The *Janthinobacterium* sp. HH01 Genome Encodes a Homologue of the *V. cholerae* CqsA and *L. pneumophila* LqsA Autoinducer Synthases

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Abstract

Janthinobacteria commonly form biofilms on eukaryotic hosts and are known to synthesize antibacterial and antifungal compounds. *Janthinobacterium* sp. HH01 was recently isolated from an aquatic environment and its genome sequence was established. The genome consists of a single chromosome and reveals a size of 7.10 Mb, being the largest Janthinobacterium genome so far known. Approximately 80% of the 5,980 coding sequences (CDSs) present in the HH01 genome could be assigned putative functions. The genome encodes a wealth of secretory functions and several large clusters for polyketide biosynthesis. HH01 also encodes a remarkable number of proteins involved in resistance to drugs or heavy metals. Interestingly, the genome of HH01 apparently lacks the N-acylhomoserine lactone (AHL)-dependent signaling system and the AI-2-dependent quorum sensing regulatory circuit. Instead it encodes a homologue of the *Legionella*- and *Vibrio*-like autoinducer (lqsA/cqsA)-synthase gene which we designated *jqsA*. The *jqsA* gene is linked to a cognate sensor kinase (*jqsS*) which is flanked by the response regulator *jqsR*. Here we show that a *jqsA* deletion has strong impact on the violacein biosynthesis in *Janthinobacterium* sp. HH01 and that a *jqsA* deletion mutant can be functionally complemented with the *V. cholerae* *cqsA* and the *L. pneumophila* *lqsA* genes.

Introduction

Janthinobacteria are Gram-negative, motile, aerobic bacteria that are commonly isolated from soil and aquatic samples. They are grouped within the family Oxalobacteraceae of the class Betaproteobacteria [1–7] and they produce a range of secondary metabolites such as violacein [8], a purple, water-insoluble pigment. Janthinobacteria are well known for their antifungal effects on frog skins. By forming biofilms on the frog skins and releasing violacein and perhaps other secondary metabolites they reduce the mortality and morbidity of their host animals significantly [9]. Furthermore it was reported that janthinobacteria affect the survival of nanoflagellates [6] and that already nanomolar amounts of violacein produced by the microbe inhibit protozoan feeding of marine biofilm bacteria [10].

It is noteworthy that up to date the genomes of only very few janthinobacteria are available. Only a single complete genome sequence has been established for *Janthinobacterium* sp. Marseille [11], even though the 4.9 Mb partial genome sequences of *Janthinobacterium* sp. strain PAMC 25724 has been announced very recently [12] and the 6.2 Mb genome sequence of strain GC3 is available as a permanent draft (DOE Joint Genome Institute). Finally the genome of the distantly related *Chromobacterium violaceum* has been published earlier [13]. Within the current publication we have established the 7.10 Mb genome sequence of a recently isolated *Janthinobacterium* sp. HH01 (from here on designated HH01). This is so far the largest known genome within the genus *Janthinobacterium*. HH01 was isolated from an aquatic source and produces violacein in stationary growth phase.

One of the striking features identified in the HH01 genome concerns the mechanism involved in cell-cell communication. Many Gram-negative bacteria employ a N-acylhomoserine lactone (AHL)-dependent quorum sensing (QS) regulatory circuit...
for intraspecies cell-cell communication. The corresponding AHL signaling molecules are synthesized through LuxS-like proteins [14,15]. In addition a second but interspecies specific signaling circuit is commonly observed in Gram-negative microbes. This QS system has been designated autoinducer 2 (AI-2) signaling circuit. It depends on the synthesis of small furanone-like molecules. AI-2 is synthesized through LuxS-like proteins [14,16]. Interestingly, HH01 lacks the synthesis genes for both of these signaling systems. However, it encodes a third type of autoinducer synthase genes previously only functionally characterized in Legionella pneumophila and Vibrio cholerae. The Legionella and Vibrio-like autoinducer synthase genes lqsA and cqsA are involved in the biosynthesis of specific α-hydroxysketone signaling molecules termed LAI-1 and CAI-1 [17–19]. The lqsA and cqsA homologue identified in the HH01 genome was designated jqsA. In addition to its identification we report on the construction of a jqsA deletion mutant. Although, we have not identified the chemical structure of the novel janthinobacterial autoinducer, which we have designated JAI-1, we provide evidence that it is involved in regulation and expression of the violacin biosynthesis genes. We also show that the jqsA deletion mutant (HH02) can be functionally complemented with the lqsA and cqsA genes and therefore might be potentially useful for the detection of homologues from other Proteobacteria.

Materials and Methods

Bacterial Strains, Plasmids and Culture Conditions

Bacterial strains and plasmids used in the present work are listed in Table S1. Primers used are listed in Table S2. Escherichia coli was grown at 37°C in lysogenic broth (LB) medium [29] (1% peptone, 0.5% yeast extract, 1% NaCl) supplemented with appropriate antibiotics. HH01 was grown in R2A medium [30] (0.05% yeast extract, 0.05% tryptone, 0.05% casamino acids, 0.05% dextrose, 0.05% soluble starch, 0.03% sodium pyruvate, 1.7 mM K2HPO4, 0.2 mM MgSO4, final pH 7.2 adjusted with crystalline K2HPO4 or KH2PO4). V. cholerae strains were incubated statically within 24-well plates and biofilm formation was scored after 24 hours of growth using a standard crystal violet approach modified from reference [31]. Unless otherwise specified, media were supplemented with antibiotics, as required, at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 12.5 μg/ml; kanamycin, 25 μg/ml; and gentamycin 10 μg/ml. For metal resistance tests copper, iron and zinc were supplied as CuCl2, 500, 600 and 700 μM; FeCl3×6H2O 1200, 1300, 1400 and 1500 μM; and ZnCl2, 400, 500 and 750 μM. Agar plates employed for assaying exoenzyme activities contained 2% of the respective substrate.

Caenorhabditis elegans Survival and Developmental Assays

All experiments were done using the C. elegans N2 strain, maintained on nematode growth media (NGM) at 20°C and fed on the E. coli strain OP50 [32]. Clean eggs and synchronized L4 larvae were obtained by bleaching as described in [32]. HH01 and the violacin-negative mutant HH5-1 (Table S1) were grown on plates and 90 μl of bacterial overnight culture were spread onto 100-mm R2A agar plates and 90 μl onto 60-mm R2A agar plates. Plates were then incubated at 20°C for three days before use in the assays. E. coli were grown overnight in LB medium at 37°C and spread onto NGM plates. For the C. elegans developmental assay clean eggs were transferred onto 100-mm R2A agar plates with either HH01, the violacin biosynthesis mutant or onto NGM plates with E. coli, the standard laboratory food for C. elegans, as control. 6 replicate plates were assayed per bacterial strain. Development was monitored for 4 days at 20°C. Finally, for C. elegans survival assay 30 L4 larvae were picked onto each of 5 replicate 60-mm agar plates per bacterial strain and incubated at 20°C. Worms were scored as dead or alive by gentle probing with a platinum wire and alive worms were transferred onto fresh plates every day. Data were analyzed using Kaplan-Meier statistics and survival curves were compared using the log-rank test. Due to multiple testing a Bonferroni correction of the p-value was made leading to a significance level of p≤0.016.

Scanning and Transmission Electron Microscopy

For scanning electron microscopy cells were fixed in paraformaldehyde (1%) and glutaraldehyde (0.25%), dehydrated by ascending alcohol series and dried at the critical point with Balzers CPD 030 Critical Point Dryer (BAL-TEC, Schalksmühle Germany). After coating samples with gold using a sputter coater SCD 050 (BAL-TEC), scanning electron micrographs were taken with a LEO 1525 (Zeiss, Jena, Germany). For transmission electron microscopy cells were fixed in 2.0% (v/v) glutaraldehyde for 2 h and 1% (w/v) osmium tetroxide overnight. The embedding was performed according to Spurr [33]. Ultrathin sections were prepared with a diamond knife DiATOME ultra 45° (Diatome AG, Biel, Switzerland) on the ultramicrotome Ultracut E (Leica-Reichert-Jung, Nußloch, Germany) and were stained with 5% uranyl acetate and lead citrate. The sample examination was done with the transmission electron microscope Leo 906E (Zeiss, Jena, Germany) equipped with a CCD camera model 794.

To visualize flagella, a small volume (3 μl) of actively growing cells from a dense suspension was dropped on a copper grid, coated with Mowital polyvinylbutyral in 0.3% chloroform. Cells were allowed to attach for about 1 minute and excess liquid was removed by absorbent paper. The grid was placed with the upper side on a drop of uranyl acetate (2%) and dried after a few seconds. Pictures were taken with a transmission electron microscope as indicated above.

Violacin Measurement

Violacin quantification was performed following a previously published protocol with minor modifications [34]. To measure the violacin amount of a freshly grown culture a 2 ml sample was centrifuged for 2 minutes at 13,000 rpm and resuspended in 0.4 ml H2Odest. After vortexing, the cells were lysed by adding 0.4 ml 10% sodium dodecyl sulfate and incubated at room temperature for 5 minutes. Violacin was quantitatively extracted from this cell lysate by adding 0.9 ml of water-saturated butanol, briefly mixing, and centrifugation at 13,000 rpm for 5 minutes in a microcentrifuge. 0.5 ml from the upper butanol phase containing the violacin was mixed with 0.5 ml water-saturated butanol, centrifuged again at 13,000 rpm for 5 min. The absorbance was measured at 585 nm in a SmartSpec 3 Plus spectrophotometer (Bio-Rad Laboratories GmbH, Munich, Germany). For complementation tests with culture supernatants 10 μl of the supernatants were sterilized by filtration. 100 μl of this culture filtrate were added to 3 ml R2A media supplemented with ampicillin and 1% of growing HH02 cultures. For complementation tests with culture extracts 1 ml of sterile filtered supernatant was extracted using ethyl acetate (1:1). 10 μl extract of a dilution series was added to an exponentially growing HH02 culture. Usually the synthesized violacin was quantified after an incubation period of 24–48 hours at 22°C. For violacin measurements with HH02...
a positive control (i.e. HH01) was always included in the experiments. To minimize variations in violacein assays, R2A culture media were prepared with nutrients from the same batch of chemicals.

Molecular Methods, Mutagenesis and Electroporation of HH01

HH01 gene cloning steps were carried out with standard methods [29]. Transformation of HH01 was conducted by electroporation: For this HH01 was grown in R2A medium overnight and then diluted in 100 ml sterile medium to an optical density (OD600 nm) of 0.1. Cells were grown to an OD600 of 0.6 at 22 °C. For the electroporation cells were placed on ice for 30 minutes prior to centrifugation at 4,000 g at 4 °C for 10 minutes. After this initial centrifugation step the cells were resuspended in 1 ml ice-cold sterile H2Odest, transferred to a pre-chilled microcentrifuge tube, washed three times with 1 ml ice-cold H2Odest, and resuspended in H2Odest, to a final concentration of 10^10 cells/ml. The cells were mixed with 1 ml EZ-Tn5<sup>TM</sup>-<KAN-2> Tnp Transposome<sup>TM</sup> and up to 1 µg of plasmid DNA, respectively and transferred to a pre-chilled 1 mm-electroporation cuvette (BIO-RAD, Gene Pulser Cuvette, E. coli Pulser Cuvette). The electroporation pulse was applied at 2.5 kV, 25 µF, 200 Ω using a Bio-Rad Gene PulserXcell, 165–2662 (Bio-Rad Laboratories GmbH, Munich, Germany). The electroporated cells were immediately mixed with 500 µl R2A medium, incubated for two hours at 22 °C and spread on selective R2A agar plates.

In order to isolate violacein negative mutants of HH01 a transposon mutagenesis library was established by applying the EZ-Tn5<sup>TM</sup>-<KAN-2> Tnp Transposome<sup>TM</sup> Kit (Epigenetix, Madison, Wisconsin, USA). The kit was used following the manufacturers instructions and 8,500 mutants were generated. A total of 50 white or cream colored mutants were selected and the manufacturers instructions and 8,500 mutants were generated. A total of about 2,400 fosmid clones were generated. This equals a three-fold coverage of the HH01 genome. Ends of 672 recombinant fosmids were sequenced using ABI 3730xl automated DNA sequencers (Life Technologies, Darmstadt, Germany), processed with Phred, and assembled using Phrap. PCR-based techniques were used to close the remaining gaps using both genomic DNA and fosmid clones as templates. Further for gap closure DNA of selected fosmids was isolated using standard protocols and sheared for the construction of small insert plasmid libraries. These were constructed with the TOPO TA Cloning<sup>TM</sup> Kit (Invitrogen) using the pCR<sup>®</sup>-TOPO<sup>®</sup> vector and previously published protocols [35].

All manual editing steps were performed using the GAGP software package v4.5 and v4.6 [36]. Coding sequences (CDS) and open reading frames (ORFs) were predicted with YACOP [37] using the ORF-finders Glimmer, Critica and Z-curv. All predicted genes were manually curated based on GC frame plot analysis, the presence of ribosome-binding sites, and comparison to known protein-encoding sequences employing the Sanger Artemis tool v13 [38]. Functional annotation was initially performed with the ERGO software tool [39] and the IMG/ER (Integrated Microbial Genomes/Expert Review) system [40]. All CDS were manually curated and verified by comparison with the publicly available databases SwissProt, EMBL (InterProScan) GenBank, COG, and Prosite using the annotation software IMG/ER (https://img.jgi.doe.gov/cgi-bin/er/main.cgi). Gene products were classified into functional categories performing a BLAST search against the COG database [41]. Comparative analyses of different organisms was done as described previously [35] using a bidirectional BLAST algorithm, combined with a global sequence alignment based on the Needleman-Wunsch algorithm [42]. ORFs were assumed to be orthologs at a similarity higher than 30% and a BLAST e-value lower than 10e-21. Visualization of the chromosomes and other DNA sequences was done by using DNA Plotter [43].

This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession AMWD00000000 in two contigs. The version described in this paper is the first version, AMWD01000000.

**Results and Discussion**

**Key Traits of Janthinobacterium sp. HH01 and Overall Organization of its Genome**

HH01 was recently isolated from a watering can at the Loki-Schmidt botanical garden of the University of Hamburg. It grew well at temperatures ranging from 10°C to 28°C and it revealed
a doubling time of approximately 1.1 h at 22°C in R2A liquid cultures under aerobic conditions. Scanning (SEM) and Transmission (TEM) electron microscopic studies indicated that the cells were rod shaped with an average length of 2–4 μm (Figs. 1A & 1B). The cell surface was covered with small outer membrane vesicle-like or bleb-like structures and their occurrence was highly reproducible in different cell preparations (Figs. 1A & 1B). They were not observed when we analysed images from the closely related *Duganella violaceinigra* (Table S1) and are therefore no preparation artefacts. However, we can only speculate about their possible role during biofilm formation, transport of DNA and or secondary metabolite export as suggested earlier [44].

The phylogenetic relationship of HH01 was established using the neighbor-joining algorithm in MEGA5 [45]. The 16S rRNA phylogenetic analysis suggested that the closest relative of HH01 was *Janthinobacterium lividum* strain EU275366. Therefore we decided to group HH01 within the genus *Janthinobacterium* (Fig. 1C).

HH01 carries a single 7.10 Mb chromosome (Table 1, Fig. 2). Using pulse field gel electrophoresis no plasmids were detected (data not shown). The overall G+C content was 64.19% and the coding density was close to 92%. The bacterial chromosome encodes approximately 5,980 ORFs including a total of 84 tRNAs encoding all essential amino acids and 20 rRNA genes (Table 1) arranged in 7 rRNA clusters. Of the identified ORFs possible functions could be assigned to almost 80% of all predicted ORFs (Table 1 and Table S3).

The likely origin of replication [47] was identified based on G+C skew, the position of chromosomal replication initiator protein (DnaA, Jab_2c34420), the DNA polymerase III beta subunit (DnaN, Jab_2c34430) and the DNA gyrase B subunit (GyrB, Jab_2c34440). The further analysis of the genome suggested that the HH01 genome differs significantly from the genomes of closely related species: *Janthinobacterium* sp. Marseille, *Janthinobacterium* sp. PAMC 25724, and between strain GC3. For instance the overall genome size with 7.10 Mb was significantly

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Figure 1. Transmission and scanning electron microscopic images of HH01 as well as a 16S rRNA-based tree. A) Transmission and B) scanning electron microscopic images of HH01. Arrows indicate observed vesicles on the HH01 outer cell surface. Scale bars of 200 nm are indicated in the images. C) 16S rRNA-based tree showing the phylogenetic affiliation of HH01. The tree was constructed using the neighbor-joining algorithm in MEGA5 [45]. Topology was evaluated by bootstrap analysis (1000 repeats, with *N. europaea* as an outgroup). Only sequences longer than 1450 nucleotides of representatives of the next relative (≥97% similarity) species validly described were included. Numbers in parenthesis indicate the corresponding GenBank entries. Bootstrap values are shown as percentages at the branch points. The scale bar represents the expected number of changes per nucleotide position.

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larger than any of the above mentioned partial or complete genomes (Table 1, Fig. 2). Also its G+C content with 64.19% is significantly different to the G+C content of Janthinobacterium sp. PAMC 25724 and Janthinobacterium sp. Marseille. However, the careful comparison of the genome of HH01 with the genomes related species suggested that larger syntenic regions occur between its genome and the genomes of Janthinobacterium sp. Marseille, PAMC 25724 and GC3 (Fig. 2). The overall genome organization is summarized in Table 1 in comparison to closely related species.

Metabolic and Catabolic Traits of HH01, Aerobic- and Anaerobic-respiration

A careful analysis of the genome data suggest that the microbe is probably capable to grow on a wide variety of different carbon and energy sources under aerobic and anaerobic conditions (Table S3). For carbon metabolism it uses most likely the Embden-Meyerhof-Parnas pathway for the degradation of glucose or other C6 carbon compounds; but also genes were identified to allow growth on C3 and C4 carbon compounds. All essential and required genes for the Embden-Meyerhof-Parnas and citric acid cycle were identified; and a number of sugar kinases were observed that are involved in the activation of the respective carbon compounds. Under anaerobic conditions it appears to be capable to use nitrate as electron acceptor. All the essential subunits of the respiratory chains of genes linked to the resistance towards heavy metals. In-
distinct clusters of genes encoding the pilMNOPQ (Jab_1c07780-Jab_1c07820); and the pilVWXYZ and funT genes (Jab_1c21010-Jab_1c21060) (Table S5). Interspersed throughout the genome many other genes possibly involved in the assembly of type 4 pili were observed. It is notable that T4P are involved in DNA uptake and the assembly of a pseudopilus appears to be required for natural competence. DNA uptake pili are one subclass of T4 pili [53,54]. The binding of the exogenous DNA is usually facilitated by the comEA locus [54]. In HH01 the corresponding homologue is encoded by comA (Jab_2c04310). Other possibly competence-
The HH01 Genome Encodes Multiple Secretion Systems

In the genome of HH01 more than 80 loci were identified that are potentially linked to protein secretion. Altogether we observed the essential genes of the twin arginine (TAT)-pathway, the Sec multimeric system as well as the genes linked to the type 1, type 2 and the type 6 secretion systems (T1SS, T2SS, T6SS). The presence of many components linked to the assembly of T4P and the observation of major competence genes might suggest that HH01 is probably capable to incorporate foreign DNA fragments from its environment.

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In HH01 the essential components of the TAT pathway (tatA/E, tatB and tatC) [53] were identified on a conserved cluster (Jab_1c00810-Jab_1c00830). In addition HH01 appears to encode all required genes to build up the Sec multimeric transport system [47]. The signal recognition particle (SRP) mediates membrane targeting of translating ribosomes displaying a signal-anchor sequence [56]. In HH01 the FtsJ (Jab_1c13340) encodes the possible signal recognition particle-docking protein and Jab_2c29130 encodes the possible Ffh protein. T1SS comprise of three proteins that transport targeted proteins across both bacterial membranes into the extracellular space [57] and several genes linked to T1SSs were identified (Table S6). T2SSs, which are broadly conserved in Gram-negative bacteria, translocate exo-proteins (e.g. cellulosomes, lipases, etc.) from the bacterial periplasm into the surrounding media and are encoded by a set of 12–16 proteins [58]. HH01 encodes more than 30 genes possibly associated with T2SSs. The majority of these is observed in two conserved clusters each coding for 11 genes essential to the T2SS (Table S6). Altogether the relative high number of T2SS-associated genes and their organization in two distinct clusters suggests that HH01 encodes two distinct sets of T2SSs. This is rather unusual compared to the other janthinobacterial genomes. While Janthinobacterium sp. Marseille lacks a T2SS about 10–12 of the essential T2SS associated genes were identified in the draft genomes of Janthinobacterium sp. strains GC3 and PAMC 25724.

T6SSs are composed of 12–25 genes and they are often involved in protein transport of effector proteins into the eukaryotic host cells. Although, they are clearly related to pathogenicity they are observed in a wide range of pathogenic as well as many non-pathogenic microbes [59,60]. The respective genes in HH01 were located in one larger cluster comprising of at least 16 genes involved in T6SS assembly (Jab_2c19030-Jab_2c19160). In addition a somewhat smaller cluster of genes possibly linked to the T6SS assembly was identified (Jab_1c15640-Jab_1c15710). Homologues were also identified in Janthinobacterium sp. GC3 and PAMC 25724 but not in Janthinobacterium sp. Marseille. It is noteworthy, that in none of the sequenced janthinobacterial species a T3SS was identified. While HH01 however, appears to lack a T4SS, detailed blast searches identified T4SS in GC3 and PAMC 25724.

In summary these data suggest that HH01 is able to effectively secrete proteins but it lacks the typical pathogen-associated systems (i.e. T3SS and T4SS).

Secondary Metabolite Gene Clusters in HH01

A secondary metabolite analysis using the AntiSMASH program [61] of the HH01 genome revealed the presence of several gene clusters linked to the biosynthesis of potentially interesting metabolites (Table S7). Two clusters were identified that are coding for phytocene synthases (Jab_2c05760 and Jab_2c05810). Additionally, seven gene clusters encoding non-ribosomal peptide synthetases (NRPS) and one gene cluster encoding a NRPS-polyketide synthase (PKS) hybrid were identified [62]. NRPS and NRPS/PKS hybrids are important classes of enzymes responsible for natural product biosynthesis and clinically used examples for compounds derived from these enzyme systems are the antibiotic daptomycin [63] and the anti-cancer compound epothilone [64].

For the clusters 2-6 the structures were predicted using standard procedures [61] (Fig. 4). Compound 2 (from cluster 2) encodes a peptide with only L-amino acids, a high number of amino acids with carboxylic acid side chains and most likely a 4-amino- benzoic starting unit. Compound 3 is an 18 amino acid peptide with almost exclusively D-amino acids at its N-terminus and L-amino acids at its C-terminus, respectively (Fig. 4). The compound from cluster 4 is very difficult to predict and might be a lipopeptapeptide with a central γ-amino acid resulting from a PKS module, which is part of the NRPS-PKS hybrid. Compound 5 might be a siderophore as a lysine monooxygenase and a formyltransferase are encoded within the cluster in addition to the NRPS, which would produce a lipoundecapeptide (11 amino acids) with a high number of serine and threonine moieties. Compound 6 is a tetrapeptide with a reduced C-terminus resulting from the C-terminal reduction (Red) domain of the NRPS. The predicted structures are summarized in Figure 4 in a linear form but it has to be mentioned that they can also be cyclic. Finally one cluster was observed coding for the synthesis of the purple pigment violacein (Fig. 4 and 5).

Altogether 2.0% of the HH01 genome is dedicated to secondary metabolism. This is significantly less compared to well-known secondary metabolite producers like Streptomyces, which encode 20–30 clusters and thereby devote 5–7% of their genomes to the synthesis of secondary metabolites [65,66]. Nevertheless, due to the presence of unusual starting units (compound 2), N-methylation domains (compound 3 and 4) and use of unusual amino acids (compound 2, 4, and 5), HH01 might be an interesting model
organism for secondary metabolite analysis. Surprisingly, cluster 3, 5, and 7 encode 4'-phosphopantetheinyl transferases (PPTase). It would be interesting whether these PPTase enzymes are specific for their individual clusters or can functionally complement each other, as usually a single PPTase is responsible for the activation of all NRPS and PKS enzymes [67]. Within this framework, the occurrence of two PPTases with the same function is already regarded as a rather unusual feature in a genome of a Gram-negative microorganism [68].

HH01 Violacein Biosynthesis Affects C. elegans Survival and Nematode Development

The violacein biosynthesis genes identified in HH01 are located on a single and highly conserved operon comprising of the viaABCDE genes (Jab_2c08810-Jab_2c08850) (Fig. 5 and Table S7). In the 5’ direction of the viaD gene several histidine sensor kinases are encoded. Similar, in the 3’ direction of viaE a possible luxQ homologue is encoded (Jab_2c08870) (Fig. 5). It is noteworthy, that the closely related and sequenced janthinobacterial strains Marseille and GC3 did not appear to encode a functional violacein operon. Violacein is a bisindole and its biosynthesis has been observed in a wide range of different bacterial genera including Janthinobacterium, Chromobacterium [1], Collimonas [69], Pseudomonas [70], Pseudoalteromonas [71] and Duganella [72]. The violacein biosynthesis has been studied in the model organism C. violaceum in much detail [73–76]. The biosynthesis is managed through the gene products of the viaABCDE genes and by using tryptophan as a starting substrate [74,75,77–79]. In HH01 the

Figure 4. Predicted structures resulting from cluster 2–6. The predicted configuration is indicated by R- or S-nomenclature. All compounds are shown in the linear form but might be cyclic (for details see text). The HH01 genome was analyzed for secondary metabolite biosynthesis gene clusters using the AntiSMASH program [61]. Additionally, the genome was manually searched for genes encoding adenylation (A) and ketosynthase (KS) domains using a local BLAST server. All identified genes and/or gene clusters encoding the respective enzymes were then manually inspected and the predicted natural products resulting from the identified enzyme activities were drawn.

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violacein biosynthesis was most pronounced in late exponential growth phase and as expected from previous studies [80] the biosynthesis was stimulated by the addition of ampicillin (data not shown).

Since it is well known that violacein has a wide range of biocidal effects [6,81–86], we were interested to test if HH01 affects survival and development of *C. elegans*. In addition we wanted to elucidate, if possibly other factors or compounds produced by HH01 would influence *C. elegans* survival and development. In our tests the majority (>99%) of nematodes was dead after three days of exposure to HH01 (Fig. 6A). Furthermore our data suggested that HH01 has a strong influence on *C. elegans* development (Figs. 6B, 6C, 6D) as the nematodes did not develop over the larval stage in the presence of HH01 (Fig. 6D). In addition, we observed decreased locomotion and increased avoidance behavior (most worms were found outside the bacterial lawn) for the worms exposed to HH01 throughout the assays (data not shown).

However, a violacein- and tryptophan-negative mutant (HH5-1, Table S1) did not affect *C. elegans* survival or development. *C. elegans* incubated with HH5-1 developed normally (Fig. 6C) and survived as well as in the presence of the *E. coli* control strain OP50 (Fig. 6B). Therefore it is highly likely that the violacein produced by HH01 itself or another compound derived from the tryptophan biosynthesis pathway was responsible for the observed phenotypes.

This hypothesis was further supported by feeding *C. elegans* with *E. coli* cells carrying extra copies of the HH01 *vioA-E* genes (Fig. S1). Interestingly, Swem et al. [87] had observed that the toxicity towards *C. elegans* was not primarily caused by the purple pigment violacein produced by *C. violaceum*. Therefore, we speculate that in *C. violaceum* possibly several pathogen-related mechanisms are expressed that significantly affect *C. elegans* survival and which are not present in HH01 and/or which are not expressed in HH01 at the same level as in *C. violaceum*.

**HH01 does not Encode a N-acyl-homoserine-lactone (AHL) Regulatory Circuit**

To analyze possible quorum sensing (QS) regulated circuits in this novel microbe detailed blast searches were performed. The HH01 genes and ORFs possibly linked to the synthesis and sensing of cell-cell communication signaling molecules are summarized in Table S8. AHLs (AI-1) are the key signaling molecules in the cell density-dependent system of gene regulation in many Gram-negative bacteria and they are usually synthesized through a LuxI like protein [15]. Employing the amino acid sequences of various LuxI homologues for blastP searches, we could not detect a homologue in the HH01 genome. Similarly *Janthinobacterium* sp. Marseille and PAMC 25724 appear to lack the AHL-dependent regulatory circuit
Table 2. ORFs identified and involved in autoinducer biosynthesis in HH01 and closely related microorganisms.

| Synthase type | Janthinobacterium sp. | C. violaceum |
|---------------|----------------------|-------------|
|               | HH01 | GC3 | PAMC | 25724 | Marseille | ATCC 12472 |
| AHL           | –    | (+) | –    | –     | –        | +          |
| Ai-II         | –    | –   | –    | –     | –        | –          |
| JAI-1         | +    | –   | +    | –     | –        | –          |

–, autoinducer synthase not detected; +, autoinducer synthase identified; (+), weak similarity observed to the known autoinducer I synthases from C. violaceum CviI and related species.
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HH01 Lacks a Gene Linked to the Synthesis of Autoinducer 2 (AI-2)

The AI-2 molecules are synthesized by a wide range of bacteria and are thought to be involved in interspecies cell-cell communication [16,92]. They are composed of a furanone like ring structure [14] (and references herein) and their synthesis is accomplished through a S-riboyl homocysteine lyase, (LuxS). Its receptor is the LuxPQ complex [93]. Interestingly, no homologue of the AI-2 synthase could be identified in our strain suggesting that HH01 most likely does not synthesize AI-2. Similarly, no such homologues were identified in any of the other janthinobacteria (Table 2). However, at least two potential sensor kinases for AI-2 molecules were identified in HH01. One of these (Jab_2c08870) was in proximity of the violacein biosynthesis genes (Fig. 5). Thus it is very well possible that HH01 senses AI-2 molecules.

The HH01 Genome Encodes a Homologue of the V. cholerae cqsA and the L. pneumophila lqsA Gene

Additional searches for possible autoinducer synthases identified a possible homologue of the V. cholerae cqsA and the L. pneumophila
lqsA genes in HH01. In *V. cholerae* and *L. pneumophila* these genes are responsible for the synthesis of the CAI-1 and LAI-1 autoinducers, respectively [17] (and references herein). The corresponding HH01 gene (Jab_2e24350) was designated *jqsA*. It is adjacent with a two-component histidine sensor kinase designated *jqsS* (Jab_2e24340) and a two-component regulator gene designated *jqsR* (Jab_2e24350) (Fig. 7). *JqsA* reveals a 63% similarity (45% identity) to the corresponding *V. cholerae* El Tor strain N16961 protein and it revealed a 61% similarity (40% identity) with the *L. pneumophila* LqsA. However, the highest similarity of *JqsA* was observed to the homologue in the *Cupriavidus necator* strain N1. JqsA is 79% similar (59% identical) to the orthologous protein in *C. necator*. A detailed phylogenetic analysis of the HH01 JqsA suggested that it grouped closely with orthologous proteins that were mostly derived from non-pathogenic environmental isolates (Fig. 7). A previous analysis by Tiaden and colleagues had already identified homologues of CqsA and LqsA in 10 different bacterial genera [17]. Surprisingly, only for the *Vibrio cqsA* and the *Legionella lqsA* gene a functional role has yet been defined [19,94]. Therefore the identification of a JqsA/JqsS homologue in HH01 increases the diversity of known CAI-1/ LAI-1-like signaling systems (Fig. 7). Since *Vibrio cholerae* lacks a LqsR homologue in its cqsA/cqsS gene cluster [17] (Fig. 7) the presence of JqsR in the HH01 gene cluster might suggest that the janthinobacterial regulatory system is overall more closely related to the *Legionella*- than to the *Vibrio*-system.

In *V. cholerae* cqsA encodes a PLP-dependent aminomethyl-CoA transferase, that is responsible for the synthesis of CAI-1, a (5′)-hydroxytridecan-4-one-like molecule. In *V. cholerae* CAI-1 is involved in the repression of virulence, in biofilm dissolution and it has been shown play a major role in natural competence [19,21,95–97]. Furthermore CAI-1 in *Vibrio harveyi* appears to be involved in the regulation of luminescence [93]. A recent study also suggested that the *V. cholerae* CAI-1 has impact on *P. aeruginosa* biofilm formation and thereby acts as an interspecies signaling molecule [98]. In *Legionella* a structurally related molecule, but biofilm formation and thereby acts as an interspecies signaling also suggested that the involved in the regulation of luminescence [93]. A recent study [19,21,95–97]. Furthermore CAI-1 in *Vibrio harveyi* appears to be involved in the regulation of luminescence [93]. A recent study also suggested that the *V. cholerae* CAI-1 has impact on *P. aeruginosa* biofilm formation and thereby acts as an interspecies signaling molecule [98].

Interestingly, in all the microorganisms carrying a cqsA, jqsA or lqsA-like autoinducer synthase gene, the synthase gene is encoded adjacent to the cognate sensor kinase (Fig. 7) [17]. The observation that the synthase is always linked to the corresponding sensor protein suggests that they might be acquired via horizontal gene transfer as part of a conserved gene cluster. It is also notable that in the sequenced partial genomes of *Janthinobacterium* sp. PAMC 25247 and GC3 a JqsA homologue was identified (Table 2). It has been shown that the synthase is always linked to the corresponding sensor protein and it revealed a 61% similarity (40% identity) with the *L. pneumophila* LqsA. However, the highest similarity of *JqsA* was observed to the homologue in the *Cupriavidus necator* strain N1. JqsA is 79% similar (59% identical) to the orthologous protein in *C. necator*. A detailed phylogenetic analysis of the HH01 JqsA suggested that it grouped closely with orthologous proteins that were mostly derived from non-pathogenic environmental isolates (Fig. 7). A previous analysis by Tiaden and colleagues had already identified homologues of CqsA and LqsA in 10 different bacterial genera [17]. Surprisingly, only for the *Vibrio cqsA* and the *Legionella lqsA* gene a functional role has yet been defined [19,94]. Therefore the identification of a JqsA/JqsS homologue in HH01 increases the diversity of known CAI-1/ LAI-1-like signaling systems (Fig. 7). Since *Vibrio cholerae* lacks a LqsR homologue in its cqsA/cqsS gene cluster [17] (Fig. 7) the presence of JqsR in the HH01 gene cluster might suggest that the janthinobacterial regulatory system is overall more closely related to the *Legionella*- than to the *Vibrio*-system.

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**Functional Verification of the HH01 jqsA Gene**

Because of the obvious similarity of JqsA with the *V. cholerae* CqsA and the *L. pneumophila* LqsA we speculated that JqsA is possibly involved in the synthesis of a novel autoinducer molecule, which we designated JAI-1. Furthermore we postulated that this novel autoinducer might be involved in the regulation of the violacin biosynthesis. To verify these hypotheses, we added extracts of *E. coli* cell supernatants overproducing the *jqsA* gene to growing cultures of HH01 and measured the violacin production over time. These tests resulted in a more than 3-fold increased violacin production after 48 h (Fig. 8A). Similarly, extracts of cell supernatants from *E. coli* cells expressing the *V. cholerae* cqsA gene resulted in a 50% increased violacin production in HH01 (Fig. 8A). No such increase was observed, when extracts of supernatants of *E. coli* cells were added that carried the empty vector as control (Fig. 8A). Furthermore, HH01 carrying extra copies of the *jqsA*, the *cqsA* or the *lqsA* genes produced statistically significant (20–50%) higher amounts of violacin compared to the parent strain (Fig. 8B). In summary, these data supported the notion that the *jqsA* gene is most likely involved in the synthesis of a novel autoinducer molecule. These data also suggested that structurally not yet defined autoinducer molecule affected the violacin biosynthesis in HH01.

To further verify the role of the *jqsA* gene for violacin biosynthesis, we constructed a *jqsA* deletion strain. The corresponding mutant was designated HH02 (Table S1) and it was strongly affected in its capabilities to synthesize violacin. It synthesized 83% less violacin compared to the parent strain (Fig. 8C). As expected, extra copies of the *L. pneumophila* lqsA and the *V. cholerae* cqsA gene partially restored the phenotype of HH02 (Fig. 8C), suggesting that these genes are functional homologues.

Although, we have not yet identified the exact structure of the proposed JAI-1 molecule, we assume that it differs from the published structure for *V. cholerae* and *V. harveyi*. This hypothesis was based on data obtained during complementation tests of *V. cholerae* and *V. harveyi* cqsA mutants. It is notable, that the HH01 derived *jqsA* gene did not complement a *V. cholerae* O1 El Tor cqsA mutant (A1552ΔcqsA) with respect to the restoration of at least three tested phenotypes: biofilm formation (lack of repression in ΔcqsA), hemagglutinin/protease (Hap) activity and natural transformation (the latter two phenotypes are positively regulated by CAI-1 in the tested *V. cholerae* strain, [21,97] (Fig. S2). Similar, *V. harveyi* mutants that were deficient in the cqsA gene could not be restored with the *jqsA* gene (data not shown). The failure of the *JqsA* synthesized autoinducer to complement the *V. cholerae* and *V. harveyi* phenotypes might be a result of the relatively high specificity and selectivity of the cqsA/cqsS system [99] and these data are in line with earlier observation reported for the *C. LAI-1* signaling molecule [18]. It is however, noteworthy, that the *L. pneumophila* lqsA expression in *V. cholerae* MM920 triggered the induction of luciferase and thereby suggesting that the signal molecule produced by LqsA is recognized by the corresponding CqsS sensor kinase.[18].

Altogether our data suggest that the HH01 jqsA gene encodes a homologue of CqsA and LqsA. Although the genome analysis and the initial tests with the *jqsA* deletion strain clearly suggest that *jqsA* is of importance for violacin biosynthesis, the final proof that it synthesizes indeed a novel autoinducer molecule has yet to be furnished. Therefore future work will now have to determine the structure of the postulated JAI-1 signaling molecule and its impact on other regulatory circuits in janthinobacteria and closely related species.

**Conclusions and outlook**

The complete genome analysis of HH01 has revealed that its genome shows a high degree of synteny with the already known janthinobacterial genomes. However HH01 also carries several unique features: First, it encodes at least 7 PKS/NRPS clusters. Second, its genome is significantly larger than any of the previously reported janthinobacterial genomes; and third it revealed the presence of an autoinducer system hitherto functionally only characterized in *Vibrio* and *Legionella*. In summary the analysis of the HH01 genome has not only given us a better understanding of the core janthinobacterial genome it has perhaps most importantly, increased the diversity of the known CqsA- and
Figure 7. Phylogenetic analysis of cqsA-, jqsA- and lqsA-like autoinducer synthases in Gram-negative bacteria. The neighbor-joining phylogenetic analysis was performed using the MEGA5 software [45] version 5.1 and comparing amino acid sequences of the corresponding synthases. Homology searches for orthologous proteins were done in the IMG genome database in September 2012 with 3,938 completed or draft bacterial genomes present. The autoinducer synthase sequences of the following strains are included, numbers in parenthesis indicate the corresponding accession number: R. eutropha H16 (YP_728640), C. necator N-1 (YP_004680649), C. taiwanensis LMG19424 (YP_001796752), C. fungivorans Ter331 (YP_004750816), P. naphthalenivorans CJ2 (YP_983733), R. tataouinensis TTB310 (YP_004671950), R. vannielii ATCC 17100 (YP_004010985), S. shabanensis E1L3A (ZP_08550556), N. mobilis Nb-231 (ZP_01127067), B. xenovorans LB400 (YP_555293), C. phaeobacteroides DSM 266 (YP_912394), C. ferrooxidans DSM 13031 (ZP_01385258), C. limicola DSM 245 (YP_001942557), P. aestuarii DSM 271 (YP_001903666), Photobacterium sp. SKA34 (ZP_01162832), V. harveyi ATCC BAA-1116 (YP_001448208), V. splendidus 12B01 (ZP_00990208). a) Marinomonas sp. is summarized for: M. mediterranea MMB-1 (ATCC 700492), M. posidonia IVIA-PO-181. b) L. pneumophila is summarized for: Philadelphia-1 (YP_096734), Paris (YP_125092) and Lens (YP_127984). Bacterial genera that have previously not been reported [17] to contain a cqsA/lqsA homologue are marked with an asterisk.

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LqsA-like autoinducer synthase proteins. Therefore HH01 might be a suitable model organism for studying the importance of the CqsA/LqsA/JqsA regulatory networks in the background of a non-pathogenic microbe. This appears tempting, since HH01 is genetically accessible and mutations can be generated with relative ease in this microbe. Within this framework it is noteworthy that with the exception of this study only little is known about the ecological role of the CqsA/LqsA/JqsA system in non-pathogenic microbes. Thus future work will now have to unravel the importance of the JqsA/JqsS regulatory circuit with relevance to its importance for survival and growth of this microorganism in its natural aquatic habitat.

Supporting Information

Figure S1 Survival of C. elegans in the presence of E. coli DH5α carrying extra copies of the HH01 vioA-E genes in pDrive. For the survival assay 30 L4 larvae were placed onto agar plates. The worms were transferred onto new plates every day and incubated at 20°C. Alive and dead worms were counted during transfer. The treatment groups were violacein expressing E. coli DH5α (n = 5) and empty vector E. coli DH5α (n = 5) as a control. For experiments with E. coli a single colony was picked, transferred into 100 ml LB medium containing 100 μg/ml ampicillin and incubated on a shaker at 37°C overnight. It was then used to seed NGM Agar plates containing 100 μg/ml ampicillin. 500 μl of the overnight culture was spread onto large plates (φ 9 cm) and 80–90 μl were pipetted into the center of small plates (φ 6 cm). The plates were then incubated at 20°C overnight before use. (TIF)

Figure S2 V. cholerae ΔcqsA mutant cannot be complemented by the HH01 jqsA. A) The enhanced biofilm formation phenotype of a V. cholerae ΔcqsA strain cannot be reverted by provision of jqsA in trans. The indicated V. cholerae

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**Figure 8. Effect of the known or assumed autoinducer molecules of different strains on the HH01 or HH02 violacein production.**

A) Effects of extracted possible JAI-1 and CAI-1 autoinducer molecules on HH01 parent strain violacein production. The autoinducers were extracted from E. coli DH5α, carrying either the jqsA or the cqsA gene in pBBR1MCS-2. Autoinducers were purified as described in the material and methods section. 10 μl of these extracts were applied to HH01 growing cultures during early exponential growth phase. The control strain carried the empty vector. B) Effects of extra copies of the HH01 jqsA, the V. cholerae cqsA and the L. pneumophila lqsA genes in the parent strain HH01. The corresponding genes were inserted into the broad host range vector pBBR1MCS-2 (Table S1). C) Violacein produced by the ΔjqsA mutant HH02, HH01 and HH02 carrying either the native jqsA, the V. cholerae cqsA or the L. pneumophila lqsA in pBBR1MCS-2. HH02 carrying an empty pBBR1MCS-2 produced similarly low amounts of violacein compared to HH02 without the empty control vector. Error bars indicate the simple standard deviations. Violacein values were calculated per ml of culture supernatant and normalized with respect to culture density at OD600 nm.
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strains were incubated statically within 24-well plates and biofilm formation was scored after 24 hours of growth using a standard crystal violet approach. The average of two independent biological replicates with triplicate samples is shown. The error bars indicate the standard deviation. B) The lowered hemagglutinin/protease (Hap) activity of the ΔcqsA strain cannot be rescued by jqsA. The respective V. cholerae strains were grown in LB medium until late exponential phase. At that time aliquots were taken from the culture and the haemagglutinin/protease (Hap) activity was measured using azocasein as a substrate. The average of two independent biological replicates with triplicate samples is shown. C) jqsA cannot restore natural transformation in a V. cholera ΔcqsA mutant. The bacterial strains were tested for chitin-induced natural transformation. Average transformation frequencies of two independent experiments are indicated on the Y-axis, <d.l., below detection limit. V. cholerae strains tested in all panels: A1552/ pBBR1MCS2-wt (WT with vector as control; lanes 1 and 2), ΔcqsA/pBBR1MCS2-mutant with vector as control; lanes 3 and 4), ΔcqsA/pBBR1MCS2-jqsA (mutant with plasmid containing jqsA gene; lanes 5 and 6), and ΔcqsA/pBBR1MCS2-cqsA (mutant with plasmid containing cqsA gene; lanes 7 and 8). Strains were grown in the absence (odd numbers) or presence (even numbers) of 1 mM IPTG.

(TIF) Table S1 Bacterial strains and plasmids used in this study. amp, ampicillin; gm, gentamycin; nal, nalidixin; km, kanamycin; cyc, cycloheximide; tet, tetracycline.

(DOCX) Table S2 Primers used for cloning and mutant construction.

(DOCX) Table S3 ORFs and genes predicted in the HH01 genome. This file contains the submission list of the Janthinobacterium sp. HH01 genome. The corresponding GenBank files are available at: DDBJ/EMBL/GenBank accession AMVD0000000. Genes/ORFs on contig 1 are indicated with jab_1xxxxx. Genes/ORFs on contig 2 are indicated with jab_2xxxxx.

(XLSX) Table S4 Predicted Genes/ORFs linked to resistance mechanisms in HH01.

(DOCX) Table S5 Predicted genes and ORFs possibly linked to cell appendages and motility in HH01. Proteins/Genes associated with Type 4 pilus assembly are in blue color.

(DOCX) Table S6 Genes/ORFs linked to protein secretion.

(DOCX) Table S7 Secondary metabolite gene clusters in HH01. NRPS (non-ribosomal peptide synthetases) and PKS (polyketide synthase) proteins are shown in bold. Adenylation (A) with specificity determined by NRPS predictor 2, thiolation (T), condensation (C), condensation/epimerization (C/E), epimerization (E), Coenzyme A ligase (CAL), methyltransferase (MT), thioesterase (TE), reduction (RED), ketosynthase (KS), acyltransferase (AT), ketoreductase (KR).

(DOCX) Table S8 HH01 genes possibly linked to cell-cell communication regulatory circuits.

(DOCX) Acknowledgments

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Author Contributions

Provided the pTS21 construct: HH LMU. Conceived and designed the experiments: CH GS WRS. Performed the experiments: CH A. Pochlein FSH MS I.KM KD A. Pohlen MB LP AB CU GT ES APR EB. Analyzed the data: CH A. Pochlein A. Pohlen FSH HS MB KJ HBB RD CS WRS. Contributed reagents/materials/analysis tools: A. Pohlen KD HS MB LP KJ EB HBB RD WRS. Wrote the paper: CS FSH WRS.

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