

Tropodithietic Acid Production in *Phaeobacter gallaeciensis* Is Regulated by *N*-Acyl Homoserine Lactone-Mediated Quorum Sensing[∇]

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The production of *N*-acyl homoserine lactones (AHLs) is widely distributed within the marine *Roseobacter* clade, and it was proposed that AHL-mediated quorum sensing (QS) is one of the most common cell-to-cell communication mechanisms in roseobacters. The traits regulated by AHL-mediated QS are yet not known for members of the *Roseobacter* clade, but production of the antibiotic tropodithietic acid (TDA) was supposed to be controlled by AHL-mediated QS in *Phaeobacter* spp. We describe here for the first time the functional role of *luxR* and *luxI* homologous genes of an organism of the *Roseobacter* clade, i.e., *pgaR* and *pgaI* in *Phaeobacter gallaeciensis*. Our results demonstrate that the AHL synthase gene *pgaI* is responsible for production of *N*-3-hydroxydecanoylhomoserine lactone (3OHC₁₀-HSL). Insertion mutants of *pgaI* and *pgaR* are both deficient in TDA biosynthesis and the formation of a yellow-brown pigment when grown in liquid marine broth medium. This indicates that in *P. gallaeciensis* the production of both secondary metabolites is controlled by AHL-mediated QS. Quantitative real-time PCR showed that the transcription level of *tdaA*, which encodes an essential transcriptional regulator for TDA biosynthesis, decreased 28- and 51-fold in *pgaI* and *pgaR* genetic backgrounds, respectively. These results suggest that both the response regulator PgaR and the 3OHC₁₀-HSL produced by PgaI induce expression of *tdaA*, which in turn positively regulates expression of the *tda* genes. Moreover, we confirmed that TDA can also act as autoinducer in *P. gallaeciensis*, as previously described for *Silicibacter* sp. strain TM1040, but only in the presence of the response regulator PgaR.

Quorum sensing (QS) is a population-density-dependent chemical communication system used by bacteria to control cellular functions through excreted small signaling molecules that interact directly to regulate the expression of sets of genes within certain bacterial species (10). By far the most intensively investigated family of intercellular signal molecules are the *N*-acylhomoserine lactones (AHLs), which are most common among Gram-negative bacteria and have become a paradigm for bacterial intercellular signaling. AHLs are synthesized by a LuxI type AHL synthase and directly bind to their cognate LuxR type transcriptional regulator proteins, thus activating the expression of target genes mediating a specific response (18, 51). In *Roseobacter* genomes a considerably high complexity of these canonical *luxI/luxR*-like genes, as well as “orphan” *luxR* genes, are present (50), which is consistent with the experimental evidence for AHL production by many members of the *Roseobacter* clade (6, 23, 32, 34, 46, 52, 53, 56). AHLs have also been detected in roseobacters obtained from samples of marine snow (23) and marine sponge (34, 52), emphasizing AHL-mediated QS as one of the most common intercellular communication mechanisms in roseobacters (20).

The various AHL compounds described for roseobacters differ from one another in chain length, ranging from C₈-HSL to C₁₈-HSL, and modifications on their acyl side chain by one or two double bounds or by a 3-hydroxyl or 3-oxo group (8, 56). Often multiple AHLs are produced by a single strain, such as

C₁₈en-HSL and *N*-3-hydroxydecanoyl-homoserine lactone (3OHC₁₀-HSL) excreted by *Phaeobacter inhibens* strain T5 (56). A new class of HSLs, the *p*-coumaryl-HSL, is produced by *Silicibacter pomeroyi* in the presence of *p*-coumaric acid (46) and seems also to be important for the host-symbiont relationship of *Phaeobacter gallaeciensis* (49).

Members of the *Roseobacter* clade and especially *Phaeobacter* spp. are often associated with diverse marine eukaryotes or organic particles (9), suggesting that surface association and colonization is central to the ecology of clade members (20, 50). Generally, the production of signaling molecules is, in addition to motility, chemotaxis, and the production of antimicrobial metabolites, a key factor in colonization success (50). However, despite the widespread occurrence of AHLs within the *Roseobacter* clade, to our knowledge, no traits regulated by AHL-mediated QS have been described thus far for these organisms.

Production of the antibiotic tropodithietic acid (TDA) is a trait of a subgroup of marine *Rhodobacteraceae* composed of the genera *Phaeobacter*, *Silicibacter*, *Ruegeria*, and *Pseudovibrio*. In all TDA-producing strains investigated thus far, the production of TDA co-occurred with the formation of a yellow-brown pigment (5–8, 19, 21–23, 38). The pigment was produced under the same conditions as TDA (6–8, 24, 38) and depends on the same genes (22). Therefore, the production of the pigment was used as a reliable indicator for TDA production in various studies (5–8, 19, 21–23, 38). For *Phaeobacter* sp. strain 27-4 it was speculated that the pigment could be a polyphenolic polymer, while in contrast to TDA the pigment has no antimicrobial activity (8). In *Phaeobacter* sp. strain Y4I a bipyridyl pigment was identified, whose production correlated with antagonistic behavior (50), but it is unknown

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype, phenotype, and/or characteristic(s)	Source or reference ^b
Strains		
<i>Phaeobacter gallaeciensis</i>		
DSM 17395	Wild-type strain	DSMZ
WP38	DSM 17395 <i>pgaI</i> ::Gm; Gm ^r	This study
WP52	DSM 17395 <i>pgaR</i> ::EZTn5; Gm ^r	This study
WP75	DSM 17395 <i>tdaA</i> ::EZTn5; Gm ^r	This study
<i>Escherichia coli</i>		
DH5 α	F ⁻ <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG</i> ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>U169 hsdR17</i> (r _K ⁻ m _K ⁺) λ ⁻	24
TransforMax EC100D pir-116	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 Δ <i>lacX74 recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ ⁻ <i>rpsL</i> (Str ^r) <i>nupG pir-116</i> (DHFR)	Epicentre
<i>Pseudoalteromonas tunicata</i>		
DSM 14096	DSM 14096	DSMZ
<i>Agrobacterium tumefaciens</i> NTL4		
	AHL biosensor strain	12
Plasmids		
pBBR1MCS-2	Broad-host-range vector; Km ^r	28
pBBR1MCS-5	Source of gentamicin resistance cassette; Gm ^r	28
pBluescript KS(+)	Cloning vector; Amp ^r	MBI Fermentas
pMB21	PCR product <i>pgaI</i> -f <i>pgaR</i> -r cloned into EcoICRI site of pBluescript KS(+); Amp ^r	This study
pMB22	Gm cloned into EcoICRI site of pMB21	This study
pMOD3gm	Gm cloned into SmaI site of EZ-Tn5pMOD<R6Kgori/MCS>	This study
pRK415iq	Broad-host-range expression vector; Tc ^r	27
pRKpgaR	PCR amplified <i>pgaR</i> ligated into EcoICRI site of pRK415iq; Tc ^r	This study
pBB0808	PCR amplified <i>lac pgaR</i> from pRK415iqpgaR cloned into pBBR1MCS-2; Km ^r	This study
EZ-Tn5pMOD<R6Kgori/MCS>	Transposon construction vector	Epicentre

^a Amp^r, ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Gm^r, gentamicin resistance.

^b DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.

whether this is the yellow-brown pigment produced by the other organisms mentioned above.

TDA seems to play an important role in colonization success (42) due to the competitive advantage over other species (5, 8, 19, 37) and was proposed to be important for the symbiosis of *P. gallaeciensis* and *Silicibacter* sp. strain TM1040 with microalga, where TDA protects the algal host from bacterial pathogens (19, 49). The competitive advantage also has practical applications, since the presence of *Phaeobacter* and *Ruegeria* spp. in turbot larval farms was found to suppress growth of marine pathogens such as *Vibrio anguillarum* (37) and enhanced survival of the larvae (45). Thus, the use of these roseobacters as probiotics in aquaculture facilities (25, 36, 38) or as antifouling agents in marine systems (15, 40) is of great interest.

Due to the correlation of TDA and AHL production in several organisms, it was speculated that the expression of TDA is controlled by AHL-mediated QS (8, 23, 32). This was supported by detection of antimicrobial activity only at high cell densities in the AHL producing *Phaeobacter* strain 27-4 (6, 7). In contrast, *Silicibacter* sp. strain TM1040 lacks genes coding for known QS systems and does not produce common AHL molecules (6, 35), but it was suggested that TDA can act as autoinducer of its own synthesis in this organism (19). Biosynthesis of TDA in *Silicibacter* sp. strain TM1040 requires the *tda* genes *tdaA*, *tdaB*, *tdaC*, *tdaD*, *tdaE*, and *tdaF* that are specifically involved in TDA biosynthesis (22), with TdaA acting as a positive regulator of *tdaCDE* gene expression (21).

In the present study we investigated the role of AHL-mediated QS in secondary metabolite production of *Phaeobacter gallaeciensis* DSM 17395. We identified genes with homology

to known *luxR-luxI* quorum-sensing systems and could show that these are essential for the synthesis of TDA. Furthermore, the results revealed that the LuxR-type transcriptional regulator in strain DSM 17395 (PgaR) and 3OHC₁₀-HSL or, alternatively, TDA are required to induce the expression of *tdaA* and subsequently for the production of TDA.

MATERIALS AND METHODS

Bacterial strains and media. The strains and plasmids used in the present study are listed in Table 1. *P. gallaeciensis* strains were routinely grown on marine broth 2216 medium (MB; BD Biosciences, Franklin Lakes, NJ). Since the pH increased in MB cultures to pH 8.6 and AHLs are not stable at high pH (58), the MB was buffered with 0.3 M HEPES (Carl Roth, Karlsruhe, Germany) and adjusted to pH 7.0 (8). Unless otherwise stated, liquid cultures of *Phaeobacter* strains were grown in Erlenmeyer flasks at 28°C on a rotary shaker at 90 rpm and were inoculated with 2% preculture grown in the same medium and under the same conditions. When required, antibiotics were added to a final concentration of 60 μ g of kanamycin/ml or 25 μ g of gentamicin/ml. *Escherichia coli* strains were used for mutagenesis and grown in Luria-Bertani (LB) medium at 37°C with shaking at 100 rpm or on corresponding solid agar medium (15 g of agar liter⁻¹). When required, gentamicin, kanamycin, ampicillin, or tetracycline were added to a final concentration of 25, 25, 250, or 10 μ g ml⁻¹, respectively.

Bioassay for AHL synthesis. For the detection of AHLs from *P. gallaeciensis* DSM 17395 and derived mutants the biosensor strain *Agrobacterium tumefaciens* NTL4(pZLR4) was used (12). The well diffusion assay was carried out as described by Ravn et al. (43) with some minor modifications. The ABT-agar was supplemented with 40 μ g of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Carl Roth)/ml. Next, 50 μ l of the test substance was added to each well. The plates were incubated 24 h at 20°C before the diameters of the AHL-induced zones surrounding the wells were measured. As a negative control, acetonitrile and MB were used. For the R-3OHC₁₀-HSL standard curve, dilutions of R-3OHC₁₀-HSL ranging from 1 to 100 nmol (1, 5, 10, 25, 50, and 100 nmol) were prepared in acetonitrile. The absence of a blue zone in the lawn indicated that the R-3OHC₁₀-HSL concentration was less than 5 nM.

Extraction and identification of AHLs from *P. gallaeciensis*. Cells of the *P. gallaeciensis* wild type and the *pgaI*- and *pgaR*-negative mutants were grown, each in 1-liter Erlenmeyer flasks with 100 ml of MB containing 2% Amberlite XAD-16 (Sigma-Aldrich, Germany). Then, 2-ml precultures were added to the medium, followed by incubation for 21 h at 28°C on a rotary shaker (90 rpm) to an optical density at 600 nm (OD_{600}) of ca. 2.0. The AHLs were extracted according to the method of Wagner-Döbler et al. (56), except that a 10:1 dichloromethane-water solvent system was used instead of methanol for the extraction of XAD-16 resin. The two phases were separated, and the organic phase was evaporated to dryness and resolved in low amount of dichloromethane (14). Analysis of the dichloromethane extracts was carried out on a GC System 7890A connected to a 5975C mass selective detector (Agilent Technologies, Palo Alto, CA) with split/splitless injector. The instrument was equipped with a fused-silica-capillary column (HP-5 ms, 30 m by 0.25 mm [inner diameter], 0.25- μ m film thickness; Agilent Technologies). The temperature conditions were as follows: 5 min at 50°C, followed by increases at 5°C/min to 320°C and operation in splitless mode. The carrier gas was helium at a flow rate of 1.2 ml min⁻¹. The injection volume was 1 μ l, and the front inlet temperature was held at 250°C. The configuration of 3OHC₁₀-HSL was determined as described previously (53).

Characterization of the antibiotic produced by strain DSM 17395. *P. gallaeciensis* DSM 17395 was incubated in MB for 20 h at 28°C and 90 rpm. Cells were removed first by centrifugation (6,000 \times g, 15 min, 4°C) and then by filtration through a 0.22- μ m-pore-size mixed-cellulose-ester membrane (Carl Roth), resulting in cell-free supernatant. The pH of the supernatant was adjusted to 3.0 with 2 M HCl, followed by extraction with 20 ml of ethyl acetate repeated two times. The vacuum-dried ethyl acetate extract was dissolved in 1 ml of acetonitrile and analyzed on a high-pressure liquid chromatography (HPLC) system (Göbel Instrumentelle Analytik, Hallertau, Germany) equipped with a diode array detector and an evaporated light-scattering detector. Samples were separated on a Nucleodur 100-5 C₁₈ EC column (Macherey-Nagel, Düren, Germany) by using an acetonitrile-water gradient system containing 0.1% trifluoroacetic acid, started with 20% acetonitrile, which was increased linearly to 100% in 25 to 30 min. Extraction and HPLC analysis were carried out by BioViotica Naturstoffe GmbH, Göttingen, Germany.

Assay of TDA production. Antimicrobial activity of *P. gallaeciensis* DSM 17395 and derived strains against *Pseudoalteromonas tunicata* DSM 14096 was determined by agar diffusion tests according to the method of Brinkhoff et al. (5) with the following modifications. Exponentially grown cultures of the target organism were adjusted to an OD_{600} of 0.0025, and 1 ml was spread onto each plate with half-strength MB 2216 agar. Cells were removed from cultures first by centrifugation (6,000 \times g, 15 min, 4°C) and then by filtration through a 0.22- μ m-pore-size mixed-cellulose-ester membrane (Carl Roth). From this cell-free filtrate, 20 μ l was applied onto an antibiotic assay disk (Rotilabo-test leaves; Carl Roth), followed by incubation for 20 h at 20°C. For the generation of standard curves, pure TDA (BioViotica Naturstoffe GmbH, Göttingen, Germany) dissolved in dimethyl sulfoxide (DMSO) at 1 mM and a standard set containing seven concentrations (1, 2.5, 5, 10, 25, 50, and 100 μ M) and DMSO as a negative control were used. For each assay, an internal standard curve was created in triplicates by calculating the ratio of TDA concentrations and the mean diameter of the zones. Only standard curves with a correlation coefficient (r^2) of >0.97 were used for quantification. The detection limit of this assay was less than 2.5 μ M.

Time course of TDA production. TDA production by *P. gallaeciensis* DSM 17395 was measured during growth on MB at 28°C and 90 rpm. Aliquots were collected at various times (Fig. 1) and used for determination of antimicrobial activity (see above). The OD_{600} was measured by spectroscopy (DU520; Beckman Instruments, Fullerton, CA). Bacterial cell counts were derived from 200 μ l of bacterial culture fixed with 2% (final concentration) formaldehyde and stored at -80°C until further processing. Bacteria were enumerated after staining with SybrGreen I by epifluorescence microscopy as described previously (31), after aggregates were dissolved with sonication. Therefore, the bacteria were thawed, diluted 1:10 in phosphate-buffered saline, and lysed by sonication on ice (Bandelin Sonopuls HD2000 with a SH 213 booster horn and an MS 73 tip) three times for 10 s with a 10-s break at 15% power. A 500- to 1,000- μ l subsample of the corresponding dilution was filtered through a black 0.22- μ m-pore-size polycarbonate filter (GE, 25-mm diameter). SybrGreen I-stained cells were counted with an Axiolab 2 microscope (Zeiss, Jena, Germany). The filtered sample volume yielded 30 to 100 stained cells in the counting grid. For each sample, 15 grids and a minimum of 500 cells per filter were enumerated.

Transformation of *P. gallaeciensis*. Cells were prepared for electroporation according to the procedure of Dower et al. (14a) with minor changes. *P. gallaeciensis* cells were grown in MB at 28°C with shaking at 90 rpm to a cell density of 7.5×10^8 cell/ml and washed four times with 10% glycerol. Subsequently, the cell pellet was resuspended in 10% (vol/vol) glycerol to yield an approximate

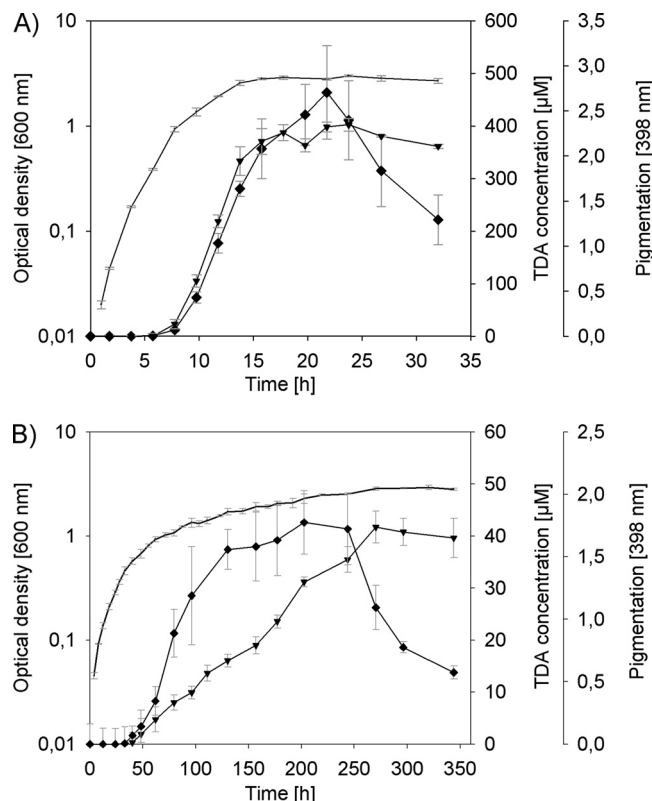


FIG. 1. (A and B) Time course of TDA and pigment production of *P. gallaeciensis* grown on MB at 28°C under shaken (A) and stagnant (B) conditions. Bacterial growth (—) is shown as absorption at 600 nm as the average of three parallel cultures. TDA production (◆) of cell-free culture fluids was measured using agar diffusion assays with *P. tunicata* as target strain ($n = 3$). The TDA concentration was calculated by the use of an internal standard curve with pure TDA ($r^2 = 0.98$). Pigmentation (▼) of cell-free culture fluids was measured by spectroscopy at 398 nm ($n = 3$).

concentration of 10^{10} to 10^{11} cells/ml. Aliquots of 40 μ l were stored at -80°C until use. For transformation, 40- μ l portions of the cells were mixed with 1 μ l of DNA, and the mixtures were electroporated at 13.75 kV/cm. Afterward, the cells were suspended in 1 ml of MB and incubated for 4 h at 28°C and 90 rpm. The cell suspension was diluted and plated on half-strength MB agar containing the appropriate antibiotic and incubated for at least 48 h at 28°C.

Construction of strains and plasmids. To construct the *pgaI* gentamicin insertion mutant, the gene, including the flanking regions, was amplified from chromosomal DNA of the *P. gallaeciensis* wild-type strain DSM 17395 in a PCR using the primers 5'-ACAGTGAACACCCCTGAATATGC-3' and 5'-GCAGC TGCCTAAATGTCAGGC-3'. The resulting PCR product was ligated into EcoICRI-digested pBluescript KS(+) to yield pMB21. The gentamicin resistance gene, amplified from pBBR1MCS-5 using a Gm primer set (28), was ligated into the EcoICRI site of pMB21, yielding plasmid pMB22. Electroporation of *P. gallaeciensis* wild-type cells with pMB22 was carried out as described above. Subsequent selection on gentamicin yielded the insertion mutant strain WP38 (*pgaI::gm*, Table 1). The insertion was verified by PCR analysis.

The *P. gallaeciensis tdaA* mutant strain WP75 and the *pgaR* mutant strain WP52 were obtained by transposon mutagenesis using the EZ-Tn5 pMOD3<R6Kgori/MCS> transposon construction vector (Epicentre, Madison, WI). For preparation of the EZ-Tn5 transposon a gentamicin-resistant gene was amplified from pBBR1MCS-5 using the Gm primer set (28) and ligated into the SmaI site of the EZ-Tn5 pMOD3<R6Kgori/MCS> transposon construction vector, giving rise to pMOD3gm. The PvuII restriction fragment of pMOD3gm was electroporated, along with the EZ-Tn5 transposase, into electrocompetent cells of wild-type DSM 17395. Transposon mutants were selected on half-strength MB agar with gentamicin. Mutants were screened for altered pigmentation compared to the wild-type by replica plating on MB agar. The locations of

TABLE 2. Primer and hydrolysis probes used in this study for qRT-PCR

Gene	Primer	Sequence (5'–3')	Amplicon (nt)	UPL probe
<i>rpoB</i>	rpoB54-f	CTGGACGAAGATGGCAAGTT	60	#54
	rpoB54-r	GTAGTCGCCGGACTGACG		
<i>pgaI</i>	pgaI26-f	CACGTGATGTGAGTGGTAATGTC	75	#26
	pgaI26-r	AATGTTGAAATTCGCATGAT		
<i>tdaA</i>	tdaA47-f	CGGATCTGGAAGTCGCTTT	63	#47
	tdaA47-r	CGTTGCGAATATCGTCCA		
<i>tdaB</i>	tdaB149-f	GCATGCCACTTGAGGTTATG	66	#149
	tdaB149-r	AAAGTTTTTCCGGCAGCA		
<i>tdaE</i>	tdaE70-f	ATGGGGCGTCTGTACTATGG	76	#70
	tdaE70-r	TGGAGAAAATCAAATGCCTCA		
<i>tdaF</i>	tdaF152-f	ATCACGATATTCTGGCCATTCT	60	#152
	tdaF152-r	CATGCGTATCGCAGACA		
<i>paaG</i>	paaG18-f	CCGAAGGCAACTGGATCA	76	#18
	paaG18-r	GCCTCATCCTGCACCTTG		
<i>paaZ2</i>	paaZ32-f	ACCTGCAAGAAAATCACACC	61	#32
	paaZ32-r	AACAGTCCCAACGCACATCT		

the insertions of the transposon were determined by rescue cloning of the candidate mutant as described by Geng et al. (22).

Complementation assays. For a complementation study, the *pgaR* gene was PCR amplified from chromosomal DNA of *P. gallaeciensis* DSM 17395 using the primers 5'-ATGGCAACCAAAGTTAATCTTGATC-3' and 5'-TTAAATGATGATCAGCCCGAGAC-3' and cloned into the EcoCRI sites of pRK415iq (27), resulting in pRKpgaR. The open reading frame of *pgaR* and the *lac* promoter were PCR amplified from pRKpgaR using the primers 5'-CCAATACGCAA CCGCTCTC-3' and 5'-TTAAATGATGATCAGCCCGAGAC-3' and ligated into the EcoCRI site of pBB1MCS-2 (28), giving rise to plasmid pBB0808. The resulting plasmid was subsequently electroporated into the *pgaR* mutant (WP52), and transformants harboring pBB0808 were selected on kanamycin. The antimicrobial activity and AHL production of WP52 pBB0808 were determined in bioassays as described above.

The mutation in *pgaI* (strain WP38) was tested for complementation by exogenous addition of culture fluid from the wild-type DSM 17395, synthetic AHLs, or pure TDA. Sterile-filtered culture fluid obtained after growth of the wild-type in MB for 14 h under shaking conditions (see Fig. 1A) was added to a culture of WP38 (4% [vol/vol]). The AHLs C₄, C₈, C₁₂, C₁₄, 3oxoC₆-AHL, and R-3OHC₁₀-HSL were prepared as described by Wagner-Döbler et al. (56), and 3OHC₁₂-HSL, C₁₀-HSL, and 3oxoC₁₀-HSL were obtained from Sigma-Aldrich. Stock solutions of the AHLs were prepared in acetonitrile and added to MB with a final concentration of 100 nM unless otherwise stated. Pure TDA was obtained from Biotoc Naturstoffe GmbH. Final concentrations of 0.1, 1, and 10 μM TDA were used for complementation analysis.

RNA preparation and quantitative real-time PCR. The wild-type and the derived mutants (WP38, WP52, and WP75) were grown in 20 ml of MB broth supplemented with 0.3 M HEPES, adjusted to pH 7.0, in a 100-ml Erlenmeyer flask at 20°C and 90 rpm. At an OD₆₀₀ of ca. 1.5, 1 ml of the culture was centrifuged, and the total RNA was extracted from the resulting cell pellet, using a High-Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. For complete removal of genomic DNA, we performed an additional DNase I degradation step with subsequent inactivation, according to the manufacturer's recommendation (Roche Diagnostics GmbH). cDNA was synthesized from DNA-free RNA with a transcript first-strand cDNA synthesis kit (Roche Diagnostics GmbH) using anchored-oligo(dT)₁₈ primer and random hexamer primer. Quantitative reverse transcription-PCR (qRT-PCR) was performed by using probes of the Universal ProbeLibrary (Roche Diagnostics GmbH) and primer pairs (TIP MolBiol, Berlin, Germany) designed for specific genes using the Universal ProbeLibrary Tool (Table 2). qRT-PCR assays were carried out using DNA probe master (Roche Diagnostics GmbH) and the following thermal cycling parameters: preincubation at 95°C for 10 min, followed by amplification in 45 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s, and finally 40°C for 30 s (1 cycle) in a LightCycler 480 detection system (Roche Diagnostics GmbH). Target cDNA levels were analyzed with the comparative cycle time (C_T) method for real-time RT-PCR, and all values were normalized relative to the expression of *rpoB*. The reported values show the average and range of three biological replicates assayed each in triplicates. Calibration curves from each gene were created, which cover a range of at least 4 orders of magnitude with a correlation coefficient (r²) of at least 0.98.

The expression of *tda* genes in the *pgaI*-negative background (strain WP38)

was also measured by qRT-PCR after exogenous addition of 10 μM TDA, as well as after the addition of 100 nM R-3OHC₁₀-HSL, using the conditions described above. Purified TDA, as well as R-3OHC₁₀-HSL, was individually added to triplicate samples and compared to uninduced samples (also in triplicates). The RNA extraction, cDNA synthesis, and qRT-PCR analysis were all performed as described above.

RESULTS

Growth-phase-dependent TDA production under shaken and stagnant culture conditions. Strain DSM 17395 produced an antimicrobial compound when grown in MB (Fig. 1). The retention time of 11.8 min and the characteristic UV spectra of the HPLC peak were identical to those of pure TDA published previously (8, 29), demonstrating that TDA is the antibacterial metabolite produced by strain DSM 17395. In this way, TDA was produced when grown in MB under shaken, as well as under stagnant culture conditions, even though TDA was produced in 10-fold-higher concentrations in shaken cultures (Fig. 1). Under both conditions, however, nearly the same maximal cell density of ca. 2 × 10⁹ cells ml⁻¹ was reached. TDA production and concomitant formation of a yellow-brown pigment was undetectable under both culture conditions during the early exponential growth phase and increased considerably during the late exponential phase, reaching maximum levels in the early stationary phase (Fig. 1). In shaken cultures, the production of the brownish pigment was found to be strictly correlated with the TDA synthesis (Fig. 1A), whereas in stagnant cultures the pigment production lagged behind the TDA production but remained below the maximal absorption observed for shaken cultures. Further experiments were carried out with shaken cultures due to the higher TDA production.

Identification of the AHL(s) produced by *P. gallaeciensis*. Increasing the amounts of pure 3OHC₁₀-HSL added to the wells in the AHL bioassay with *A. tumefaciens* NTL4 caused an increase in the diameters of the induced blue zones surrounding the wells, which showed a high correlation coefficient (r² = 0.998) (Fig. 2A). A concentration-dependent induction of *A. tumefaciens* was also observed when pure TDA was applied (Fig. 2A). This means that the induction by sterile filtered culture fluid of the wild-type strain DSM 17395 (Fig. 2B) could be due to TDA and/or AHLs. To find out whether AHLs were produced by strain DSM 17395, a sterile-filtered culture fluid of a TDA-negative mutant (strain WP75) was used. A clearly induced zone surrounding the well was visible in the bioassay with this strain (Fig. 2B), verifying that AHLs were produced by *P. gallaeciensis*.

For identification of the produced AHL(s), a gas chromatography-mass spectrometry (GC-MS) analysis of a dichloromethane extract from culture fluid of *P. gallaeciensis* was performed. The GC-MS analysis was difficult due to presence of large peaks of diketopiperazines from the amino acid-containing MB (56) that masked the AHLs (Fig. 3). The results shown in Fig. 3A revealed, however, presence of a compound showing the typical fragmentation pattern of AHLs: *m/z* 143 and 102 (11, 56) at 42.5 min. Identification of the AHL as 3-hydroxy-HSL was supported by the prominent ion at *m/z* 172 at the same retention time (56). The AHL present in the extract was R-3OHC₁₀-HSL, which was unambiguously identified by its mass spectrum and its gas chromatographic retention index, that correspond to those of synthetic 3OHC₁₀-HSL (Fig. 3D).

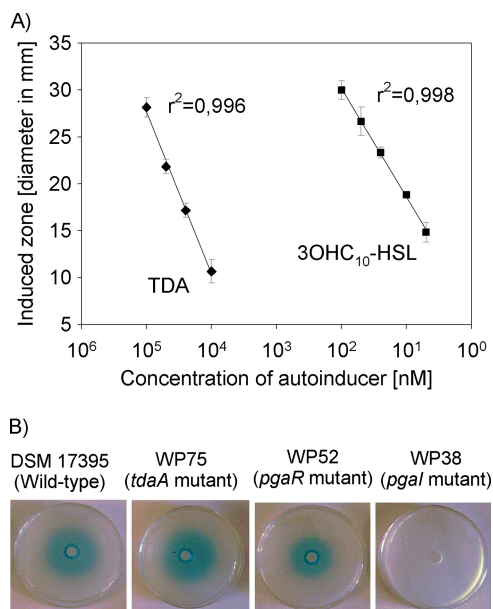


FIG. 2. *A. tumefaciens* NTL4 well diffusion assay. (A) Relationship between the concentration of 3-*N*-hydroxy-decanoyl-L-homoserine lactone (3OHC₁₀-HSL) and TDA in well diffusion assays and resulting diameters of induced blue zones. Error bars indicate standard deviations ($n = 3$). (B) Well diffusion assays with sterile-filtered culture fluids from late-exponential-phase cultures of the wild-type strain DSM 17395, the *tdaA* mutant strain WP75, the *pgaR* mutant strain WP52, and the *pgaI* mutant strain WP38. The culture fluids were added to wells in agar containing *A. tumefaciens* NTL4 and X-Gal. Blue zones surrounding the wells indicate induced β -galactosidase activity.

The absolute configuration proved to be pure “(R)-” as described for *Phaeobacter inhibens* T5.

The *pgaI* gene product catalyzes the synthesis of R-3OHC₁₀-HSL. The genome of *P. gallaeciensis* comprises genes coding for a common LuxR-LuxI-type QS system with an AHL synthase PgaI (accession number ZP_02146368) and a LuxR family transcription factor PgaR (accession number ZP_02146367). The derived amino acid sequence of *pgaR* showed 34% identity to RaiR of *Rhizobium etli* (accession number O54452), which was predicted to be a typical LuxR-type QS regulator (44) and harbored a characteristic helix-turn-helix domain and a typical autoinducer binding domain. The derived amino acid sequence of *pgaI* shows 36% identity to the AHL-synthase RaiI of *Rhizobium etli* (accession number O54451), which is required for the synthesis of autoinducer molecules that are involved in the restriction of nodule number (44).

Culture fluids of mutants lacking *pgaI* (strain WP38) or *pgaR* (strain WP52) were analyzed with the AHL assay and GC-MS analysis. It was found that the sterile-filtered supernatant of the *pgaR* mutant gave a positive signal in the AHL bioassay, whereas the supernatant of the *pgaI* mutant was negative (Fig. 2B). The GC-MS analysis confirmed these results because the typical fragmentation pattern of AHLs, as found for the wild type (Fig. 3A), was also found in extracts of the *pgaR* mutant (Fig. 3B), but not for the *pgaI* mutant (Fig. 3C). Thus, it is obvious that *pgaI* is responsible for the synthesis of R-3OHC₁₀-HSL, and PgaI can still produce R-3OHC₁₀-HSL in a *pgaR*-

negative background, indicating that PgaR is not essential for the production of R-3OHC₁₀-HSL.

The *pgaI*-*pgaR* QS system is essential for TDA production in *P. gallaeciensis*. Cell-free culture fluids of the *pgaI* and the *pgaR* mutant grown in MB were tested for antimicrobial activity to determine whether TDA production is influenced by the *pgaR*-*pgaI* QS system. Culture fluids of both mutants did not inhibit *Pseudoalteromonas tunicata* in agar diffusion assays, suggesting that they did not produce TDA in contrast to the wild-type (Fig. 4B). Due to the coincident production of TDA with the formation of a yellow-brown pigment, mutations in *pgaI* and *pgaR* also resulted in loss of pigmentation (Fig. 4C). TDA and pigment production were restored for the *pgaI* mutant by the addition of sterile-filtered supernatant from the wild type (Table 3), as well as by the exogenous addition of R-3OHC₁₀-HSL (Table 3 and Fig. 4). The wild-type phenotype was restored dependent on the concentration of R-3OHC₁₀-HSL, i.e., TDA synthesis was restored starting at a concentration of 0.5 nM (Table 3). The exogenous addition of other synthetic AHLs than R-3OHC₁₀-HSL to MB cultures of the *pgaI* mutant failed to restore TDA and pigment production, indicating that the induction is highly specific to R-3OHC₁₀-HSL (Table 3). However, pigment and TDA production were also restored in cultures of the *pgaI* mutant by the addition of 1 μ M pure TDA (Fig. 4 and Table 3).

The fact that PgaR is also required for production of the pigment and TDA was confirmed by recovery of the wild-type phenotype for strain WP52 (*pgaR* mutant) by a plasmid-borne copy of *pgaR* (pBB0808). Culture fluid of WP52 carrying pBB0808 showed the same antimicrobial activity as the wild type (Fig. 4B), whereas pigment production was not completely restored (Fig. 4C). In contrast to the *pgaI* mutant, the wild-type phenotype of the *pgaR* mutant could not be restored by the exogenous addition of pure R-3OHC₁₀-HSL or TDA.

QS can play a crucial role in bacterial growth, such as was shown for *Yersinia pseudotuberculosis* or *Rhodobacter sphaeroides* (1, 39). We measured growth of the wild type, the *pgaI* mutant (strain WP38), and the *pgaR* mutant (strain WP52) in MB to test whether QS affects the growth behavior of *P. gallaeciensis*. Figure 4A shows that the *pgaI* and *pgaR* mutants reached lower ODs compared to the wild type, but both strains reached the same cell density as the wild type (ca. 2×10^9 cells ml⁻¹). The difference between the ODs is based on the absorption of the yellow-brown pigment present in the cell-free culture fluid of the wild type. The absorption at 600 nm of a sterile-filtered culture fluid of the wild type after an incubation time of 14 h was 0.6, and the absorption of the cells suspended in fresh MB was 1.8, which is similar to the maximal ODs reached by the *pgaI* and *pgaR* mutants. In all complementation experiments in which the TDA and thus pigment production was restored as well (Fig. 4B and C), strains reached the same maximal OD as the wild type (Fig. 4A). The wild-type-like multicellular star-shaped growth behavior, as previously described for *Phaeobacter* sp. strain 27-4 (8) and other members of the *Roseobacter* clade (6, 55), was also observed in liquid cultures of the *pgaI* and *pgaR* mutants (data not shown). Thus, our results suggest that QS does not affect the growth and aggregation behavior of *P. gallaeciensis*.

Influence of *pgaR* on the expression of genes essential for TDA biosynthesis. Since the *pgaR* and *pgaI* mutants pro-

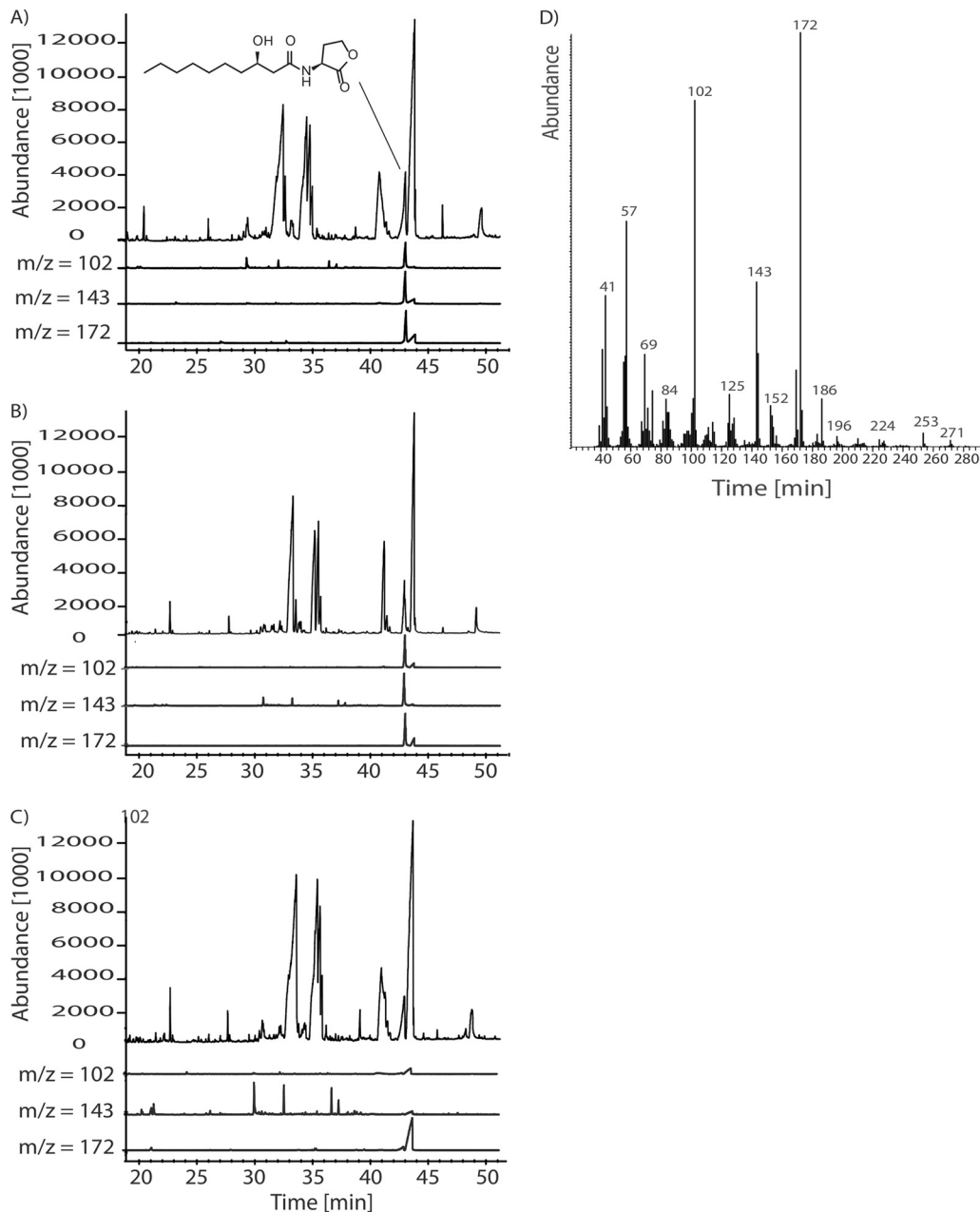


FIG. 3. (A to C) Total ion chromatograms and the characteristic ion traces m/z 102, 143, and 172 (slightly offset for improved visibility) of culture extracts of the *P. gallaeciensis* wild-type strain DSM 17395 (A), *pgaR* mutant strain WP52 (B), and *pgaI* mutant strain WP38 (C). (D) Mass spectrum of R-3OHC₁₀-HSL/peak at 42.6 min.

duced no TDA (Fig. 4B), we assumed that the *tda* genes *tdaA* to *tdaF*, whose expression is essential for the synthesis of TDA in *Silicibacter* sp. strain TM1040 (22), are QS controlled in *P. gallaeciensis* DSM 17395. The *tda* genes *tdaA*, *tdaB*, *tdaE*, and *tdaF* and *paaZ2*, as well as *paaG*, are also known to be essential for TDA biosynthesis in strain DSM 17395 (S. Thole et al., unpublished data). Therefore, qRT-PCR was used to compare the transcription levels of the above mentioned genes in the wild-type and the *pgaR* mutant strain WP52. Measurements of these transcripts showed that the genes *tdaB*, *tdaE*, *tdaF*, and *paaZ2* were clearly

downregulated in the *pgaR* mutant compared to the wild type (Fig. 5A). Transcription of *paaG* was not affected in the *pgaR* mutant as expected, because *paaG* is essential for the primary metabolism and not specific to TDA biosynthesis (22). Figure 5A also shows that the transcription level of *pgaI* was 5-fold higher in the wild type than in the *pgaR* mutant, suggesting that the transcription of *pgaI* is slightly positively regulated by PgaR. Interestingly, the expression of *tdaA*, coding for a transcriptional regulator (19, 21), decreased by a factor of 51 in the *pgaR* mutant compared to the transcription level of the wild type, indicating that tran-

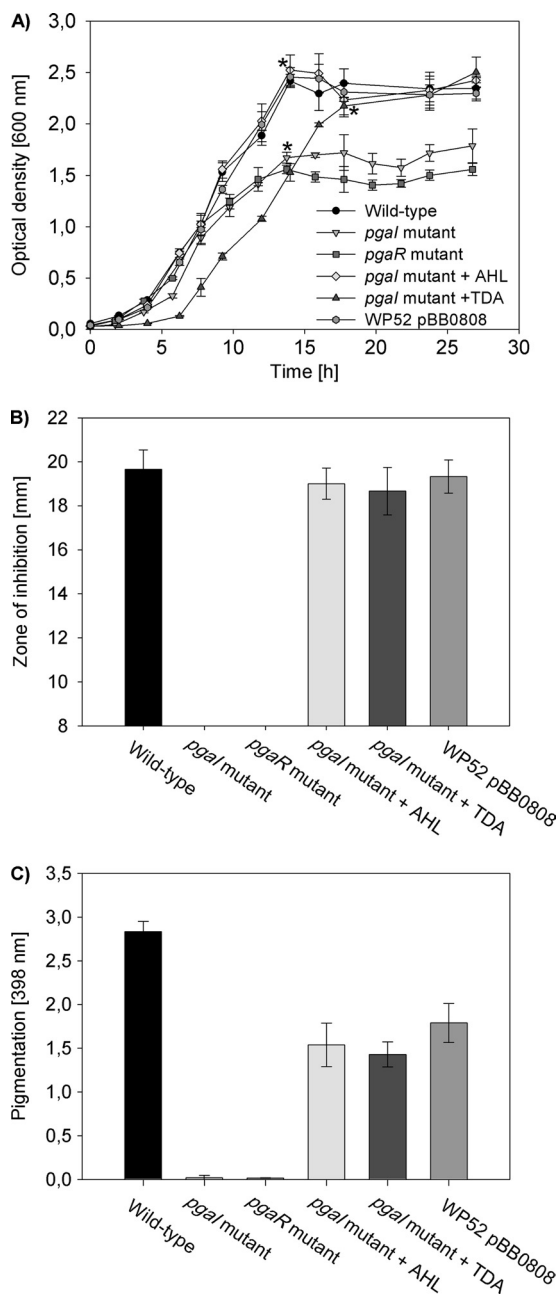


FIG. 4. Growth, antimicrobial activity, and pigmentation of *P. gallaeciensis* DSM 17395 and derived mutants. The results demonstrate that the PgaI-PgaR QS system of *P. gallaeciensis* is essential for the TDA and pigment production. (A) Growth curves of *P. gallaeciensis* wild-type strain DSM 17395, *pgaR* mutant strain WP52, strain WP52 carrying pBB0808, *pgaI* mutant strain WP38, and strain WP38 in the presence of either added 100 nM R-3OHC₁₀-HSL or 10 μ M TDA. The cultures were grown at 28°C in MB under shaken conditions. Growth was measured by OD₆₀₀ in triplicate cultures. The asterisks indicate points in time where culture fluids were taken for the analysis shown in panels B and C. (B) Zone of inhibition against *P. tunicata* determined in agar diffusion assays from cell-free culture fluids of different *P. gallaeciensis* strains at indicated points in time ($n = 3$). (C) Pigmentation of different *P. gallaeciensis* strains measured by spectroscopy at 398 nm ($n = 3$).

TABLE 3. Relative concentration of TDA produced by the *pgaI* mutant strain WP38 grown in MB medium after the addition of AHLs, TDA, or culture fluids

Exogenous addition (final concn)	Relative amt (%) of TDA ^a
C ₄ -HSL (100 nM).....	0
3oxoC ₆ -HSL (100 nM).....	0
C ₈ -HSL (100 nM).....	0
C ₁₀ -HSL (100 nM).....	0
C ₁₂ -HSL (100 nM).....	0
3OHC ₁₂ -HSL (100 nM).....	0
C ₁₄ -HSL (100 nM).....	0
3oxoC ₁₀ -HSL (100 nM).....	0
R-3OHC ₁₀ -HSL (0.1 nM).....	0
R-3OHC ₁₀ -HSL (0.5 nM).....	62 (15)
R-3OHC ₁₀ -HSL (1 nM).....	90 (27)
R-3OHC ₁₀ -HSL (100 nM).....	87 (18)
TDA (0.1 μ M).....	0
TDA (1 μ M).....	72 (21)
TDA (10 μ M).....	65 (15)
DMSO.....	0
Acetonitrile.....	0
Culture fluids	
Wild type.....	83 (13)
<i>pgaR</i> mutant (WP52).....	72 (18)
<i>pgaI</i> mutant (WP38).....	0
MB.....	0

^a The values are the average relative percentages of TDA from three independent cultures compared to the wild-type percentage; the range \pm is indicated in parentheses.

scription of *tdaA* is also PgaR dependent in *P. gallaeciensis* (Fig. 5A).

Expression of the *tda* genes was measured in a *tdaA*-negative background to verify that *tdaA* is essential for transcription of the *tda* genes in *P. gallaeciensis*. Therefore, we used the *tdaA* mutant strain WP75 (Table 1), which is unable to produce TDA. The results of the qRT-PCR revealed that *tdaB*, *tdaE*, and *tdaF* were strongly downregulated in a *tdaA*-negative genetic background and confirmed that TdaA is a transcriptional activator for the *tda* genes in *P. gallaeciensis* (Fig. 5A). Thus, it is not surprising that a decrease of the *tdaA* transcription level in the *pgaR* genetic background results in a decrease of transcripts of the other *tda* genes (Fig. 5A).

R-3OHC₁₀-HSL or TDA act as autoinducers depending on RaiR. Expression analyses in the *pgaI*-negative mutant strain WP38 revealed that the transcription levels of the *tda* genes, including *tdaA* (28-fold), were clearly reduced, indicating that R-3OHC₁₀-HSL induces the expression of *tdaA* (Fig. 5B). Addition of R-3OHC₁₀-HSL to cultures of the *pgaI* mutant resulted in an increase in *tdaA* transcription up to the wild-type level (Fig. 5B). We investigated the *tdaA* expression in the *pgaI* mutant after the exogenous addition of TDA since the exogenous addition of TDA also restored pigment and TDA production (Fig. 4B and C). The addition of TDA resulted in a *tdaA* transcription level comparable to that of the wild type (Fig. 5B). Thus, the expression of *tdaA* requires R-3OHC₁₀-HSL or TDA for maximal transcription.

DISCUSSION

Monitoring TDA production as a function of growth (Fig. 1) showed that TDA accumulated during the transition of the exponential to the stationary phase. This suggests that produc-

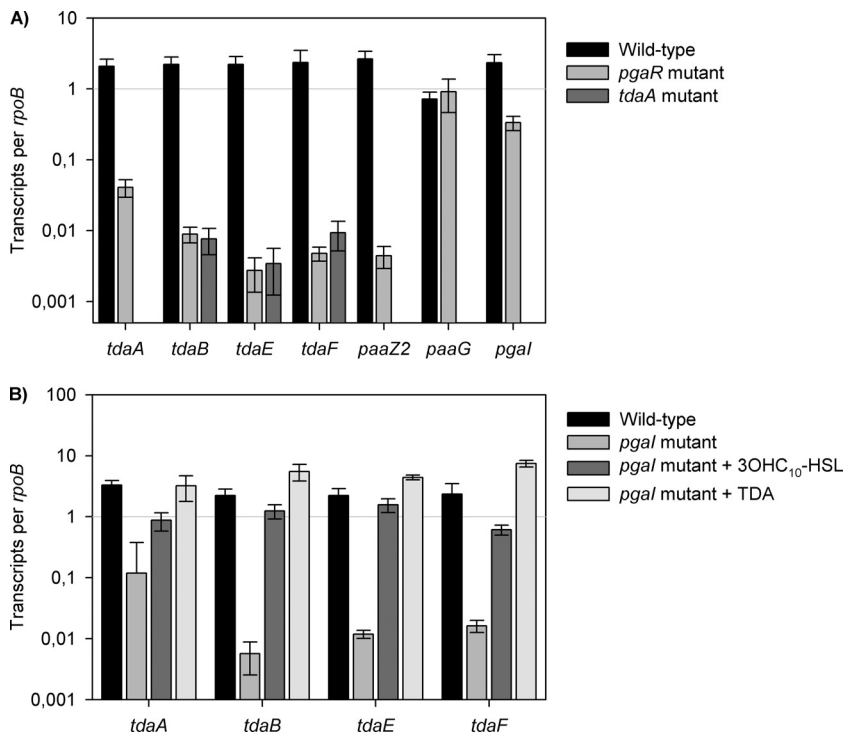


FIG. 5. Effects of disrupting *pgaR*, *pgal*, and *tdaA* genes on the expression of genes essential for TDA biosynthesis at the RNA level. (A) Relative transcription levels of *tdaA*, *tdaB*, *tdaE*, *tdaF*, *paaZ2*, *paaG*, and *pgal* from wild-type *P. gallaeciensis* DSM 17395 (black bars), the *pgaR* mutant strain WP52, and the *tdaA* mutant strain WP75 grown in liquid MB and determined by using qRT-PCR analysis of reverse-transcribed RNA samples (see Materials and Methods for details). (B) Relative transcription levels of *tda* genes of the wild type and the *pgal* mutant strain WP38 grown without or in the presence of either exogenously added R-3OHC₁₀-HSL or TDA. The transcription is shown as the relative expression of each target gene compared to *rpoB*. The error bars are derived from three independent cultures assayed each in triplicate.

tion of TDA in *P. gallaeciensis* strain DSM 17395 is cell density dependent and typifies the production of a classical secondary metabolite. Furthermore, this was a first hint that production of TDA could be controlled in a quorum-dependent manner as previously postulated for *Phaeobacter* sp. strain 27-4, due to the production of AHLs and TDA at high cell densities (8). Growth of strain DSM 17395 under static conditions lead to a decreased growth rate and resulted in 10-fold-lower level of TDA compared to cultivation under shaken conditions (Fig. 1B). It was shown that different *Phaeobacter* spp. produced TDA when grown under shaken and stagnant conditions, whereas *Ruegeria* spp. expressed this phenotype only during stagnant conditions (37), which was also observed for *Silicibacter* sp. strain TM1040 and *Phaeobacter* sp. strain 27-4 (7, 8). Other than DSM 17395, only *Silicibacter pomeroyi* DSS-3, of those that have been assessed to date, can produce elevated levels of TDA under shaken conditions (6). The striking discrepancy between these strains in TDA production indicates differences in response to environmental conditions and in the regulation of TDA production.

Production of AHLs was previously shown for several members of the *Roseobacter* clade (6, 23, 32, 46, 56). An influence of the AHLs on hydrolytic enzymatic activities and antibiotic production in marine snow was already suggested for these bacteria by Gram et al. (23). However, no traits or genetic elements regulated by AHL-mediated QS were identified yet,

although the genome sequences provide some obvious clues (50).

In our study we could demonstrate that PgaI is an AHL-synthase that produces R-3OHC₁₀-HSL in *P. gallaeciensis* (Fig. 3), an AHL that is also produced by other members of the genus *Phaeobacter* (e.g., *Phaeobacter* sp. strain 27-4 and *Phaeobacter inhibens* strain T5), which were shown to produce TDA, too (5, 8, 56). In addition to these strains, AHLs were often detected in other members of the *Roseobacter* clade that showed antimicrobial activity, like *Roseovarius* spp., *S. pomeroyi* DSS-3 (6), *P. gallaeciensis* 2.10 (41), and *Phaeobacter* sp. strain Y4I (50). Bacteria of the *Silicibacter-Ruegeria* subgroup that were isolated from marine sponges were all identified as AHL producers (34). However, there is not always a correlation between AHL production and antimicrobial activity since *Silicibacter* sp. strain TM1040 produces no common AHL molecules (6, 35) but TDA (22). This suggests a different regulation mechanism for the production of TDA in *Silicibacter* sp. strain TM1040 compared to the AHL-producing strains, including *P. gallaeciensis* DSM 17395. Differences between these two organisms are also reflected by production of highest TDA concentrations of strain TM1040 under static conditions, whereas strain DSM 17395 produces 10-fold-higher amounts of TDA at shaken culturing conditions (Fig. 1). As a conclusion, the data mentioned above suggest adaptation of the strains to different ecological niches.

In both QS-negative mutants (WP38 and WP52, Table 1), the expression of *tdaA* was clearly reduced compared to the wild type (Fig. 5), indicating that the regulatory protein TdaA, which is required for TDA synthesis, was regulated by the *pgaR-pgal* QS system. In *Silicibacter* sp. strain TM1040 it was shown that the expression of *tdaAB* was not affected by the type of culturing; only the expression of *tdaCDEF*, and therefore *tdaA*, was suggested to be expressed constitutively in *Silicibacter* sp. strain TM1040 (19). Nevertheless, *tdaA* positively controls the expression of other *tda* genes, and *tdaA* is essential to respond to extracellular signaling molecules for the induction of TDA biosynthesis in *P. gallaeciensis* and *Silicibacter* sp. strain TM1040.

In the *pgaI* mutant strain WP38, the wild-type phenotype was restored by addition of R-3OHC₁₀-HSL but not by the exogenous addition of other AHLs (Table 3). This indicates a high level of specificity of the PgaR receptor protein toward R-3OHC₁₀-HSL in *P. gallaeciensis*. However, TDA production, as well as the expression of *tdaA*, was also restored in cultures of the *pgaI* mutant by addition of pure TDA (Fig. 4B and 5B). In *Silicibacter* sp. strain TM1040 it was shown that the addition of TDA induces expression of *tdaC* and *tdaF*, whereas *tdaA* seems to be expressed constitutively (19). Furthermore, we showed that in *P. gallaeciensis* TDA production induced by either R-3OHC₁₀-HSL or TDA depends on the response regulator PgaR. Here, PgaR is necessary to induce the expression of *tdaA*, and TdaA consequently induces the expression of *tda* genes (Fig. 5A). For *Silicibacter* sp. strain TM1040 it was hypothesized that TDA is necessary for the maximal activity of TdaA (19), which subsequently induces the expression of *tdaCDEF* (21). However, the findings that TDA is not required for heterologous *tdaC* expression in *E. coli* and that TDA is not necessary for binding of TdaA to *tdaC* (21) were contradictory to this hypothesis and were already discussed by Geng and Belas (21).

Induction of the monitor system of *A. tumefaciens* (Fig. 2A) indicates that TDA could also act as an autoinducer in bacteria other than roseobacters. *A. tumefaciens* is known to respond to many AHLs of different length and substitutions of the acyl-chain moiety and therefore displays a broad detection window, which depends on the specificity of the TraR receptor protein (12, 16, 59). Biosensor strains are often used for AHL detection, but it was also observed that they can be activated by compounds other than AHLs, such as diketopiperazine or mimic compounds (2, 26). Therefore, a direct detection method such as HPLC coupled with MS, as shown in this and previous studies (8, 53, 56), is necessary to unequivocally demonstrate the presence of AHLs. Despite the fact that TDA and R-3OHC₁₀-HSL do not show structural similarity and the only superficial resemblance are the ring structure and the presence of polar and apolar sides in both compounds, it is possible that TDA competes for the same PgaR-binding side as R-3OHC₁₀-HSL. Similar findings were described for diketopiperazine, which antagonize the AHL-mediated induction of bioluminescence (26). TraR of *A. tumefaciens* as well as PgaR of *P. gallaeciensis* respond significantly better to 3OHC₁₀-HSL than TDA. In both strains a 2,000-fold-higher concentration of TDA was necessary compared to R-3OHC₁₀-HSL (Table 3; Fig. 2A). This is in accordance with the observation that a LuxR homolog responds best to the AHL produced by its

cognate LuxI homolog (26). Furthermore, the threshold concentration of the signal molecule depends on the nature of the signal, the transport and diffusion rate (4), which obviously varies considerably between TDA and R-3OHC₁₀-HSL. It is also possible that both classes of signal molecules, TDA and R-3OHC₁₀-HSL, can act as signals in a hierarchical regulatory cascade that controls the production of TDA. This was often observed such as in the plant pathogen *Ralstonia solanacearum*, where a non-AHL signal, a 3-hydroxypalmitic acid methyl ester modulates pathogenicity in conjunction with AHLs (13, 17).

In several bacteria production of secondary metabolites is regulated hierarchically and *luxR-luxI* systems are often part of such networks (30, 33, 47). These QS signaling systems are often global regulators of gene expression that allow ordered expression of hundreds of genes (48). Often global regulatory genes control antibiotic production and cause pleiotropic effects, like *afsR*, *afsB*, *bldA*, and *ndgR* of *Streptomyces coelicolor* (3, 57). With our study we demonstrate that the antibiotic and pigment production in *P. gallaeciensis* is coordinately regulated via the *pgaR-pgal* QS system, such as the carbapenem antibiotic and prodigiosin pigment in *Serratia* sp. strain ATCC 39006 (54). The pleiotropic phenotype could be due to the activation of the *tda* operon, which encodes proteins involved both in the synthesis of TDA and pigment (22). We hypothesize that the *pgaR-pgal* QS system is located relatively high in the hierarchy of regulation in *P. gallaeciensis*, whereas TDA production directly depends on TdaA. The production of TDA in strain DSM 17395 is obviously regulated by a complex regulatory network, which may act at different levels in a regulatory cascade. Such a complex regulatory web would be consistent with the need of *P. gallaeciensis* to sense, integrate, and respond to a variety of environmental and physiological signals and to adapt to and colonize different niches and environments.

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