Comparative Genomics Reveals Insights into Induction of Violacein Biosynthesis and Adaptive Evolution in Janthinobacterium

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ABSTRACT Violacein has different bioactive properties conferring distinct selective advantages, such as defense from predation and interspecific competition. Adaptation of Janthinobacterium to diverse habitats likely leads to variation in violacein production among phylogenetically closely related species inhabiting different environments, yet genomic mechanisms and the influence of adaptive evolution underpinning violacein biosynthesis in Janthinobacterium are not clear. In this study, we performed genome sequencing, comparative genomic analysis, and phenotypic characterization to investigate genomic factors regulating violacein production in nine Janthinobacterium strains, including a type strain from soil and eight strains we isolated from terrestrial subsurface sediment and groundwater. Results show that although all nine Janthinobacterium strains are phylogenetically closely related and contain genes essential for violacein biosynthesis, they vary in carbon usage and violacein production. Sediment and groundwater strains are weak violacein producers and possess far fewer secondary metabolite biosynthesis genes, indicating genome adaptation compared to soil strains. Further examination suggests that quorum sensing (QS) may play an important role in regulating violacein in Janthinobacterium: the strains exhibiting strong potential in violacein production possess both N-acyl-homoserine lactone (AHL) QS and Janthinobacterium QS (JQS) systems in their genomes, while weaker violacein-producing strains harbor only the JQS system. Preliminary tests of spent media of two Janthinobacterium strains possessing both AHL QS and JQS systems support the potential role of AHLs in inducing violacein production in Janthinobacterium. Overall, results from this study reveal potential genomic mechanisms involved in violacein biosynthesis in Janthinobacterium and provide insights into evolution of Janthinobacterium for adaptation to oligotrophic terrestrial subsurface environment.

IMPORTANCE Phylogenetically closely related bacteria can thrive in diverse environmental habitats due to adaptive evolution. Genomic changes resulting from adaptive evolution lead to variations in cellular function, metabolism, and secondary metabolite biosynthesis. The most well-known secondary metabolite produced by Janthinobacterium is the purple-violet pigment violacein. To date, the mechanisms of induction of violacein biosynthesis in Janthinobacterium is not clear. Comparative genome analysis of closely related Janthinobacterium strains isolated from different environmental habitats not only reveals potential mechanisms involved in induction of violacein production by Janthinobacterium but also provides insights into the survival strategy of Janthinobacterium for adaptation to oligotrophic terrestrial subsurface environment.

KEYWORDS Janthinobacterium, violacein, adaptation, evolution, comparative genomics, oligotrophic, subsurface, genomics
 Phylogenetically closely related microorganisms are often present in diverse environmental habitats. To adapt to such diverse habitats, bacteria evolve differently (1), leading to variations in cellular function, metabolism, and secondary metabolite biosynthesis. Genomic signatures of these adaptive changes (e.g., gene gain/loss) can provide insight into the selective pressures acting on these microbes and the niches they inhabit (2, 3). *Janthinobacterium* has been observed in lake sediments (4), soil (5), surface water (6), and seawater (7), as well as harsh environments such as Antarctica (8–10) and oligotrophic groundwater (11), and is a good candidate for genomic based studies of ecophysiology.

The most well-known secondary metabolite produced by *Janthinobacterium* is the purple-violet pigment violacein, for which *Janthinobacterium* was aptly named (janthinus meaning "violet" or "violet-blue" in Latin). Violacein possesses a wide range of biological activities, including antibacterial, antiviral, antifungal, antiparasitic, antiprotozoal, anti-inflammatory, antitumor, antieukemic, antioxidant, and antiulcerogenic (12–18). With such a diverse range of activities, ecophysiological function of violacein may be multifaceted. Violacein can confer protection from protist predators such as nematodes and bacterivorous nanoflagellates (19–21). Given its antibacterial properties, violacein may also play a role in interspecific competition (22). Adaptation of *Janthinobacterium* strains to various environments may lead to adaptive changes in their violacein production potential. For example, a strain of *Janthinobacterium* isolated from a high-altitude glacial stream was found to lack typical violet pigmentation, indicating adaptation and survival strategy in harsh aquatic ecosystem of high altitude where the organisms need to be tolerant to cold temperature, strong radiation, and low nutrients rather than predation or competition (6).

Genomic mechanisms regulating violacein biosynthesis appear to vary widely. In bacteria, the bisindole violacein is formed by the condensation of two tryptophan molecules through the sequential action of five enzymes, encoded by the operon *vioABCDE* (23, 24). Quorum sensing (QS) has been shown to play an important role in violacein biosynthesis in violacein-producing bacteria *Chromobacterium* (25), *Duganella* (21), and *Janthinobacterium* (26). The QS signal molecules consist mainly of *N*-acyl-homoserine lactones (AHLs), autoinducing peptides (AIPs), and autoinducer-2 (AI-2), of which AHLs are produced mainly by Gram-negative bacteria (27), AIPs are produced by Gram-positive bacteria (28), and AI-2 can be produced by both (29). In AHL-producing bacterium *Chromobacterium violaceum* (30), biosynthesis of violacein is positively regulated by the AHL CviI/R QS system (25). *Duganella* sp. HH01 (previously known as *Janthinobacterium* sp. HH01) lacks the synthesis genes for both AHLs and AI-2, and its violacein biosynthesis is strongly affected by a novel janthinobacterial autoinducer synthesized by the enzyme *jqsA* (21). The importance of *jqsA* gene for violacein production in *Janthinobacterium* was later confirmed in the strain *Janthinobacterium* sp. HH102 (26), which also lacks the genes for synthesis of AHLs and AI-2. These results suggest that in phylogenetically distant taxonomic groups, genomic mechanisms involved in violacein biosynthesis are quite variable, but whether this is due to phylogeny or differential selective pressures from their respective environments is unclear.

By examining a phylogenetically close group of bacteria inhabiting diverse environmental niches, such as *Janthinobacterium*, we can gain insights into underlying habitat-based selection for violacein production. Here, we performed genome sequencing, comparative genomic analysis, and phenotypic characterization of nine *Janthinobacterium* strains isolated from surface soil and terrestrial subsurface ecosystem (sediment and groundwater). We conducted laboratory phenotypic characterization in various carbon sources since it has been reported that environmental conditions, including carbon source (31–34), can influence violacein induction. We also performed a preliminary study of the spent media of *Janthinobacterium* strains to test if the potential QS signaling molecules in the spent media may lead to induction of violacein biosynthesis. Results from this study can provide insights into potential genomic mechanisms involved in violacein biosynthesis in *Janthinobacterium* strains and their survival strategy and genome evolution for adaptation to oligotrophic terrestrial subsurface environment.
RESULTS AND DISCUSSION

Genome identity, phylogenetic relationship, and pangenome of Janthinobacterium.

To unravel genome evolution and molecular mechanisms involved in violacein biosynthesis in Janthinobacterium, we sequenced, assembled, and analyzed the genomes of the type strain J. lividum ATCC 12473 isolated from soil as well as eight Janthinobacterium strains isolated in this study from Oak Ridge Reservation Field Research Center (FRC) sediment and groundwater. Our isolates have smaller genome sizes and fewer predicted protein-encoding genes than the type strain ATCC 12473 (Table 1).

We compared the violacein biosynthesis essential genes vioABCDE among the nine Janthinobacterium genomes. Although the sequences are not identical among the strains, each of the genomes has all five full-length genes (Fig. S1). Also, the upstream JqsR (response regulator) binding site is conserved among all the strains (Fig. S1). This evidence suggests that all of the nine strains have the potential for violacein production.

A phylogenetic tree of our isolates together with the type strain ATCC 12473 was constructed based on the 49 highly conserved Clusters of Orthologous Groups (COG) domains (35). Our subsurface isolates cluster within the Janthinobacterium genus and are closely related to the type strain J. lividum ATCC 12473 (Fig. 1a). Further phylogenetic analysis based on average nucleotide identity (ANIm) was performed to assess the overall genome sequence similarity among our isolates and the type strain ATCC 12473. A hierarchal clustering based on the distance matrix of ANIm divergence values was computed and visualized as a heatmap (Fig. 1b). Our subsurface isolates show high similarities (ANIm > 92%) to the type strain ATCC 12473. The isolate EB271-G4-7A is the closest to the type strain with ANIm value of 98.73%, indicating that this isolate and the type strain ATCC 12473 can be identified as members of the same species (ANI cutoff for same species: 95%) (36). The other isolates are less similar to the type strain ATCC 12473 (ANIm 92.02 to 93.62%), suggesting that these isolates belong to different species from the type strain.

The pangenomic analysis of 50 Janthinobacterium genomes, including the 9 genomes sequenced in this study and 41 publicly available genomes, results in 20,556 gene clusters which are grouped into four bins based on their occurrence across genomes: core gene clusters (n = 1,433), soft core gene clusters (n = 1,413), shell gene clusters (n = 5,745), and cloud gene clusters (n = 11,965) (Fig. 2). “Core” genes are genes present in all 50 genomes, “soft core” genes are genes present in 48 or 49 genomes, “cloud” genes are genes present in 1 or 2 genomes, and the remaining genes are shell genes. It is worth noting that the violacein biosynthesis essential genes vioABCDE are not core genes—they are missing in 17 of the 50 Janthinobacterium genomes (Table S1). Interestingly, the Janthinobacterium strains reported so far from cold habitats do not harbor vioABCDE genes in their genomes (Table S1), indicating that these strains have abandoned the violacein production capability (which is beneficial for survival in competitive environment) for adaptation to harsh cold ecosystems.

The relatively small core genome in Janthinobacterium pangenome supports the concept of a Janthinobacterium “open pangenome” (6). Typically, the sympatric species that live

| Strain     | Source         | NCBI accession no. | Genome size (mbp) | GC content (%) | No. assembled contigs (≥1,000 bp) | No. protein-coding genes | No. genes with function prediction |
|------------|----------------|--------------------|-------------------|----------------|----------------------------------|--------------------------|-----------------------------------|
| ATCC 12473 | Type strain    | JACYMN0000000000   | 6.69              | 62.45          | 30                               | 5,911                    | 4,661                             |
| FW305-129  | FRC groundwater| JACYMM0000000000   | 6.23              | 63.15          | 41                               | 5,493                    | 4,410                             |
| GW458P     | FRC groundwater| JADBGP0000000000   | 6.29              | 63.36          | 26                               | 5,648                    | 4,394                             |
| GW460P     | FRC groundwater| JACYML0000000000   | 6.25              | 62.95          | 41                               | 5,594                    | 4,427                             |
| GW460W     | FRC groundwater| JACYMJ0000000000   | 6.25              | 62.95          | 42                               | 5,594                    | 4,426                             |
| FW305-128  | FRC groundwater| JACYMK0000000000   | 6.30              | 62.75          | 53                               | 5,622                    | 4,431                             |
| EB271-G4-7A| FRC sediment   | JACYMI0000000000   | 6.43              | 62.71          | 13                               | 5,736                    | 4,568                             |
| EB271-G4-3-1| FRC sediment  | JACYMH0000000000   | 6.15              | 63.16          | 45                               | 5,529                    | 4,437                             |
| EB271-G4-3-2| FRC sediment  | JACYMG0000000000   | 6.15              | 63.17          | 29                               | 5,505                    | 4,431                             |
in multiple environments of mixed microbial communities tend to have large genomes and an open pangenome, since they have multiple ways of exchanging genetic material, and hence continuously extend their total set of genes. On the other hand, the allopatric species that live in an isolated and restricted niche usually have smaller genomes and a closed pangenome, because the niche would hamper the ability of those species to obtain foreign genes by the lack of mechanisms for gene exchange and recombination (37, 38). *Janthinobacterium* has been reported as a symbiont (39), which may explain why it has an open pangenome. The open pangenome of *Janthinobacterium* and the discrepancy in the number of unique genes among *Janthinobacterium* strains strongly support that *Janthinobacterium* species have extremely flexible genetic content, allowing them to adapt to diverse environments and conditions. These genetic features of *Janthinobacterium* may lead to variation in violacein production among the strains from different environmental habitats.

**Phenotypic variations in carbon utilization and violacein production among the *Janthinobacterium* strains.** In this study, we assessed the growth of the nine *Janthinobacterium* strains and their potential in violacein production using 15 different carbon sources representing different classes of substrates. The growth (optical density at 600 nm [OD600]) of strains reached stationary phase between 48 to 72 h, when the purple color indicating violacein biosynthesis began to be perceptible in cultures. Maximum violacein concentration was detected at late-growth phase (92 or 171 h). This parallels previous observations by Pantanella et al., who reported that the production of violacein in *J. lividum* DSM 1522 began during the stationary phase of growth and reached its maximum during the early-death phase (31).

All *Janthinobacterium* strains tested in this study can utilize carbohydrates (including 4 monosaccharides and 2 disaccharides), glycerol, mannitol, glutamic acid, glucose-6-phosphate, and malic acid but not glutamine (Fig. 3). All strains except for the isolate FW305-129 can also grow when supplied with the sugar alcohols sorbitol and xylitol, indicating that these strains vary in genetic potential for metabolism of polyols and likely other carbon substrates. We searched for sorbitol and xylitol dehydrogenase genes in the Prokka-annotated *Janthinobacterium* genomes sequenced in this study and found that two of our isolates (FW305-129 and GW458P) did not have the sorbitol dehydrogenase gene. We further constructed a hidden Markov model (HMM) from previously published *Janthinobacterium* sorbitol dehydrogenase protein sequences and utilized the HMM to search for homologous sequences. The sorbitol dehydrogenase gene is highly conserved among the type strain ATCC 12473 and our isolates, with the
exceptions of strains FW305-129 and GW458P (Fig. S2). Although we did not find annotated xylitol dehydrogenase proteins in the Janthinobacterium strains sequenced in this study, their capabilities of growing with xylitol (except for the strain FW305-129) suggest that their polyol dehydrogenases may exhibit broad specificity to polyols (including xylitol), which has been demonstrated previously in the yeast Meyerozyma caribbica 5XY2 (40). Further isolation and characterization of these proteins are required to evaluate this hypothesis.

Among the carbon sources tested in this study, ribose, maltose, glycerol, and mannitol generally induce higher yield of violacein than did other carbon substrates (Fig. 3), demonstrating the impact of carbon source on violacein biosynthesis. Pantanella et al.
reported similar results in which violacein production in *J. lividum* DSM 1522 was enhanced by glycerol but inhibited by glucose. We also observe that the growth of *Janthinobacterium* is not necessarily correlated with violacein production. For example, all strains can grow in malic acid with maximum OD_{600} up to 0.4 to 0.7, but no violacein was detected under this growth condition.

Although our *Janthinobacterium* isolates from the terrestrial subsurface are phylogenetically closely related to the type strain *J. lividum* ATCC 12473, they exhibit phenotypic variations in both carbon utilization and violacein biosynthesis under the culture conditions tested in this study. Compared to the type strain ATCC 12473, which is the strongest violacein producer (maximum violacein concentration measured to be 26 mg/L when amended with mannitol), our isolates from the terrestrial subsurface sediment and groundwater show weaker capabilities in producing violacein (Fig. 3). Only one isolate, EB271-G4-7A, exhibits comparable potential in violacein biosynthesis (maximum concentration 21 mg/L in maltose) to the strain ATCC 12473, while the others produce much less violacein with maximum concentration of 8 mg/L for the isolate FW305-129, 2 to 4 mg/L for the isolates GW458P, GW460P, and EB271-G4-3-1, and below the detection limit (0.2 mg/L) for the isolates FW305-128, GW460W, and EB271-G4-3-2.

Comparative genomics reveals secondary metabolite biosynthetic gene cluster diversity in *Janthinobacterium*. To understand the genomic underpinning of the phenotypic variation in violacein production among the nine *Janthinobacterium* strains, we investigated their secondary metabolite biosynthetic genes by using antiSMASH to search for putative biosynthetic gene clusters (BGCs). We identified 32 BGCs in the type strain ATCC 12473 isolated from soil and much fewer BGCs in our isolates from the oligotrophic terrestrial subsurface: 16 to 18 BGCs in the isolates from sediment and 6 to 11 BGCs in the isolates from groundwater. Typically, one BGC is responsible for the production of one or several similar compounds with bioactivities that vary only in terms of strength and/or specificity (41). The relatively small numbers of BGCs identified in our isolates suggest that these *Janthinobacterium* strains living in subsurface...
environments are less capable of producing diverse secondary metabolites compared to their relative (i.e., \textit{J. lividum} ATCC 12473) living in soil.

Of the BGCs found, some are present in all strains, appearing to indicate an important or essential role, whereas others are found only in some strains. The widespread BGCs include those for the production of indole (violacein biosynthesis genes \textit{vioABCDE}), acyl amino acids, terpene, and bacteriocin (Fig. 4). On the other hand, a biosynthetic pathway for cyanobactin was predicted exclusively in the isolate FW305-129, and the pathways for aryl polyene pigments were found in four of nine strains (Fig. 4). Interestingly, only the strains ATCC 12473 and EB271-G4-7A, which are the two strong violacein producers compared to the other strains in our phenotypic characterization, were predicted to have the BGCs for the production of QS signaling homoserine lactone molecules, i.e., AHLs (Fig. 4).

It is worth noting that none of our isolates from the terrestrial subsurface contain the gene clusters linked to the biosynthesis of nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), which are prevalent in \textit{Janthinobacterium} strains (26), including the type strain ATCC 12473 (Fig. 4). NRPS and PKS are responsible for biosynthesis of a multitude of secondary metabolites, including many important drugs such as the antibiotic daptomycin and the anti-cancer compound epothilone (42, 43). Also, the clusters encoding tropodithietic acid, which functions as an antibiotic and a signaling molecule (44), are observed only in the type strain ATCC 12473 and sediment-derived isolates but not in any groundwater-derived isolates (Fig. 4).

Secondary metabolites are produced most abundantly by microorganisms in crowded and competitive habitats (45). In less competitive environments, biosyntheses of some secondary metabolites are rendered useless and are eventually lost (38). Analyses of BGCs in our isolates from FRC sediment and groundwater show that those strains possess fewer antibiotics-related BGCs compared to the type strain ATCC 12473 isolated from soil. This is likely an adaptive strategy, since production of diverse antibiotics provides diminished benefits for the survival of organisms inhabiting low-nutrient oligotrophic environment. Here, microbial interactions may shift from competition to cooperation as environmental stress (e.g., low nutrients) increases (46). Conversely, this could also indicate other unknown important roles of secondary metabolites for survival in these oligotrophic settings.

\textbf{The QS systems in \textit{Janthinobacterium}.} Our previous phenotypic characterization and putative BGCs analyses show that the strains ATCC 12473 and EB271-G4-7A, which possess AHL production genes in their genomes, are stronger in violacein production than other strains, indicating that QS signaling molecules are correlated with induction of violacein biosynthesis in \textit{Janthinobacterium}. So far, the QS systems that have been verified to affect violacein production in violacein-producing bacteria include AHL QS
system reported in C. violaceum (25) and Janthinobacterium QS (JQS) system in Duganella (21) and Janthinobacterium (26). Therefore, we further examined the nine Janthinobacterium genomes obtained in this study for their QS systems.

We identified two AHL synthase genes (lasI, KBase coding sequence identifier NNGPKELA_05099, and anoI, NNGPKELA_05806) in the genome of the type strain ATCC 12473 which exhibits the most potent violacein production. The strain EB271-G4-7A possesses only one AHL synthase gene, lasI (JAJFNNGN_01224) (Fig. 5). None of the other seven Janthinobacterium strains, which are relatively weak violacein producers, contains either of these AHL synthase genes. This evidence supports the potential roles of AHLs in augmenting production of violacein in Janthinobacterium.

To put these results into more context, we compared with 41 publicly available Janthinobacterium genomes. We found that lasI and anoI rarely exist in Janthinobacterium (Fig. 5, Table S1). In addition to these two AHL synthase genes (lasI and anoI), we identified an unnamed AHL synthase gene in 3 publicly available Janthinobacterium genomes (Table S1). AHL-related quorum quenching genes pvdQ and quiP have a more variable distribution: pvdQ is present in all of our 9 genomes and some of the other 41 genomes, whereas the quiP is not in our genomes but dispersed across others (Table S1). The homoserine lactone efflux protein rhtB is present in all genomes analyzed herein (Table S1). For C. violaceum, the AHL binds cytoplasmic CviR and forms a stable protein-ligand complex (AHL: CviR complex), which consequently activates the transcription ofvio operon by binding to its promoter site (25). For Janthinobacterium, only two of our strains presented here harbor genes as candidate AHL-sensing transcriptional regulators: 3 genes in ATCC 12473 (NNGPKELA_01319, NNGPKELA_05100, NNGPKELA_05484) and 2 genes in EB271-G4-7A (JAJFNNGN_01201, JAJFNNGN_03005). All of these five proteins have an N-terminal AHL-binding domain (Pfam identifier PF03472) and a C-terminal DNA-binding domain (Pfam identifier PF00196). None of these five regulators exhibits HMM-detectable homologues to the CviR, but they have some similarity to LasR and other QS regulators. The rest of our strains presented herein have no genes encoding regulators with the AHL-binding domain.

As for other QS systems, all of the nine Janthinobacterium genomes obtained in this study have the JQS system (Fig. S3), consisting of the autoinducer synthase gene jqsA, the sensor kinase/phosphatase gene jqsS, and the response regulator gene jqsR (21). The JQS system is also present in other public available Janthinobacterium strains, except
the strain Marseille (Table S1). The jqsA/S/R genes are highly similar among the 49 strains (9 strains sequenced in this study and 40 public available strains), with the identical amino acid residues to be >68% for JqsA, >53% for JqsS, and >69% for JqsR. Similarly to previously reported Janthinobacterium strains (26), none of our isolates or the type strain ATCC 12473 codes for a luxS homolog, necessary for the synthesis of the QS signal molecule AI-2. Given that the JQS system is highly conserved across Janthinobacterium, unlike the AHL QS system, it is likely involved with essential processes.

Based on the above finding that the type strain ATCC 12473 and the isolate EB271-G4-7A have both AHL QS system and JQS system, which may explain their augmented viola- cein production under the culture conditions tested in this study compared to that of other FRC Janthinobacterium strains which only have the JQS system, we tested the effects of ATCC 12473 and EB271-G4-7-A spent media on viola- cein biosynthesis in other strains. We observed that the growth media amended with spent media of ATCC 12473 or EB271-G4-7-A induced viola- cein production in some Janthinobacterium strains (Fig. S4), supporting the potential role of AHLs in inducing viola- cein production in Janthinobacterium. However, this hypothesis requires further validation via advanced instruments, such as liquid chromatography-mass spectrometer, to confirm the presence and concentrations of AHLs in spent medium or identify other extracellular metabolites of interest, which are out of scope of this study. Future studies may also consider an alternative approach to test this hypothesis via generation of knockout mutants in the AHL synthase genes, which can provide further evidence to verify the involvement of AHLs in the induction of viola- cein biosynthesis in Janthinobacterium.

Conclusions. In this study, we performed comparative genomic and phenotypic analyses of nine Janthinobacterium strains to uncover the potential genomic mechanisms involved in viola- cein biosynthesis in Janthinobacterium. Despite their phylogenetically close relationship, these strains vary in carbon utilization and viola- cein production under the culture conditions tested in this study. Our analyses show a habitat-related pattern of secondary metabolite genes in these Janthinobacterium strains and that their potential of viola- cein production is related to the type of QS system in their genomes. The strains containing both AHL QS and JQS systems exhibit higher potencies in viola- cein production compared to the strains containing only the JQS system, suggesting the potential role of AHLs in augmenting production of viola- cein in Janthinobacterium. Violacein production was low in strains from the oligotrophic aquatic environment (groundwater), and in general, these strains have the lowest capacity for secondary metabolite production. This may indicate that the selective advantage of high viola- cein production is greater in productive surface-associated systems, such as biofilms, which aligns with previous work on viola- cein aiding in biofilm predation defense (20). However, although clearly more tightly regulated in the subsurface strains, all the eight oligotrophic subsurface strains retained the genes for viola- cein production, unlike the cold-adapted Janthinobacterium strains (6). This suggests that there is still a selective advantage to maintaining the capability to produce viola- cein under certain conditions.

MATERIALS AND METHODS

Janthinobacterium strains. The type strain J. lividum ATCC 12473, isolated from soil in Michigan, USA, was purchased from American Type Culture Collection (Manassas, VA). The other eight Janthinobacterium strains were isolated in this study from uncontaminated sediment and groundwater at Oak Ridge Reservation Field Research Center (FRC), Oak Ridge, Tennessee (Table 1). The sediment was collected 3 to 3.6 m below ground surface at well EB271, and groundwater was sampled from wells FW305, GW458, or GW460. Briefly, sediment and groundwater samples were shipped immediately to the experimental laboratory facilities with ice packs after collection. An aliquot (20 μL) of groundwater or water extract from the sediment was spread over the surface of 1/10 diluted Reasoner’s 2A (R2A), tryptic soy broth (TSB), or Luria-Bertani (LB) agar plates. Bacterial colonies were streaked again if deemed necessary, until single axenic colonies were obtained. Single colonies grown on the plates were picked and used for 16S rRNA gene sequencing for bacterial identi- fication as described previously (47). The purified isolates were preserved in 17% vol/vol glycerol at −80°C until further use.

Genome sequencing, assembly, and annotation. Genomic DNA of all nine Janthinobacterium strains were extracted using a PureLink genomic DNA minikit (Invitrogen, Thermo Fisher Scientific) following the manufacturer’s protocol and then sequenced on a Novaseq 6000 S4 sequencer (Illumina, San Diego, CA, USA) with 2 × 150 bp paired-end reads. Adapter-related reads, low quality reads, and N-containing reads were
 trimed off using an in-house developed software and assembled into contigs using SPAdes (v3.13.0) in KBase (48). Annotation was performed using the Joint Genome Institute’s Integrated Microbial Genomics (IMG) annotation pipeline (49, 50) and Prokka (v1.12) in KBase (51).

**Phylogenetic analysis.** Phylogenetic analysis was conducted using the “Insert Set of Genomes Into SpeciesTree” app (v2.2.0) in KBase, which uses a select subset of 49 core, universal genes defined by Clusters of Orthologous Groups (COG) gene families to construct a species tree (35). Briefly, a subset of public KBase genomes closely related to the *Janthinobacterium* genomes in this study was selected based on the relatedness determined by alignment similarity to the 49 COG domains. Then, the *Janthinobacterium* genomes were inserted into curated multiple sequence alignments (MSA) for each COG family. The curated alignments were trimmed using GBLOCKS to remove poorly aligned sections of the MSAs. The MSAs were concatenated, and the approximate maximum-likelihood phylogenetic tree including the 9 *Janthinobacterium* genomes sequenced in this study and 10 genomes identified in the previous step was reconstructed using FastTree2 (v2.1.10) (35).

**Comparative genomic analyses.** Overall genome similarity among the nine *Janthinobacterium* genomes sequenced in this study was assessed with average nucleotide identity (ANI) calculated using dRep (https://github.com/MrOlm/drep).

To compare various genes and proteins, including sorbitol dehydrogenase and AHL synthases, Prokka-annotated genes were identified in KBase. Organisms lacking a Prokka-annotated sorbitol dehydrogenase were further evaluated for possession of an *sdh* gene using a hidden Markov model (HMM) via HMMER (v3.3) (http://hmmer.org). The HMM was built with five sorbitol dehydrogenase protein sequences from published *Janthinobacterium* strains (accession numbers ATD62391.1, AYMY77587.1, STQ93531.1, PHV03862.1, and WP_086147751.1), and a cutoff score was assigned based on scores from reference *Janthinobacterium* sequences from the NCBI genome database (cutoff E value of 1e−113, cutoff score of 375). The HMM was then used to search for homologous sequences in the genomes using the HMMER suite default parameters. The sorbitol dehydrogenase protein sequences were aligned using MUMmer (52) (v4=0.0), and alignments were visualized in JalView (53) (v2). Genomic context of each AHL-coding gene was visualized using the “Feature Context” feature from the Prokka (v1.12) output in KBase.

**Pangenome analysis.** We acquired 41 isolate genomes of *Janthinobacterium* from the National Center for Biotechnology Information (NCBI), with the accession numbers listed in Table S1. A pangenome including 50 *Janthinobacterium* genomes (9 genomes sequenced in this study and 41 publicly available genomes) was constructed using anv’i’o (version 6.2) (54, 55) following the anv’i’o workflow for pangenome analysis.

**Violacein production in *Janthinobacterium* strains in the presence of different carbon sources.** Nine *Janthinobacterium* strains were streaked from glycerol stocks on R2A agar plates. A single colony grown on the plate was picked and incubated aerobically in R2A broth at 27°C to early-stationary phase. A 3-mL aliquot was harvested and centrifuged at 10,000 × g for 3 min. The supernatant was removed, and the pellet was washed three times with minimal medium (Table S2) before resuspension in 3 mL of minimal medium. The carbon sources tested in this study include four monosaccharides (D-ribose, D-glucose, D-fructose, D-mannose), two disaccharides (D-maltose, D-cellobiose), four sugar alcohols (glycerol, xylitol, D-sorbitol, D-mannitol), three α-amino acids (D-glutamic acid, L-asparagine, L-glutamine), and two common metabolites (D-glucose-6-phosphate and L-malic acid). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The carbon stock solutions (400 mM) were prepared with MilliQ water and filter-sterilized (0.2-μm pore size, polyethersulfone, Thermo Fisher Scientific).

Violacein biosynthesis assay was performed in 96-well plates, with each well containing 180 μL of minimal medium, 10 μL of bacterial culture (washed and resuspended), and 10 μL of carbon stock solution (final carbon concentration: 20 mM). The 96-well plates were incubated at room temperature (22°C) in the dark without shaking. For each strain, nine experimental replicates were included for each carbon source as well as the negative control (without carbon amendment), with three sacrificial replicates harvested at 50, 92, and 171 h for violacein measurement. The growth of bacteria (i.e., OD₆₀₀) was measured twice a day via microplate spectrophotometer (BioTek, VT, USA).

To prepare the spent media of *J. lividum* ATCC 12473 and *Janthinobacterium* sp. EB271-G4-7A, we grew these two strains in R2A broth and then minimal medium (with 20 mM maltose) as described above. The spent medium was prepared by filtering the culture with 0.2-μm polyethersulfone filter at the late-growth phase (96 h). We studied the effects of the two spent media on violacein biosynthesis in other *Janthinobacterium* strains by growing the test strain (*n* = 3) in a medium containing 50% spent medium of *J. lividum* ATCC 12473 or *Janthinobacterium* sp. EB271-G4-7A and 50% minimal medium (with 20 mM maltose) in a 96-well plate as described above. For each test strain, we also included a control group in which the strain was grown in just the minimal medium (with 20 mM maltose). After 171 h, the cultures in the 96-well plates were harvested for violacein measurement as described below.

**Extraction and quantification of violacein.** The biosynthesized violacein was attached mostly to biofilm in the culture due to its hydrophobicity. Therefore, to quantify violacein, bacterial cells in the 96-well plates were harvested by centrifugation at 5,000 × g for 20 min. The colorless supernatant was removed, and pellets were lysed via freeze-thaw cycles and then resuspended in 0.2 mL 100% ethanol followed by centrifugation at 5,000 × g for 20 min. An aliquot of 150 μL supernatant (which was purple in color) was transferred to a new 96-well plate, and the extracted violacein was quantified by measuring the absorbance at 580 nm (56) via microplate spectrophotometer (BioTek, VT, USA). The calibration curve of violacein was prepared by dissolving violacein standard (≥98%, Sigma-Aldrich) and performing a serial dilution with 100% ethanol, resulting in a quantification range of 0.2 to 50 mg/L.
Secondary metabolite analysis. The presence of biosynthetic gene clusters (BGCs) in the Janthinobacterium genomes was analyzed using standalone-lite antiSMASH version 5.1.1 with default parameters.

Data availability. The nine Janthinobacterium genomes sequenced in this study were deposited in the GenBank database with accession numbers listed in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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