Nonbioluminescent Strains of *Photobacterium phosphoreum* Produce the Cell-to-Cell Communication Signal *N*-(3-Hydroxyoctanoyl)homoserine Lactone

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Bioluminescence is a common phenotype in marine bacteria, such as *Vibrio* and *Photobacterium* species, and can be quorum regulated by *N*-acylated homoserine lactones (AHLs). We extracted a molecule that induced a bacterial AHL monitor (*Agrobacterium tumefaciens* NT1 [pZLR4]) from packed cod fillets, which spoil due to growth of *Photobacterium phosphoreum*. Interestingly, AHLs were produced by 13 nonbioluminescent strains of *P. phosphoreum* isolated from the product. Of 177 strains of *P. phosphoreum* (including 18 isolates from this study), none of 74 bioluminescent strains elicited a reaction in the AHL monitor, whereas 48 of 103 nonbioluminescent strains did produce AHLs. AHLs were also detected in *Aeromonas* spp., but not in *Shewanella* strains. Thin-layer chromatographic profiles of cod extracts and *P. phosphoreum* culture supernatants identified a molecule similar in relative mobility (R_f value) and shape to *N*-(3-hydroxyoctanoyl)homoserine lactone, and the presence of this molecule in culture supernatants from a nonbioluminescent strain of *P. phosphoreum* was confirmed by high-performance liquid chromatography–positive electrospray high-resolution mass spectrometry. Bioluminescence (in a non-AHL-producing strain of *P. phosphoreum*) was strongly up-regulated during growth, whereas AHL production in a nonbioluminescent strain of *P. phosphoreum* appeared constitutive. AHLs apparently did not influence bioluminescence, as the addition of neither synthetic AHLs nor supernatants delayed or reduced this phenotype in luminescent strains of *P. phosphoreum*. The phenotypes of nonbioluminescent *P. phosphoreum* strains regulated by AHLs remains to be elucidated.

Photobacterium phosphoreum is a marine psychrotolerant bacterium commonly found free living in temperate waters or in high cell densities in fish gastrointestinal tracts and in specific light organelles in deep-sea fish (30, 47, 48). *P. phosphoreum* is a bioluminescent bacterium (17, 46, 48) which can also be found in dim and dark versions; loss of bioluminescence among bioluminescent species is frequently observed (16, 30, 31, 53). It is unknown if all nonbioluminescent *P. phosphoreum* strains have ever been able to produce light, although it has been shown that at least part of the essential *lux* genes is present in both the luminous and nonluminous strains (17). *P. phosphoreum* belongs to the *Vibrionaceae*, which harbor several bioluminescent species, including *Vibrio fischeri* and *Vibrio harveyi*. These two species regulate bioluminescence through a circuit popularly referred to as quorum sensing (22, 50, 54). In brief, quorum sensing involves the production and release of communication molecules, which accumulate in the surrounding environment and allow the bacteria to coregulate specific phenotypes. The communication molecules regulating bioluminescence in *V. fischeri* are *N*-acylated homoserine lactones (AHLs). Quorum sensing enables *V. fischeri* to up-regulate bioluminescence when situated in its symbiont *Euprymna scolopes* and abolish light production when in the free-living

state in the sea. Quorum sensing allows many other gram-negative bacteria to regulate different phenotypes, most of them involved in pathogenesis or symbiosis. It appears that bioluminescent *P. phosphoreum*, although closely related to *V. fischeri*, does not produce AHLs (20, 21, 29, 38).

P. phosphoreum also plays an important role in food microbiology, where it acts as the most important spoilage bacterium of packed, chilled fish fillets (11, 12, 52). Its ability to use trimethylamine oxide (TMAO) as a terminal electron acceptor facilitates its growth in packed fish products, where it reduces this compound to trimethylamine (TMA), imparting to the product a spoiled, fishy flavor. We are currently investigating the potential involvement of quorum sensing in food products in which spoilage is caused by growth of and metabolism by gram-negative bacteria.

It has recently been demonstrated that quorum-sensing signals, AHLs, can be extracted from several food products, including fish (smoked and fresh), vegetables, and meats (4, 26, 27), and are produced by several gram-negative food spoilage bacteria (4, 7, 8, 23, 26, 44, 45). In some food products, AHL production does not influence the decay in food quality (4, 8), yet in other cases, the production of degradative enzymes, which have a negative influence on the quality of milk and vegetable products, can be AHL regulated (7, 8, 44).

The present study investigates the production of AHLs in packed fish and identifies the AHL-producing bacteria. The nature of the AHL found in the fish and the AHL produced by *P. phosphoreum* is elucidated, and the AHL and light production kinetics in *P. phosphoreum* are investigated.

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TABLE 1. Strains and plasmids used in the study

Species	No. of strains	Strain	Plasmid	Antibiotic resistance	Source or reference
<i>A. tumefaciens</i>	1	NT1	pZLR4	Gentamicin, kanamycin	49
	1	LRF6	pZLR4	Gentamicin, kanamycin, tetracycline	This study
<i>C. violaceum</i>	1	CV026		Kanamycin	37
<i>P. phosphoreum</i>	1	P100			11, 14, 15, 16; P. Dalgaard, unpublished results
	1	FR7			
	157				
	1	LRF1			This study
	1	LRF2			This study
<i>S. putrefaciens</i>	16				This study
	67				
	1	ATTC8071			
	1	NCTC10695			
	1	ATCC33224			
	1	NCTC10735			
	1	NCIMB1733			
	1	CCUG12947			
	1	CCUG12948			
	1	CCUG15766			
	1	CCUG22946			
	1	NCIMB12577			
	1	NCIMB12580			
	1	NCIMB10473			
	26				24
<i>Shewanella algae</i>	10				24
	1	IAM14159			
	1	NCTC10738			
	1	NCIMB12582			
<i>E. coli</i>	1	S17 λ pir	pBSL199	Tetracycline, ampicillin	1
			pNU121	Ampicillin	42
	1	S17 λ pir	pBSL180	Kanamycin, ampicillin	1
	1	HB101	pRK600	Chloramphenicol	34
	1	CC118 λ pir	pUTTet	Tetracycline	34
	1	BW20767	pRL27	Kanamycin	35
	1	MT102			T. Hansen, unpublished results

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used are listed in Table 1. *Agrobacterium tumefaciens* NT1 (pZLR4) (49) and *Chromobacterium violaceum* CV026 (37, 49) were used for the detection, identification, and quantification of AHLs as previously described (45). *A. tumefaciens* NT1 (pZLR4) was grown in ABTG medium (9), and *C. violaceum* CV026 was grown in LB₅ medium (3). Strains of *P. phosphoreum* isolated in this study (18 strains) as well as 159 strains isolated from previous storage trials with cod and salmon were grown in GMB containing TMAO (18) or on modified Long and Hammer agar (L&H) (13, 52) at 15°C. *Shewanella* strains from this study (67 strains), 36 strains previously isolated from spoiling fish, and 15 strains from culture collections were grown on iron agar (IA; Oxoid CM867) (25) or in brain heart infusion broth (Oxoid CM225) at 25°C and 200 rpm for aeration. Studies of bioluminescence and AHL production kinetics in *P. phosphoreum* were carried out by using strain P100 (nonbioluminescent; NCIMB 13481) isolated from CO₂-packed cod fillets and strain FR7 (bioluminescent) isolated from vacuum-packed cod fillets (11). A gene library of *P. phosphoreum* strain P100 was constructed with *Escherichia coli* strain MT102. P100 (NCIMB 13481) and FR7 were identified previously as *P. phosphoreum* by using 156 phenotypic characteristics (14). The non-bioluminescent strain (P100) had above 99% 16S rDNA similarity with the type strains of *P. phosphoreum* and also contained *luxA* with a 730-bp fragment sequence typical of *P. phosphoreum* (17).

Storage trial with packed, chilled cod. Fresh fillets of cod (*Gadus morhua*) were obtained from a Danish filleting factory and transported on ice to the laboratory where they arrived 1 day after the fish were caught. Fillets were packed in 120-g portions either in a vacuum (25 mbar) or with approximately 4 volumes of a modified atmosphere consisting of 40% CO₂ and 60% N₂ and stored at approximately 2°C. A laminar film with low gas permeability was used for packing (NEN/LLDPE 75; Amcore Flexibles, Horsens, Denmark).

Enumeration of bacteria from cod samples. At regular intervals during storage, samples of cod fillets were withdrawn and aerobic plate counts were determined on L&H plates incubated at 15°C. Luminescent bacteria were counted on L&H plates after 2 to 3 days of incubation, and aerobic colonies on L&H plates were counted after 5 to 7 days. H₂S-producing organisms were enumerated as

black colonies in pour-plated IA incubated at 25°C for 3 to 5 days. A *P. phosphoreum*-specific count was obtained by using a conductometry-based method (13).

The level of AHL-producing bacteria was estimated by using a replica-plating procedure in which the L&H plates were replicated onto indicative agar plates containing *A. tumefaciens* NT1 (pZLR4) imbedded in ABT agar supplied with 50 µg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/ml or *C. violaceum* CV026 embedded in LB₅ medium (4, 27). The replica plates were incubated at 20°C for 1 to 5 days, and colonies causing a blue color (cleavages of X-Gal by *A. tumefaciens* NT1 [pZLR4]) or a purple pigment (produced by *C. violaceum* CV026) in the surrounding agar were interpreted as being AHL positive.

Identification of bacteria. Strains isolated from the replica plating procedure as potential AHL producers were identified by standard biochemical tests. Strains were grown in a liquid medium, and shape and motility were determined by phase-contrast microscopy (×1,000 magnification). Colonies from agar plates (24 to 48 h) were tested for Gram reactions (with 3% KOH), catalase reactions (3% H₂O₂), oxidase reactions (BBL Dryslide Oxidase slides; Becton and Company, Sparks, Md.), the ability to ferment and oxidize glucose (Merck 10282 supplemented with a total of 1% NaCl), the ability to grow in the presence of vibriostaticum (2,4-diamino-6,7-di-isopropylteridine, 0/129 DD15, 150 µg; Oxoid 129150) and bioluminescence when grown aerobically on L&H plates with 1% NaCl at 15°C. Vibriostaticum-sensitive isolates were further tested for metabolism of arginine, lysine, and ornithine; growth without NaCl; growth at 0°C and 35°C; assimilation of D-mannitol; production of TMA from TMAO; and production of gas from glucose. All tests were carried out as previously described (2, 11, 14). The phenotypic tests selected for identification as *P. phosphoreum* are a subset of tests from several studies where numerous phenotypic characteristics and sequencing of 16S rDNA and *luxA* were used to identify type strains and environmental isolates of the photobacteria (14, 17). To verify the scheme, 16S rDNA sequences (1,333 to 1,497 bp) were determined for one luminous *P. phosphoreum* isolate (FR7) and for three nonluminous *P. phosphoreum* isolates (NCIMB 13478, NCIMB 13479, and P50) previously isolated from packed cod and identified by the selected phenotypic tests (14, 17). 16S rDNA sequences were determined by the BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie Universiteit Gent, Ghent, Belgium. The four 16S rDNA sequences have been deposited in the EMBL, GenBank, and DDBJ nucleotide databases. Gram-negative and catalase- and oxidase-positive mobile rods resistant to vibriostaticum were tentatively identified as *Aeromonas*, and these isolates all produced gas from glucose. Strains were identified as *Shewanella* spp., being gram-negative mobile rods positive for catalase and oxidase but unable to ferment or oxidize glucose and having the ability to form TMA from TMAO and to produce H₂S on IA. These strains usually formed pink- to orange-pigmented colonies on IA (52).

Extraction of AHLs from cod filets. AHLs were extracted from approximately 10 g of the homogenized cod fillet by using three times as much acidified (0.5% formic acid) ethyl acetate (approximately 30 ml) as fish sample. The extracts were filtered through a Whatmann 4 filter and evaporated under a stream of nitrogen, dissolved in 1 ml of acidified ethyl acetate, and stored at -20°C.

Identification by TLC and quantification of AHLs. AHL production in bacteria isolated as presumed AHL producers based on the replica plating procedure was verified in a well diffusion assay. Indicative plates were prepared as for the replica plating procedure, by using both *A. tumefaciens* NT1 (pZLR4) and *C. violaceum* CV026. Wells were cut into the agar in which 60 µl of sterile-filtered supernatant from an outgrown culture (24 to 48 h as described above) of the presumed AHL producer or 60 µl of ethyl acetate extract was added (45). AHLs were tentatively identified directly from sterile supernatants derived from outgrown bacterial cultures or from ethyl acetate extractions of the culture supernatants or cod filets by separating them by thin-layer chromatography (TLC) and subsequently developing the TLC plates by using an indicative agar containing *A. tumefaciens* NT1 (pZLR4) (45, 49). A semiquantification of AHLs was performed by preparing a well diffusion assay based on *A. tumefaciens* NT1 (pZLR4). Increasing concentrations of *N*-(3-hydroxyoctanoyl)homoserine lactone (OH-C₈-HL; provided by John Nielsen, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark) was added to the wells, and a standard curve correlating the size of the induced halo surrounding the well to the amount of AHL was constructed and used for estimating the concentration of AHL in a sample (23, 45).

Confirmation of AHLs by HPLC-ESI⁺-high-resolution mass spectrometry (HRMS). Bacterial ethyl acetate extracts were evaporated to dryness in a vacuum, redissolved in 10 times less methanol-water (1:1), and analyzed on two different columns; 1 µl was injected in an Agilent 1100 high-pressure liquid chromatograph (HPLC) equipped with either a Phenomenex (Torrance, Calif.)

Luna II C₁₈, 3-µm particle, 50- by 2-mm column and a Phenomenex 2-mm C₁₈ SecurityGuard precolumn or a Phenomenex Polar RP Phenyl, 3-µm particle, 150- by 2-mm column and a Phenomenex 2-mm Polar RP SecurityGuard precolumn. The HPLC was coupled to an LCT orthogonal time-of-flight mass spectrometer (MS; Micromass, Manchester, United Kingdom), and equipped with a Z-spray electrospray ionization (ESI) source and a LockSpray probe (using leucine encephalin as lock mass) (41). The MS was operated in the positive-ion ESI (ESI⁺) mode and tuned to a resolution of >6,500 (at half-peak height), and data were collected as centroid data from *m/z* 100 to 900 as previously described (41). Analyses were performed with water (MilliQ) containing 10 mM ammonium formate and 20 mM formic acid (both analytical grade) and acetonitrile (gradient grade) containing 20 mM formic acid. A flow rate of 0.3 ml/min was used, starting with 5% acetonitrile (Luna C₁₈ column) or 0% acetonitrile (Polar RP column), going to 100% in 25 min, holding for 3 min, returning to 5% acetonitrile in 4 min, and then equilibrating by 7 min. To verify that no AHLs could be detected in a luminescent photobacterium (FR7), the following reference standards were coanalyzed on both columns: OH-C₈-HL, *N*-butanoyl-homoserine lactone (C₄-HL), *N*-hexanoyl-homoserine lactone (C₆-HL), *N*-octanoyl-homoserine lactone (C₈-HL), *N*-(3-oxohexanoyl)homoserine lactone (O-C₆), *N*-(3-oxooctanoyl)homoserine lactone (O-C₈), and *N*-(3-oxodecanoyl)homoserine lactone (O-C₁₀). Besides checking for the extracts for the [M+H]⁺ and [M+Na]⁺ (±0.01 *m/z*) adducts of these reference standards, we also inspected the chromatograms for peaks resembling same adducts of *N*-decanoyl-homoserine lactone (C₁₀-HL), *N*-dodecanoyl-homoserine lactone (C₁₂-HL), *N*-tetradecanoyl-homoserine lactone (C₁₄-HL), *N*-(3-oxododecanoyl)homoserine lactone (O-C₁₂-HL), *N*-(3-oxotetradecanoyl)homoserine lactone (O-C₁₄-HL), *N*-(3-hydroxyhexanoyl)homoserine lactone (OH-C₆-HL), *N*-(3-hydroxydecanoyl)homoserine lactone (OH-C₁₀-HL), and *N*-(3-hydroxydodecanoyl)homoserine lactone (OH-C₁₂-HL).

AHL production kinetics. Growth of *P. phosphoreum* strain P100 in GMB or in juice from cod (10, 11) was monitored by enumeration on L&H or (only for GMB) by absorbance measurements at 600 nm. Samples were withdrawn and analyzed for AHL content by using a well diffusion assay with OH-C₈-HL as the standard.

Studies of bioluminescence. The nonbioluminescent, AHL-producing *P. phosphoreum* strain P100 was cross-streaked (on L&H at 15°C) against the luminescent, non-AHL-producing *P. phosphoreum* strain FR7 to determine potential AHL inhibition or induction of bioluminescence. *P. phosphoreum* strain FR7 was grown in GMB supplemented with 10% sterile supernatant from an outgrown culture of *P. phosphoreum* strain FR7 or P100 to test the presence of either an inducing factor produced by the luminescent *P. phosphoreum* strain FR7 or an inhibiting factor from *P. phosphoreum* strain P100. Finally, *P. phosphoreum* strain FR7 was grown in the presence of 1 µM OH-C₈-HL or 1 µM *N*-oxo-hexanoyl-homoserine lactone (O-C₆-HL; Sigma-Aldrich 142537-62-6) to test any effect from AHLs on light production. Light production was measured by using a Wallac scintillation counter (MicroBeta 1450-405 511).

Genetic manipulation techniques used with *P. phosphoreum*. Transposon mutagenesis was performed by using Tn5 and Tn10 transposons in an attempt to isolate, sequence, and knock out the AHL synthetase enzyme encoded by an *luxI* or *ainS* homologue from *P. phosphoreum* strain P100 (32). *P. phosphoreum* was mixed with a plasmid donor (pBSL199, pBSL180, or pRL27), and the mixture was plated on an L&H plate for 24 h. The cells were removed, resuspended in peptone saline, and plated on selective L&H plates supplied with appropriate antibiotics (typically 50 µg of gentamicin/ml) to remove the donor and another antibiotic to select for transposon mutants (see Table 1). Several incubation temperatures and incubation times (15, 20, 25, 30, and 37°C for 0 to 6 h) were used for the heat treatment tests of the *P. phosphoreum* before it was mixed with the donor. A gene library of *P. phosphoreum* strain P100 was created by cutting chromosomal DNA (purified by using the QIAGEN genomic DNA Tip 100/G System; Hilden, Germany) with *Bcl*I restriction enzyme for 1 h. The fragments (purified by using GFX PCR DNA purification kit; Amersham Biosciences, Little Chalfont, Buckinghamshire, England) were inserted into pNU121 (42), and clones containing inserts were selected on LB₅ medium supplemented with 5 µg of tetracycline/ml. Outgrown colonies were screened for AHL production by replica plating on indicative plates containing *A. tumefaciens* NT1 (pZLR4) LRF6, which is a tetracycline-resistant variant of *A. tumefaciens* NT1 (pZLR4) created by using the Tn10-tet transposon delivery vector pBSL199 (1) encoding tetracycline resistance.

Nucleotide sequence accession numbers. The four 16S rDNA sequences identified in this study (for nonluminous *P. phosphoreum* isolates NCIMB 13478, NCIMB 13479, and P50 and for luminous *P. phosphoreum* isolate FR7) have been deposited in the EMBL, GenBank, and DDBJ nucleotide databases under accession numbers AJ746357 to AJ746360, respectively.

TABLE 2. Production of AHLs and bioluminescence by *P. phosphoreum*, *Aeromonas* spp., and *Shewanella* spp. isolated from fish or obtained from culture collections^a

Species	Source	No. of strains	Bioluminescence	No. positive for AHLs	Isolated in	
					This study	Previous study
<i>P. phosphoreum</i>	Cod, VP	1	+	0	X	
	Cod, VP	5	—	5	X	
	Cod, MAP	2	+	0	X	
	Cod, MAP	10	—	8	X	
	Cod, VP, or MAP	14	+	0		X
	Cod, VP, or MAP	46	—	17		X
	Salmon, VP, or MAP	57	+	0		X
	Salmon, VP, or MAP	42	—	18		X
<i>Aeromonas</i> spp.	Cod, VP	9	—	8	X	
	Cod, MAP	8	—	1	X	
<i>Shewanella</i> spp.	Cod, VP	43	—	0	X	
	Cod, MAP	24	—	0	X	
	Strain collection ^b	51	—	0		X

^a VP, vacuum packed.^b Strains previously isolated from iced, vacuum-packed, or MAP fish or strains from culture collections (ATTC, NCTC, NCIMB, ACAM, IAM, and CCUG).

RESULTS

Bacterial and chemical changes in packed cod fillets. Aerobic plate counts increased from approximately 10^5 CFU/g to approximately 10^8 CFU/g during 16 days of storage, while the level of *P. phosphoreum* increased from approximately 10^3 to 10^7 CFU/g. The levels of AHL producers reached close to 10^7 CFU/g after 16 days. Levels of trimethylamine and total volatile nitrogen increased to 40 to 60 and 80 to 100 mg of N/100 g, respectively (data not shown). Ethyl acetate extracts of both vacuum-packed and modified-atmosphere-packaged (MAP) cod fillets elicited a positive response only in an *A. tumefaciens* NT1 (pZLR4)-based well diffusion assay from day 7 onward, reaching a maximum at approximately day 16. No response was seen with the *C. violaceum* assay.

Identification of AHL-producing bacteria among the spoilage microbiota. Ninety-seven potential AHL-producing colonies were isolated from L&H plates based on their ability to elicit a response in the replica plating procedure. These isolates represented both package conditions and all sampling points from day 12 onward (approximately six isolates per sampling). Most colonies eliciting a positive response on the replica plates were detected by *A. tumefaciens* NT1 (pZLR4). However, these colonies were difficult to isolate from the master plates, as they constituted only approximately 1% of the microbial community. All isolates were therefore tested for AHL production as single cultures by using a well diffusion assay. Twenty-three percent (22 of 97) of the isolates were AHL positive in this screening, and these isolates were all identified as *P. phosphoreum* or *Aeromonas* spp., while the rest of the isolates were either non-AHL-producing *P. phosphoreum* or *Shewanella* spp. (Table 2). To further confirm that AHL production is common among spoilage isolates of *P. phosphoreum* but not among *Shewanella* spp., we also tested isolates from previous storage trials (Table 1) as well as 51 *Shewanella* spp. from strain collections (Table 2). Clearly, compounds evoking a response in the *A. tumefaciens* assay were detected with *P. phosphoreum* but not *Shewanella* strains. The 16S rDNA sequences from nonluminescent and AHL-positive *P.*

phosphoreum strains (NCIMB 13478, NCIMB 13479, and P50) and from the luminous and AHL-negative *P. phosphoreum* strain (FR7) showed 99.2 to 99.4% similarity with the type strain of *P. phosphoreum* (ATCC 11040^T). The 16S rDNA sequence similarity between the nonluminescent *P. phosphoreum* P100 and the luminous isolate FR7 was 99.9%.

TLC profiling of AHLs. When TLC profiled, the sterile filtered supernatants from *P. phosphoreum* were shown to contain a component with an R_f of ~0.2. In contrast, ethyl acetate extracts contained a compound with an R_f of ~0.4 (similar to that of synthetic OH-C₈-HL). However, when OH-C₈-HL was added to a supernatant from the non-AHL-producing *P. phosphoreum* strain FR7, it changed the mobility to an R_f of ~0.2, similar to that of supernatants derived from AHL-producing *P. phosphoreum*. Ethyl acetate extracts of cod fillets contained a compound similar to ethyl acetate extracts of pure cultures (R_f ~ 0.4), but the R_f changed when the extract was added to *P. phosphoreum* culture supernatants (Fig. 1). More than 20 presumed AHL-producing *P. phosphoreum* isolates and all AHL-containing extracts from cod were separated on TLC, and the same AHL-inducing spot was found in all isolates (results not shown). The AHL produced by *Aeromonas* had a similar mobility and shape as OH-C₈-HL, and a few *Aeromonas* strains produced, in addition, a molecule which migrated slower than the OH-C₈-HL. We therefore conclude that pure cultures of *P. phosphoreum* and *Aeromonas* spp. did indeed produce the AHL compound detected in spoiling cod fillets.

Confirmation of AHLs by HPLC-ESI⁺-HRMS. The presence of OH-C₈-HL in *P. phosphoreum* strain P100 was detected by HPLC-ESI⁺-HRMS (Fig. 2), with the retention time and ESI⁺ spectra identical to those for the OH-C₈-HL reference standard. The detection was further validated by the calculated masses (<1 mDa deviation) of the [M+H]⁺ and [M+Na]⁺ adducts as well as the [M-H₂O+H]⁺ ion (Fig. 2). An extract of the bioluminescent, non-AHL-producing strain FR7 was also analyzed by HPLC-ESI⁺-HRMS on two different reversed-phase columns. A search was made for the [M+H]⁺ and [M+Na]⁺ adducts of C₄ to C₁₂ AHLs with nonsubstituted,

FIG. 1. TLC profiles of cell-to-cell communication signals found in spoiling cod fillets and produced by *P. phosphoreum*. Plates illustrate profiles of extracts (panel a, lanes 1, 4, and 7; panel b, lanes 1, 4, and 6) as well as supernatants or extracts added to supernatants. (a) Lane 1, *N*-(3-hydroxyoctanoyl)homoserine lactone; lane 2, extract of vacuum-packed cod fillets added to the supernatant from *P. phosphoreum* strain LRF1; lane 3, the supernatant from *P. phosphoreum* strain LRF1; lane 4, extract of vacuum-packed cod fillets; lane 5, extract of MAP cod fillets added to the supernatant from *P. phosphoreum* strain LRF2; lane 6, the supernatant from *P. phosphoreum* strain LRF2; lane 7, extract of vacuum-packed cod fillets; lane 8, the supernatant of *P. phosphoreum* strain P100. (b) Lane 1, *N*-(3-hydroxyoctanoyl)homoserine lactone; lane 2, *N*-(3-hydroxyoctanoyl)homoserine lactone added to the supernatant from *P. phosphoreum* strain FR7 (non-AHL producing); lane 3, *N*-(3-hydroxyoctanoyl)homoserine lactone added to the supernatant from *P. phosphoreum* strain P100 grown in GMB; lane 4, extract of *P. phosphoreum* strain P100 grown in GMB; lane 5, the supernatant from *P. phosphoreum* strain P100 grown in ABTG medium; lane 6, extract of *P. phosphoreum* strain P100 grown in ABTG medium.

OH-substituted, or O-substituted side chains (OH-C₈-HL, C₄-HL, C₆-HL, C₈-HL, O-C₆-HL, O-C₈-HL, and O-C₁₀-HL available and coanalyzed) and did not indicate the presence of AHLs.

Kinetics of AHL and light production in *P. phosphoreum*. To elucidate the potential role of AHL molecules as quorum-sensing regulators in *P. phosphoreum*, we focused on an obvious phenotype: bioluminescence. However, it became apparent after the testing of the 18 *P. phosphoreum* strains from this study and the 159 *P. phosphoreum* strains isolated from previous trials with cod and salmon that not a single bioluminescent *P. phosphoreum* strain produced detectable AHL, while 47% (48 of 103) of the nonluminescent strains gave a positive response with *A. tumefaciens* NT1 (pZLR4) (Table 2).

The amount of AHL, as estimated by the well diffusion assay, increased at the same rate as growth in the nonbioluminescent AHL-producing *P. phosphoreum* strain P100 in a standard laboratory growth medium (Fig. 3a) as well as in buffered juice from cod fillets (results not shown). The light production in the bioluminescent non-AHL-producing *P. phosphoreum* strain FR7 appeared to be strongly up-regulated in late exponential phase (Fig. 3b).

Since we did not find any bioluminescent and AHL-producing *P. phosphoreum* isolates, we tested whether AHLs had an influence on the light production or whether we could detect an inducing molecule in the luminescent *P. phosphoreum*. Several attempts were made to induce light production in the luminescent *P. phosphoreum* strain FR7 by using sterile-filtered supernatants from outgrown cultures of *P. phosphoreum* strain FR7, but they did not result in an earlier up-regulation of the bioluminescent phenotype. The possible inhibitory effect of AHLs on light production was tested. The AHL-producing *P. phosphoreum* strain P100 was cross streaked against the luminescent *P. phosphoreum* strain FR7, but no inhibition of light was seen. Sterile-filtered supernatants from outgrown cultures of *P. phosphoreum* strain P100 as well as synthetic O-C₆-HL and OH-C₈-HL were added to *P. phosphoreum* strain FR7; however, similar to the results of the cross-streaking experiment, neither inhibition nor delay of light production was found.

Finally, attempts to generate AHL-negative mutants of *P. phosphoreum* strain P100 (to determine putative quorum-regulated phenotypes) by using Tn5 and Tn10 mini-transposons failed. Subsequently, a gene library of *P. phosphoreum* P100 was constructed in *E. coli*. The library (25,000 colonies) was screened for luminescent colonies by using decanal as substrate and was screened for AHL-producing clones by using a replica assay, but none were found.

DISCUSSION

The present study is, to our knowledge, the first to demonstrate that *P. phosphoreum* strains produce acylated homoserine lactones. Interestingly, the compound was discovered only in nonbioluminescent strains (Table 2). Previous screenings for AHL production by *P. phosphoreum* have focused on luminescent strains (20, 21, 29, 38), and this fact probably explains why AHL production in *P. phosphoreum* hitherto has not been reported.

The standard TLC procedure for identification of AHLs

FIG. 2. HPLC-ESI⁺-MS chromatograms. The lower left chromatogram is the total ion current (TIC) chromatogram from the *Photobacterium phosphoreum* strain P100 sample. The middle and the upper chromatograms are the extracted ion chromatograms corresponding to the masses of the [M+H]⁺ adducts of *N*-(3-hydroxyoctanoyl)homoserine lactone (OH-C₈-HL) for the *P. phosphoreum* strain P-100 sample and a OH-C₈-HL reference standard, respectively. The electrospray mass spectrum for OH-C₈-HL is shown (lower right) along with the molecular structure and the calculated masses (calc. mass) for the three adducts. TOF, time of flight; ES⁺, positive-ion ESI.

used in this and many other studies (6, 23, 33, 49) resulted in the identification of two different molecules when supernatants from *P. phosphoreum* were compared with extracts of the same supernatants or extracts from cod. The reason for this apparent difference was that the bacterial supernatant altered the mobility of the molecule and thereby dramatically changed the tentative identification. Hence, it is advisable to extract bacterial culture supernatants before TLC profiling and identification.

Only a single circular spot ($R_f \approx 0.4$) was detected by TLC in extracts from cod fillets packed in a vacuum or modified atmosphere and from all strains of *P. phosphoreum*. The component did not tail, comigrated with the OH-C₈-HL reference standard, and was unambiguously identified for *P. phosphoreum* strain P100 by HPLC-ESI⁺-HRMS (Fig. 2). It was necessary to lower the content of the organic phase significantly, compared to that used in the study of Morin et al. (40), in the HPLC analysis of both the C₁₈ and the phenyl (with polar capping) columns. The molecule, found in cod fillets, also comigrated with OH-C₈-HL, indicating that *P. phosphoreum* could be the producer of this compound in packed cod fillets. Furthermore, the concentration of the component reached measurable levels as *P. phosphoreum* reached 10⁶ CFU/g at a time when the aerobic plate count level had been at that level or higher levels for several days, strengthening the hypothesis that *P. phosphoreum* is the producer of the cell-to-cell signaling compound. This specific AHL has, to our knowledge, been found previously only in *Pseudomonas fluorescens* (49) and hydroxyl-substituted AHLs are identified only in three other species: *Xenorhabdus nematophilus* (19), *Vibrio anguillarum* (39), and *V. harveyi* (5). Interestingly, the *Aeromonas* spp. isolated in this study produced a compound with similar properties in TLC (results not shown).

FIG. 3. Production of cell-to-cell communication signals in *P. phosphoreum* strain P100 measured as OH-C₈-HL equivalents (a) and bioluminescence (luminescence counts per second; LCPS) (b) in *P. phosphoreum* strain FR7 grown in GMB at 5°C.

The AHL production kinetics of *P. phosphoreum* was investigated and the concentration of AHL in withdrawn samples was estimated based on standard curves generated by using OH-C₈-HL (45). Production kinetics showed that the presumed AHL is produced constitutively by *P. phosphoreum* strain P100, although this species is closely related to *V. fischeri*, in which the production of O-C₆-HL is regulated in an autoinducible loop. This finding adds *P. phosphoreum* to the growing list of bacterial species in which AHL production appears to be constitutive (26, 28, 36, 45, 51).

None of the luminescent strains of *P. phosphoreum* produced AHLs, and this finding allowed us to speculate that AHL negatively regulates the bioluminescent phenotype. For instance, some *V. fischeri* autoinducer analogs may reduce bioluminescence in *P. phosphoreum* strain 8265 (21). There seems to be an overlap in the biochemical pathways in the synthesis of AHL and the aldehyde (tetraaldehyde in the case of *P. phosphoreum*) used to fuel the bioluminescent reaction. Both compounds use acyl-acyl carrier protein from the fatty acid biosynthesis pathway, although the acyl used for OH-C₈-HL synthesis is probably octanoyl, while it is presumably tetradecanoyl for tetraaldehyde (43). However, we could not show experimentally that AHLs influence bioluminescence, since the addition of synthetic O-C₆-HL, OH-C₈-HL, or supernatants derived from *P. phosphoreum* strain P100 had no effect on luminescence for *P. phosphoreum* strain FR7. Similarly, *V. fischeri* autoinducer analogs had no effect on bioluminescence for *V. harveyi* strain MAV or *Photobacterium leiognathi* strain 721 (21). The bioluminescence of *P. phosphoreum* strain FR7 was, however, strongly up-regulated in the late exponential growth phase, indicating the presence of a specific regulatory circuit. The presence of specific luminescence-inducing molecules, such as a new type of autoinducers, could not be proven in this study since we did not find an early up-regulation of the bioluminescent phenotype in *P. phosphoreum* strain FR7 supplemented with supernatants derived from outgrown cultures of *P. phosphoreum* strain FR7. Although we did not find any AHLs in bioluminescent *P. phosphoreum*, they may, in principle, produce types of AHLs that are not recognized by the monitors and the HPLC-MS method used in this study. *A. tumefaciens* NT1 pZLR4 responds primarily to oxo-substituted AHLs and nonsubstituted AHLs (except C4) (49), whereas *C. violaceum* CV026 produces predominantly violacein in response to nonsubstituted AHLs but also responds to oxo-substituted compounds (37). As is evident from our study, NT1 also reacts when exposed to OH-substituted AHLs; hence, when used in combination, they will cover most of the well-known AHLs.

We do not know, at present, if *P. phosphoreum* uses AHL in a quorum-sensing regulatory circuit or what the affected phenotype(s) is (if any). If AHLs are used as quorum-sensing molecules, they may play a role in the natural environment of the bacterium, e.g., in the gastrointestinal tract of fish. In this environment, they may up-regulate activities, e.g., chitinase activity, and hence facilitate the degradation of crustaceans. However, both luminescent and nonluminescent strains are chitinolytic (11), but AHL regulation may allow a more specific up-regulation in late exponential growth phase. Although we did find a slight up-regulation of chitinase activity in *P. phosphoreum* in late exponential phase (data not shown), we were

unable to create any mutations in *P. phosphoreum*, which would have allowed us to investigate the regulatory function of AHLs.

In conclusion, our study adds the marine bacterium *P. phosphoreum* to the growing list of AHL-producing bacteria. In many of these bacteria, the regulatory function of the signals is unknown, and further studies are needed to determine the role of OH-C₈-HL in quorum sensing in *P. phosphoreum*.

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