*Anabaena* sp. XPORK4C), [KP715721](#) (*Anabaena* sp. BECID30), and [KP715722](#) (*Anabaena* sp. XPORK13A)].

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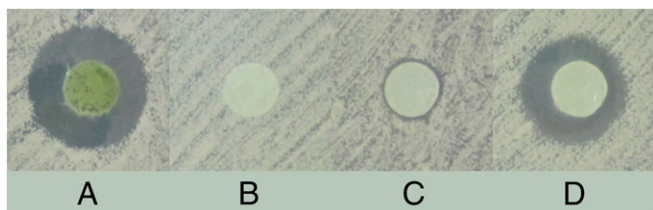


Fig. 1. Disk diffusion assay showing synergistic antifungal activity against *C. albicans* HAMBI 261 of anabaenolysin B and cyclodextrin. Whole-cell methanol extract from *Anabaena* sp. XPORK1D (A); a fraction containing cyclodextrins (B); anabaenolysin B (C); and a fraction containing cyclodextrins and anabaenolysin B combined (D).

chromatography (HPLC) to screen the methanol extracts from the strains, and detection was based on the characteristic UV spectrum of the anabaenolysins resulting from three conjugated double bonds in the hydroxyamino fatty acid (16). We then tested strains containing the characteristic UV spectra against bacteria (data not shown) and fungi to verify the activity of the cell extracts. Extracts containing anabaenolysins exhibited antifungal activity against *Candida albicans* and *Aspergillus* spp. (SI Appendix, Table S2). However, bioassays of isolated anabaenolysin B against *C. albicans* showed a much smaller zone of inhibition (Fig. 1C). Combining isolated anabaenolysin B with a fraction containing hydrophilic compounds from the same *Anabaena* sp. XPORK1D strain restored the stronger antifungal activity (Fig. 1D). Efforts to identify the synergistic compound led to the detection of α -, β -, and γ -cyclodextrins (Fig. 2).

Anabaenolysins. We detected anabaenolysins A–D (Fig. 2) in 11 strains of the genus *Anabaena* isolated from the brackish water of the Baltic Sea (Fig. 3 and SI Appendix, Table S3). Each strain produced roughly similar amounts of only one major anabaenolysin variant. These 11 cyanobacterial strains were isolated from benthic, epiphytic, epilithic, and sediments environments (Fig. 3). The cells contained almost all of the anabaenolysins and very little of the lipopeptides were present in the culture medium (SI Appendix, Fig. S1). A phylogenetic tree based on the 16S rRNA gene indicates that most of the anabaenolysin producers are grouped with other *Anabaena* spp. strains isolated from the Baltic Sea (Fig. 3). However, *Anabaena* sp. XPORK15F is more distantly related to the other anabaenolysins producers (Fig. 3). Comparison of the 16S rRNA gene sequences shows low identity of *Anabaena* sp. XPORK15F and other anabaenolysin producers (94%).

Anabaenolysins A, B, and C were the major variants present (Figs. 2 and 3), but we also detected minor variants (present in quantities of a small percentage of the main variant) of anabaenolysins in these *Anabaena* strains (data not shown). We determined the two previously unidentified variants of anabaenolysins C and D from *Anabaena* sp. XPORK13A (Fig. 2). Abl-C (1.94 mg) was purified from 2.4 g of dried biomass resulting to minimum of 0.8‰ of Abl-C in the *Anabaena* sp. XPORK13A dry cell mass. NMR and amino acid analysis indicated the presence of a glutamine instead of glycine in position 3 (SI Appendix, Figs. S2 and S3 and Table S4). Anabaenolysin D lacks a double bond in the α -hydroxy- β -amino fatty acid (AHFA¹) (Fig. 2). The presence of single bond is shown as smaller mass (by two units) and in the NMR signals δ_C 27.9 ppm and δ_H 1.26 ppm for 17-CH₂, and δ_C 13.6 ppm and δ_H 0.85(t) ppm for 18-CH₃, from anabaenolysin D, which demonstrates that the last double bond is saturated.

Cyclodextrins. We selected *Anabaena* sp. XSPORK2A for the isolation of the synergistic compound. A sharp peak containing different variants of cyclodextrins was isolated by high-performance liquid chromatography (HPLC) and analyzed by HPLC-ion-trap mass spectrometry (ITMS), ultra-performance liquid chromatography-quadrupole time of flight mass spectrometry (UPLC-QTOF), and

nuclear magnetic resonance (NMR) (Fig. 2 and SI Appendix, Figs. S4 and S5 and Table S5). NMR showed that the cyclodextrin fraction contained mainly di- and triAc- α -cyclodextrins as well as mono- and diAc- β -cyclodextrins. The results showed the presence of glucose units, acetyl groups in 6-OH groups, and the absence of free anomeric carbon and proton signals (SI Appendix, Fig. S6 and Table S6). Also, ¹H and ¹³C signals were typical for cyclodextrin but different from linear glucooligosaccharide signals. Accurate masses of sodiated di- and triAc- α -cyclodextrin ions m/z 1079.3291 (Δ 1.7 ppm) and m/z 1121.3383 (Δ 0.4 ppm) provided elemental compositions of C₄₀H₆₄O₃₂Na and C₄₂H₆₆O₃₃Na, respectively, which confirms the cyclodextrin structures. We detected cyclodextrins in all anabaenolysin-producing strains (Table 1). The cyclodextrins had a diverse chemical structures with mono-, di-, and triacetylated- α - as well as non-, mono-, and diacetylated- β -cyclodextrins, formed by six and seven hexoses and decorated with acetyl (Ac) groups (Fig. 2 and Table 1). Most of the strains also produced tetra-Ac- α -cyclodextrins, tri-Ac- β -cyclodextrins, and nonacetylated γ -cyclodextrins. Acetyl groups were always bound to the 6-OH of the glucose unit.

Biosynthetic Genes of Anabaenolysins and Cyclodextrins. Genome mining identified a putative 23-kb anabaenolysin gene cluster (*abl*) in the partial genome of *Anabaena* sp. XSPORK2A (Fig. 4). The putative *abl* gene cluster encodes hybrid NRPS-PKS

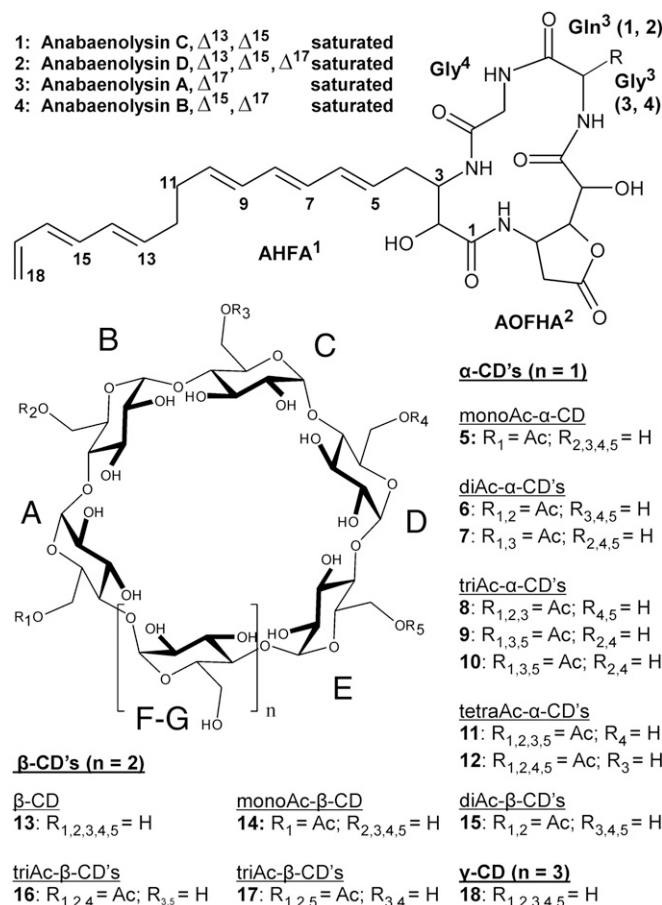


Fig. 2. Structures of anabaenolysins A–D (compounds 1–4) and α -, β -, and γ -cyclodextrins (compounds 5–18). The newly discovered anabaenolysins C and D differ mainly in the glutamine in position 3 from anabaenolysins A and B, which have a glycine molecule in position 3. Ac, acetyl; AHFA¹, (5E,7E,9E)-3-amino-2-hydroxyoctadeca-5,7,9,17-tetraenoic acid; AOFHA², (3-amino-5-oxotetrahydrofuran-2-yl)(hydroxy)acetic acid; CD, cyclodextrin.

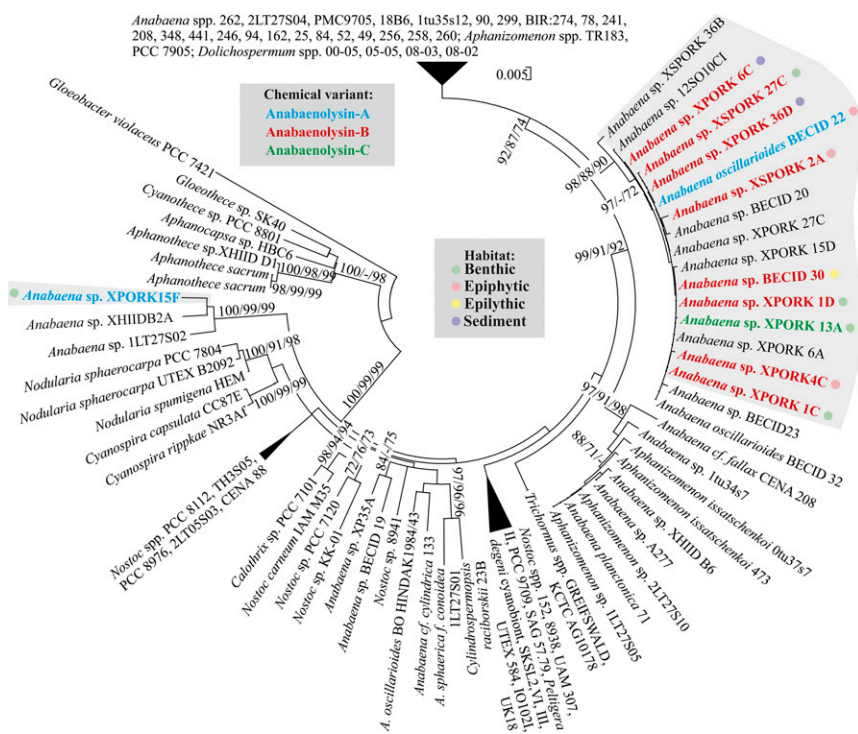


Fig. 3. Phylogenetic tree of cyanobacteria based on 16S rRNA gene sequences. The phylogenetic tree was constructed by using neighbor-joining, maximum parsimony, and maximum likelihood methods using 1,000 bootstraps. Strains producing anabaenolysins are marked according to the main variant produced: blue (anabaenolysin A), red (anabaenolysin B), and green (anabaenolysin C).

enzymes and a fatty acid desaturase (Fig. 4). AblA is a 278-kDa NRPS protein predicted to be involved in the incorporation of the 3-amino-2-hydroxyoctadecanoic acid, a hydroxyl group, an asparagine, and a methyl group into the growing anabaenolysin peptide (Fig. 4 and *SI Appendix, Table S7*). AblB is a 171-kDa PKS and is likely to incorporate a malonyl-CoA precursor unit and another hydroxyl group. AblC is a 35-kDa delta fatty acid desaturase (FADS) predicted to be responsible for the formation of double bonds in the 3-amino-2-hydroxyoctadecanoic acid. AblD and AblE are, respectively, 124-kDa and 157-kDa NRPS proteins that incorporate L-glycine into the peptide intermediate (*SI Appendix, Table S8*), and a thioesterase domain present in the latter catalyzes the cyclization and release of the compound. CamA encodes a 75-kDa protein containing two S-layer homology domains and a glycoside hydrolase domain with an unclear role in anabaenolysin biosynthesis. However, this enzyme may play a role in the formation of cyclodextrins. The anabaenolysin gene cluster is surrounded upstream by proteins unlikely to be involved in

anabaenolysin biosynthesis (e.g., peptidoglycan binding protein, transposase, lysin, and transmembrane sensor-like protein) and downstream by DNA repair and response regulator proteins (*SI Appendix, Table S8*).

The *abl* gene cluster was found in the draft genomes of two additional anabaenolysin producers, *Anabaena* sp. XPORK13A and *Anabaena* sp. XPORK15F (Fig. 4). The only genetic region with lower similarity is the adenylation domain of AblD, which encodes the NRPS that incorporates the amino acid in position 3 of the anabaenolysin gene clusters (*SI Appendix, Table S9*). XSPORK2A produces anabaenolysin B with glycine in position 3, whereas XPORK13A produces a variant with glutamine in this position (Fig. 4). The downstream response regulator proteins from both strains are similar, but the upstream proteins vary, and the XPORK13A strain encodes more proteins with unknown function (*SI Appendix, Table S8*).

Anabaena sp. XPORK13A and XSPORK2A strains are more closely related to one another than to the XPORK15F strain based

Table 1. Relative (%) abundance of the cyclodextrins (CDs) identified in the anabaenolysin-producing strains

Strain	α -CDs					β -CDs					γ -CD
	monoAc- α -CD	diAc- α -CD	triAc- α -CD	tetraAc- α -CD	SUM	β -CD	monoAc- β -CD	diAc- β -CD	triAc- β -CD	SUM	
XPORK1C	<1	31	54	1	86	3	4	6	ND	13	<1
XPORK1D	<1	37	47	1	86	3	4	6	ND	14	<1
XSPORK2A	<1	18	45	5	68	7	12	11	<1	30	2
XPORK4C	2	56	15	ND	73	4	12	11	ND	27	<1
XPORK6C	2	42	42	<1	87	4	5	4	<1	13	<1
XPORK13A	1	49	41	<1	92	2	3	3	<1	7	<1
XPORK15F	4	46	24	<1	75	2	5	17	1	25	<1
XSPORK27C	<1	57	35	<1	92	<1	2	5	<1	8	<1
XPORK36D	<1	28	52	4	84	4	4	8	<1	16	<1
BECID22	ND	18	54	3	76	<1	6	17	<1	24	<1
BECID30	<1	31	52	<1	84	1	6	8	<1	16	ND

The relative abundance of the main cyclodextrin produced is in bold. Relative abundances were calculated from the peak areas of the extracted sodiated cyclodextrin ion chromatograms. ND, not detected.

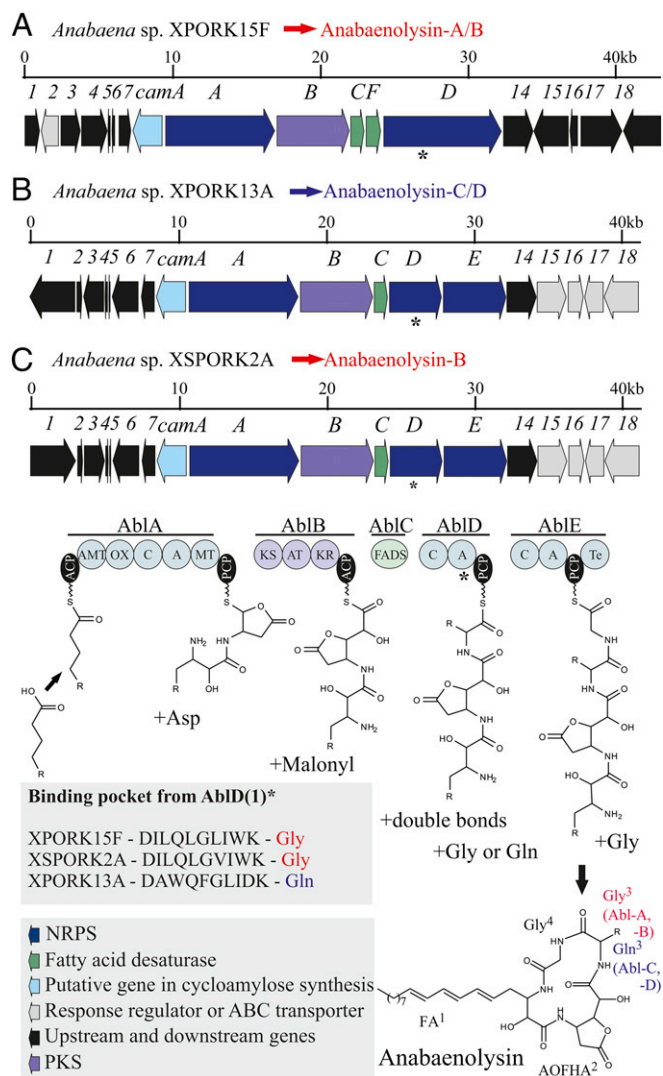


Fig. 4. Putative anabaenolysin gene clusters from *Anabaena* spp. XPORK15F (A), XPORK13A (B), and XSPORK2A (C). The main variant of anabaenolysin (A or B in red, C and D in blue) produced per strain is indicated in the figure. The main difference between the variants is the glycine (A/B) or glutamine (C/D) in position 3. The signature of the binding pockets from the adenylation domain of AblD(1) appears in the figure, and an asterisk (*) denotes the AblD(1) region in the gene cluster.

on the 16S rRNA gene phylogenetic tree (Fig. 3). The 24.3-kb anabaenolysin gene cluster of *Anabaena* sp. XPORK15F encodes a second 35-kDa fatty acid desaturase (AbIF). This strain contains the 276-kDa NRPS protein AblF, which corresponds to both AblD and AblE proteins in the XSPORK2A and XPORK13A anabaenolysin gene clusters (Fig. 4). Comparison of the entire anabaenolysin gene cluster showed less similarity to the sequences of XPORK15F than to those of the XSPORK2A strain (62% of coverage and 79% of identity). Nevertheless, these two strains synthesize the same chemical variant of anabaenolysin, anabaenolysin B (Figs. 2 and 3). *Anabaena* sp. XPORK15F also synthesizes anabaenolysin A, the main chemical variant produced by this strain, which lacks a saturated bond in the fatty acid chain characteristic of anabaenolysin B (16). Proteins upstream and downstream of the anabaenolysin gene cluster differ in both XPORK15F and XSPORK2A strains (SI Appendix, Table S9).

A phylogenetic tree based on the sequences of the condensation domains present in the anabaenolysin gene cluster indicates that

the first condensation domain of AblA is grouped with domains containing a monooxygenase and/or aminotransferase domains prior to them, whereas the condensation domains of AblD and AblE are grouped with other LCL condensation domains (SI Appendix, Fig. S8). How the 3-amino-2-hydroxyoctadecanoic acid is activated and incorporated into anabaenolysin remains unclear. However, we detected FAAL encoded at distant locations in the three *Anabaena* spp. genomes (data not shown). The adenylation domain in the AblA is predicted to activate asparagine (NRPSpredictor2) or aspartic acid (PKS/NRPS Analysis) (SI Appendix, Table S7). The AOFHA moiety, which is the 2-(3-amino-5-oxotetrahydrofuran-2-yl)-2-hydroxyacetic acid (Fig. 2) in the anabaenolysin structure, is predicted to be derived from an aspartic acid. The methyltransferase-like domain embedded in the adenylation domain might be involved in the formation of AOFHA. However, the exact biosynthetic mechanism underpinning the biosynthesis of the AOFHA is unclear and remains to be elucidated (Fig. 4).

The CamA has a domain predicted to act as an α -amylase. A phylogenetic tree based on diverse α -amylases shows that CamA sequences are more closely related to sequences predicted to be 4- α -glucanotransferases and amylo- α -1,6-glucosidases (SI Appendix, Fig. S8). We found similar amino acid sequences in the genomes of *Anabaena* sp. 90 (71–84% identity and 100% coverage) and *Anabaena* sp. 1tu33S10 (70–82% identity and 100% coverage) (data not shown). However, methylated and disparately derivatized cyclodextrins were present only in *Anabaena* sp. 90 cell extracts (data not included); no cyclodextrin glucanotransferase (CGTase) sequences were present in the three partial genomes obtained from *Anabaena* sp. XSPORK2A, XPORK13A, or XPORK15F.

Discussion

Synergistic Antifungal Activity. Cyanobacteria are known to produce lipopeptides with antifungal activity, such as nostofungicidine (18), laxaphycins (19–21), lobocyclamides (22), and hassallidins (14, 23, 24). In this study, we show that anabaenolysins have a weak antifungal activity (Fig. 1). Isolated anabaenolysins are known to permeabilize mammalian cells, which contain cholesterol (17), and it could also make the ergosterol containing fungal cells permeable. The structure of these two sterols differs only by two double bonds and a methyl group.

We found that all anabaenolysin producers also produce cyclodextrins, which increased the antifungal activity of anabaenolysins (Fig. 1D). Previously, lipopeptides, such as laxaphycins (19–21) and lobocyclamides (22), showed synergistic antifungal activity. Portoamides A and B have also shown synergistic allelopathic activity inhibiting the green microalgae *Chlorella vulgaris* (25). However, the combination of two chemically different variants of the same compound improved their antifungal or allelopathic activity. In the present study, the two unrelated compounds worked in synergy to produce a potent antifungal activity. Previous studies have shown that structurally unrelated compounds produced by *Streptomyces* spp., such as the β -lactam antibiotic cephamycin C and clavulanic acid, an inhibitor of β -lactamases, functioned synergistically (26).

Cyclodextrins are known to improve the solubility of compounds because of the hydrophobic and hydrophilic portions of their cone-shaped structures (1). The hydrophobic portion of a compound penetrates the hydrophobic inner cavity of a cyclodextrin molecule, resulting in an inclusion complex that increases hydrophilicity/water solubility. This feature makes them an important tool in drug delivery (1, 4, 7). β -cyclodextrins are also used to extract cholesterol from the plasma cell membrane (27) and, in a similar way, could act on the ergosterol present in the fungal membrane. Most of the cyclodextrins produced by *Anabaena* (73–92%), are α -cyclodextrins, which are capable of forming inclusion complexes with hydrocarbon chains similar to those present in anabaenolysins (2). A small percentage of the cyclodextrins produced by *Anabaena* (7–30%), are β -cyclodextrins, which are capable of forming inclusion complexes with membrane sterols. *Anabaena*-producing

α -cyclodextrins may form inclusion complexes with anabaenolysins to increase their solubility so that the concentration of anabaenolysins against the target increases. β -cyclodextrins produced by the same *Anabaena* strains could target fungal ergosterol-containing membranes by extracting ergosterol from the membrane, thereby changing the native function of the membrane. Commercially available amphotericin B and cyclodextrins used together showed an increase of 60% in antifungal activity compared with amphotericin B alone (7). Another example of improved efficacy in the treatment of oral candidosis in patients with AIDS comes from the use of commercially available itraconazole in complex with cyclodextrins (28). Cyclodextrins produced by the cyanobacterium *Tolythrix byssoides* blocked the antifungal activity of the tolythrycins produced by this cyanobacterium (29). However, in this study we showed that *Anabaena* spp. from the Baltic Sea produce both cyclodextrins and anabaenolysins and that anabaenolysin antifungal activity improves in the presence of cyclodextrins.

Benthic Cyanobacteria Producing Anabaenolysins and Cyclodextrins.

Cyanobacteria isolated from terrestrial and marine, brackish, and freshwater environments produce lipopeptides (9, 30–32). In the present study, anabaenolysins were detected in benthic cyanobacteria isolated from the Baltic Sea (Fig. 3). We used HPLC to analyze 151 methanol extracts from 14 different genera of cyanobacteria (SI Appendix, Table S1). However, only *Anabaena* species produced anabaenolysins and cyclodextrins (Fig. 3 and Table 1). Anabaenolysins A and B were first described as products of *Anabaena* spp. XPORK15F and XSPORK27C (16). We detected previously unidentified variants of anabaenolysins C and D produced by *Anabaena* sp. XPORK13A. Clarifying whether *Anabaena* strains exclusively produce anabaenolysins and determining the geographical distribution of anabaenolysin producers will require further analysis with a broader range of cyanobacterial genera and a larger selection of habitats.

The rRNA gene phylogeny groups most anabaenolysin producers together with nonproducers (Fig. 3). However, *Anabaena* sp. XPORK15F is located in a different part of the phylogenetic tree (Fig. 3). Previous studies indicate that benthic cyanobacteria are genetically more diverse than planktonic ones (33, 34).

Anabaenolysin producers were found to contain differently acetylated variants of α - and β -cyclodextrins as well as nonacetylated β - and γ -cyclodextrins. *Anabaena* sp. 90 can produce methylated and separately derivatized cyclodextrins, which have been described as products of *T. byssoides* (29). Putative genes involved in the synthesis of cyclodextrins were reported from *Nostoc* sp. PCC 9229 (35). In this study, bioinformatic analysis aided the discovery of a new cyclodextrin producer known as planktonic *Anabaena* sp. 90. However, our results indicate that *Anabaena* strains present in the benthic habitat of the Baltic Sea are the only known producers of anabaenolysins.

Potential Biosynthetic Gene Cluster of Anabaenolysin and Cyclodextrins.

Many of the cyanobacterial bioactive natural products reported to date are synthesized by a hybrid of NRPS and PKS pathways (36). We discovered in the partial genomes of *Anabaena* spp. XSPORK2A, XPORK13A, and XPORK15F a putative anabaenolysin gene cluster (Fig. 4). The anabaenolysin gene cluster consists of a hybrid NRPS/PKS, one or two fatty acid desaturase(s), and a hypothetical protein (Fig. 4). The incorporation of the fatty acid chain into the anabaenolysin is unclear. The phylogenetic tree groups the condensation domain of AblA with other condensation domains that come after aminotransferases and/or monooxygenases. Previous studies indicate that condensation domains are involved in the incorporation a β -hydroxyl fatty acid into lipopeptides (37–39). Surfactin, lichenysin, fengycin, and arthrofactin are examples of lipopeptides that are produced by using a starter condensation domain (37). Fatty acids, activated by FAAL enzyme, can be incorporated into nonribosomal peptide synthesis (40). The lipidation of daptomycin involves the DptE FAAL enzyme and the DptF acyl

carrier protein (40). The anabaenolysin gene cluster contains an acyl carrier protein in the AblA (Fig. 4). *Anabaena* sp. XSPORK2A has three FAALs, and XPORK13A and XPORK15F have two FAALs encoded elsewhere in its genome. However, a full understanding of anabaenolysin lipidation will require further analysis. The adenylation domain of AblA is predicted to incorporate an aspartic acid (SI Appendix, Table S7). The methyltransferase-like activity embedded in this domain could result in the formation of AOFHA. AOFHA formation may occur in a manner homologous to Ahp (3-amino-6-hydroxy-2-piperidone) formation in anabaenopeptilides, where it is predicted to form from glutamine, thus involving an unusual methyltransferase-like domain (41). This methyltransferase-like domain is putatively involved in the heterocyclization of anabaenolysins and anabaenopeptilides. Future biochemical characterization will improve our understanding of the function of these enzymes.

The presence of a gene encoding the putative α -amylase CamA in the anabaenolysin gene cluster suggests that *Anabaena* may coordinate the production of anabaenolysins and cyclodextrins. Phylogenetic analysis shows CamA sequences to be more closely related to 4- α -glucanotransferases, which produce cycloamyloses (e.g., ref. 42). The action of 4- α -glucanotransferases synthesizes larger cycloamyloses, whereas cyclodextrin glucanotransferases (CGTases) synthesize cyclodextrins (5, 6, 41). Phylogenetic studies based on the α -amylase (glycoside hydrolase 13 family) help to predict the function of enzymes among this family, but may be unreliable for a small number of proteins (43). Recombinant 4- α -glucanotransferases from *Synechocystis* sp. PCC 6803 could convert sucrose and amylopectin in cycloamyloses (42, 44). Cyclodextrins are known to act synergistically to improve the potency of a range of antifungal compounds (e.g., ref. 7). In this study, we show that living organisms use the synergistic application of cyclodextrins and lipopeptides to produce an antifungal effect. Interestingly, we found an α -amylase-encoded gene, which may be involved in cyclodextrin synthesis, upstream of the putative anabaenolysin biosynthetic gene cluster. The presence of enzymes involved in the synthesis of anabaenolysins and cyclodextrins in the same gene cluster indicates that they may be cosynthesized and act together. Lipopeptides with a unique chemical structure, such as anabaenolysins, can be used topically to treat fungal infections. Our knowledge of anabaenolysin biosynthetic genes improves the potential for further modifications in the chemical structure or for the heterologous expression of this peptide.

Materials and Methods

Details of cyanobacterial strains (SI Appendix, Table S1) and their cultivation as well as DNA isolation, 16S rRNA gene sequencing, and phylogenetic tree construction are presented in SI Appendix, SI Materials and Methods (primers in SI Appendix, Table S10). Accession numbers of 16S rRNA genes obtained in this study are as follows: KP715718 (*Anabaena* sp. XPORK1C), KP715719 (*Anabaena* sp. XPORK1D), KP715720 (*Anabaena* sp. XPORK4C), KP715721 (*Anabaena* sp. BECID30), and KP715722 (*Anabaena* sp. XPORK13A). Genome sequences of *Anabaena* spp. XSPORK2A, XPORK13A and XPORK15F were obtained and the biosynthetic gene clusters annotated as detailed in SI Appendix, SI Materials and Methods. The anabaenolysin gene clusters were deposited into the GenBank (NCBI) with the following accession numbers: *Anabaena* sp. XPORK15F (KP7161740), *Anabaena* sp. XPORK13A (KP7161741), and *Anabaena* sp. XSPORK2A (KP7161742). Detection of anabaenolysins and cyclodextrins was based on UV spectrum and mass spectrometry (SI Appendix, SI Materials and Methods). The disk diffusion bioassay was used to detect the antifungal activity (SI Appendix, SI Materials and Methods). Previously unidentified anabaenolysins (C and D) were identified based on amino acid analysis, mass spectrometry, and NMR. Mass spectrometry and NMR solved the structures of cyclodextrins.

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