Scientific Research Publishing

Advances in Biological Chemistry, 2024, 14, 171-186
https://www.scirp.org/journal/abc
ISSN Online: 2162-2191
ISSN Print: 2162-2183

Sequencing the Genome of a Marine Bacterium Called *Pseudoalteromonas ulvae* TC14 and Molecular Characterization of Its Communication System (Quorum Sensing)

Emmanuel Gozoua[1,2,3], Dan Eude Kpannieu[4,5]*, Thomas Koffi[1,6], Rose Koffi-Nevry[3], Claudine Baraquet[2], Maelle Molmeret[2], Yves Blache[2]

[1]UFR Sciences de la Mer, Université polytechnique de San Pedro (UPSP), San-Pédro, Côte d'Ivoire
[2]Laboratoire Matériaux Polymeres Interface Environnement Marin, Université de Toulon, La Garde, France
[3]UFR Sciences et Technologie des Aliments, Université Nangui Abrogoua, Abidjan, Côte d'Ivoire
[4]UFR Environnement, Laboratoire des Sciences et Techniques de l'Eau (LSTE), Université Lorougnon Guédé de Daloa, Daloa, Côte d'Ivoire
[5]Laboratoire de Chimie Physique et Microbiologie Pour Les Matériaux et L'environnement, Université de Lorraine, Villers-Lès-Nancy, France
[6]Unité de Recherches en Signaux, Imagerie et Instrumentation Optoélectronique (UR-SIIO), Ecoles d'ingénieurs en Construction Navale (ECN) et BTP, Université Polytechnique de San Pedro (UPSP), San Pedro, Côte d'Ivoire.
Email: *kpannieude1@gmail.com

How to cite this paper: Gozoua, E., Kpannieu, D.E., Koffi, T., Koffi-Nevry, R., Baraquet, C., Molmeret, M. and Blache, Y. (2024) Sequencing the Genome of a Marine Bacterium Called *Pseudoalteromonas ulvae* TC14 and Molecular Characterization of Its Communication System (Quorum Sensing). *Advances in Biological Chemistry*, **14**, 171-186.
https://doi.org/10.4236/abc.2024.146013

Received: October 3, 2024
Accepted: November 22, 2024
Published: November 25, 2024

Copyright © 2024 by author(s) and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).
http://creativecommons.org/licenses/by/4.0/

Open Access

Abstract

The communication system known as quorum sensing (QS) in gram-negative bacteria regulates biofilm formation and many other functions. The particularity of *Pseudoalteromonas ulvae* TC14 seems to be the absence of a LuxI inducer, and therefore the lack of production of small molecules of Acyl homoserine lactone (AHL). Previous studies had shown that it does not harbor the inducer of the regulatory molecule (AHL). Nevertheless, it is able to regulate these same functions (biofilm, violacein) via AHLs. This could mean the presence of specific receptors for these induction molecules, belonging to the LuxR family. The aim of this study was to test these hypotheses using molecular analysis. Genome sequencing of the *P. ulvae* TC14 strain was carried out by Molecular Research LP (MR DNA), using the Illumina Hiseq 2500 method. The results revealed the presence of 2,293,242 base pairs, *i.e.* 100% of the genomic volume. The number of coding gene sequences was 1983, and the Guanine + Cytosine (G+C) percentage of the base number was 41.55%, revealing stability in the sequenced genome. In order to verify the identity of the sequenced genome, a phylogenetic analysis based on RNA16S was carried out. This analysis resulted in 93% homology with the previously sequenced and characterized species *Pseudoalteromonas tunicata*,

showing that it belongs to the genus *Pseudoalteromonas*. To these comparative results should be added those derived from genome analysis based on nucleotide percentage using the tools available at https://img.jgi.doe.gov/. The results showed that *Pseudoalteromonas tunicata* D2 has the highest percentage nucleotide identity (ANI) (75.7913%), followed by *Pseudoalteromonas flavipulchra* NCIMB2033 (72.2736%) and *Pseudoalteromonas phenolica* KCTC 12086 (71.6685%). Next, the search for the various genes involved in QS was carried out using sequence comparisons via the BLAST method. For LuxI, sequence templates from the genomes of Vibrio, *Chromobacterium*, Shewanella or even other *Pseudoalteromonas* species were screened against the TC14 genome. This yielded no conclusive results synonymous with the non-presence of LuxI in TC14 as assumed by previous research. The search for LuxR, on the other hand, gave rise to the presence of eight presumptive sequences. Molecular characterization of the presumptive LuxR sequences was carried out to assess their gene expression. This characterization showed expression of these LuxR homologous sequences in a range from o.11 to 5.33 picograms (pg). Even if these sequences were not analyzed in depth, the retro-transcription technique nevertheless showed a minimum of activity, which could enable us to distinguish them from inactive sequences. The next step was to compare the LuxRs found in TC14 with the so-called Solo LuxRs, which act autonomously. This study shows that *P. ulvae* TC14 is a bacterium with a particular LuxR-based communication system.

Keywords

Quorum Sensing, Acyl Homoserine Lactone, Violacein, Biofilm

1. Introduction

Microorganisms have the capacity to adapt rapidly to environmental conditions. To achieve this, they rely on a communication mechanism based on small, diffusible molecules such as acylhomoserine lactones (AHLs), furanosylborate diesters (AI2s), cis-unsaturated fatty acids (DSF family signals) and peptides (Williams and [1]. This mechanism, commonly known as quorum sensing (QS), is most often dependent on a visible, measurable signal such as bioluminescence in *Vibrio harveyi*. QS enables the bacteria to recognize a certain cell density through the visible signal produced by the bacteria [2]. This process leads to a preferred mode of life, the biofilm. By definition, biofilms are heterogeneous structures made up of bacterial populations embedded in an extracellular matrix, attached to natural or artificial surfaces. The main characteristics of biofilms and the techniques used to study them have been studied on several occasions. They have led to the establishment of a five-stage model for biofilm formation: reversible adhesion of bacteria from the planktonic phase to a surface, irreversibility of adhesion corresponding to the synthesis of structures on the bacterial surface, formation of microcolonies, then development of these microcolonies reflecting the maturation stage of the biofilm and colonization of new surfaces [3]. QS is the mechanism used by both Gram-negative and Gram-

positive bacteria to regulate the expression of various genes in a synchronized, cell-density-dependent manner [4]. It is involved in numerous physiological processes including virulence factor production [5], bioluminescence emission, pigment production [5], biofilm formation and motility [6]. In Gram-negative bacteria, QS involves the production of and response to small signaling molecules called N-acyl homoserine lactones (AHLs) [7]. In this study, we will focus on the species *Pseudoalteromonas ulvae* (TC14) isolated from the Toulon harbor. This biofilm-forming bacterium produces a purple-red substance called violacein. The choice of this species is based on the fact that it appears to regulate biofilm and violacein formation through QS [8]. In addition, the absence of LuxI synthase in this species, according to previous studies, suggests its inability to produce AHLs. The hypothesis that it would accept exogenous AHLs to regulate biofilm and violacein was tested in this study. A complete sequencing of the *P. ulvae* genome (TC14) will be carried out to detect the genes of interest. This will be followed by characterization of the genes involved in QS, to gain an overall view of the bacterium's communication system.

2. Material and Methods

2.1. Genome Sequencing and Whole Genome Analysis of *P. ulvae* TC14

The complete TC14 genome and all information relating to genome extraction parameters were submitted to Molecular Research LP DNA laboratory (USA) for sequencing. For TC14 DNA extraction, 10 ml of TC14 overnight culture was performed. DNA extraction was performed using QIAGEN's QIAquick gel extraction kit (50). DNA samples were stored at −20˚C RNA was extracted using an RNeasy mini kit (50). RNA was then purified using the Rneasy clean up kit (50). PCR was performed and a 1% agarose gel was visualized to verify the absence of DNA contamination. All RNA samples obtained were stored at −80˚C.

2.2. RNA Retranscription and Single-Stranded DNA Production (qRT-PCR)

RNA reverse transcription and single-stranded DNA production from our RNA samples were performed using the High capacity cDNA reverse transcription kit from thermo Fisher scientific. NAOH and HCL solutions were successively used during this 1N step for the separation of cDNA from the bacterial membrane. Our cDNA samples were then purified using the Qiaquick PCR purification kit from QIAGEN. Quantitative PCR was carried out using the Lightcycler 480 instrument from Roche for the first samples. White 96-well plates and SYBR GREEN I Master reagent, also supplied by Roche, were used. Results were analyzed using LightCycler® 480 SW 1.5.1 software. The experiment was continued using a Bio-rad instrument and the Bio-rad SYBR GREEN reagent. Quantitative PCR was performed using Roche's Lightcycler 480 instrument for the first samples. White 96-well plates and SYBR GREEN I Master reagent, also supplied by Roche, were used. Results were analyzed using LightCycler® 480 SW 1.5.1 software. The experiment was continued

using a Bio-rad device and the Bio-rad SYBR GREEN reagent.

2.3. Bacterial Culture under Planktonic Conditions (*P. ulvae* TC14)

Bacterial strains were isolated from cryotubes previously stored at −80˚C. Bacteria were started on agar plates (VNSS, MBA and LBA). Whatever the culture conditions (planktonic or sessile), a 5 or 10 mL preculture was performed from one or more clones in a 30 mL or 60 mL flask. Precultures are carried out overnight at 20˚C with 120 rpm agitation for *P. ulvae* TC14 strains. For biosensor strains CV026 and *Vibrio harveyi*, precultures were incubated at 30˚C with 120 rpm agitation. Biosensors are test bacteria with a visible, measurable signal enabling a given trait to be studied in another bacterium. A 25 mL culture (OD600 = 0.1) was then prepared from the precultures in a 250 mL Erlenmeyer flask and incubated at 20˚C for TC14 and 30˚C for CV026 and *Vibrio harveyi*. The culture was then incubated until the post-exponential growth phase, *i.e.* the beginning of the stationary phase, a phase conducive to the production of QS molecules [9] such as AHLs [10] and quinolones [11].

2.4. Extraction and Assay of Violacein

Extraction of the purple pigment from *P. ulvae* TC14 was performed according to the protocols described by [12]. One milliliter of bacteria cultured under planktonic or sessile conditions in VNSS, MB or LB media was centrifuged at 6000 g for 10 min in a mini centrifuge (Eppendorf mini Spin). After centrifugation, the culture supernatant was discarded, and the bacterial pellet suspended in 1 mL absolute ethanol (Fischer, France). The resulting ethanolic suspension was subjected to ultrasonication in an ultrasonic bath (Bransonic Ultrasonic Cleaner) at 245 Watts for 20 minutes, with the aim of lysing the cells until they became completely white (after around 20 min). After this, the ethanolic extract was separated from the pellet by centrifugation at 10,000 g for 20 min, and the absorbance of the violet pigment was measured at 575 nm with a spectrophotometer (Thermo Scientific, Genesys 20).

2.5. Bacterial Culture and Genome Extraction Conditions

An overnight culture of *P. ulvae* TC14 was carried out at 20˚C in VNSS with shaking at 120 rpm. A volume of 2 mL was collected and centrifuged for 3 min at 8,000 rpm. DNA was then extracted from the pellet using QIAGEN's QIAamp genomic DNA kits. The concentration of the DNA obtained was measured using a Nanodrop 2000c spectro from Thermofisher Scientific. A 50 µL volume of genomic DNA with a concentration of 338.8 ng/µL, conditioned in distilled water, was obtained and stored at −20˚C. The DNA was then sent to Molecular Research Laboratory (USA) for sequencing.

2.6. Sequencing and Genome Assembly

Sequencing was performed using the Nextera DNA sample preparation kit

(Illumina), following the manufacturer's user guide. Initial DNA concentration was assessed using the Qubit® dsDNA HS test kit (Life Technologies). This method has been described by Rothberg J. [10] as the most suitable for sequencing long genomes. Thus, 50 ng of DNA were used for sequencing. The sample underwent simultaneous fragmentation and addition of adapter sequences. These adapters are used in a cycle-limited PCR (5 cycles) in which single indices were added to the sample. After genome preparation, the final DNA concentration was measured using the Qubit® dsDNA HS test kit (Life Technologies) and the average genome size was determined using the Agilent 2100 bioanalyzer (Agilent Technologies). The library was diluted (to 10.0 pM) and its paired end sequenced for 500 cycles using the HiSeq system. Genome assembly was performed using NGEN V12 Ngen sequence analysis software, annotation with RAST and coverage > 50x.

2.7. Design (or Preparation) of Primers from the Various Genes Identified

Primers were designed for luxR in the QS system (no genes homologous to luxI were found) and for certain genes homologous to *C. violaceum* vio genes involved in violacein production. Eight putative LuxR genes were detected in the *P. ulvae* TC14 genome. In addition to the LuxR genes, those of the violacein operon (vio operon). A total of 14 genes (8 LuxR, 3 vio operon genes and the csgD, dgc and tda genes described in previous work as genes involved in biofilm formation via QS) were selected for analysis. From the sequences of these genes, primers were defined using NCBI's Primer-blast site (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). These primer sequences were supplied to Eurogentec, Belgium for oligonucleotide production. Primers obtained at 50 μM diluted in miliQ water were stored at −20˚C.

2.8. PCR Verification of Primers

The various primers were verified by PCR on the *P. ulvae* TC14 genome. PCR was performed with 5 μL of each primer fragment in the presence of 0.2 μg of genomic DNA using the Promega Madison WI (USA) kit. Bands were visualized by electrophoresis on a 1Kb gel containing 1% agarose. Bands were visualized using an ETNA (European Technological Nettwork Alliance) variable-wavelength UV Translumination device.

2.9. Quantitative Reverse Transcription PCR (RTqPCR) Technique

RTqPCR is a technique for performing quantitative PCR on RNA samples. The RNA is reverse-transcribed using a reverse transcriptase to synthesize complementary DNA (cDNA). The aim was to check the level of the above-mentioned genes at different growth times in *P. ulvae* TC14 and eventually to be able to speculate on which LuxR gene is likely to respond to the presence of AHLs. Total RNAs were therefore extracted from the purified *P. ulvae* TC14 genome, and the

cDNAs produced were synthesized and quantified by retrotranscription using a High Capacity cDNA kit (Themofischer Scientific). These cDNAs were then quantified by PCR using the primers described above.

2.10. RNA Extraction from *P. ulvae* TC14

Samples were taken following growth kinetics from a 25 mL planktonic culture of TC14 (OD600 = 0.1) in an Erlenmeyer flask (250 mL). Sampling times ranged from 1 to 33 h, in line with the different growth phases of *P. ulvae* TC14. Nine 1 mL culture samples, taken at 1 h, 3 h, 5 h, 7 h, 10 h, 15 h, 23 h, 28 h and 33 h, were placed in Ependorf tubes. A volume of 2 mL of RNAprotect Bacteria reagent (QIAGEN) was added and centrifuged at 8000 rpm for 10 min. The resulting pellets were stored at −80˚C. Total RNA was extracted using the RNeasy mini kit and purified using the RNeasy clean up kit. RNAs were assayed using Nanodrop. A PCR step was performed to verify RNA purity. RNA samples were then stored at −80˚C. Three biological replicates were carried out for this study.

2.11. cDNA Production and Purification

Single-stranded complementary DNA was produced using the High capacity CDNA reverse transcription kit. A 10µL volume of RNA is added to 10µL of a master mix containing reverse transcriptase. The resulting mixture is processed according to a program based on a series of incubations optimized according to the protocol of High capacity cDNA Reverse Transcription kits. The incubation program for retro-transcription of RNA into cDNA was carried out in four successive steps. Each step was assigned a specific temperature. Thus, four successive temperatures were observed:

- 25 0C for 10 minutes.
- 37 0C for 120 minutes.
- 85 0C for 10 minutes.
- 40C for storage.

2.12. RT q-PCR Technique

96-well plates were used for cDNA quantification. Each cDNA sample was diluted to a concentration of 0.2 ng/µL, and genomic DNA was prepared to establish the standard range. A cascade dilution was made from a 2 ng/µL genomic DNA sample, and a batch of 6 genomic DNA samples with concentrations ranging from 2 to 0.00002 ng/µL was obtained and used as the standard range. The experiment was carried out by adding 5 µL of each sample to wells according to an established plate plan. Control wells were set up by adding water instead of DNA. Volumes of 5 µL of primer and 10 µL of Master mix containing Sybr green (for genomic DNA and cDNA labelling) were added to each well. Plates were read in an RT-qPCR device (lightcycler480 or Bio-Rad) and results processed using the corresponding software (lightcycler480 SW 1.5.1 or Bio-Rad CFX Maestro).

3. Results and Discussion

3.1. Complete Genome Sequencing of *P. ulvae* TC14

The genome of *P. ulvae* TC14 was sequenced by Molecular Research LP (MR DNA), using the Illumina Hiseq 2500 method. A total of 2,293,242 base pairs were sequenced, *i.e.* 100% of the genomic volume. The number of coding gene sequences is 1983, and the G+C percentage of the base number is 41.55%. All this information is summarized in **Table 1** and figure below.

Table 1. Statistical data of *P. ulvae* TC14 genome.

Characteristics	*Numbers*	*Percentage of genomic volume*
DNA, total number of base pairs	4,586,484	100%
DNA, number of coding bases	4,114,901	89.72%
DNA, G+C number of bases	1,905,705	41.55%
Number of scaffolds	14	100%
Total number of genes	4110	100%
Number of coding genes	3966	96.50%
Number of RNA genes	144	3.50%
rRNA	41	1%
5S rRNA	12	0.29%
16S rRNA	13	0.32%
23S rRNA	16	0.39%
Number of tRNA genes	100	2.43%

3.2. Phylogenetic Analysis of *P. ulvae* TC14 Based on the rRNA16S Gene

Bacteria lend themselves very well to phylogenetic analysis, as many genomes have been fully sequenced and their number continues to grow. Their genomes are small and contain little repetitive DNA in contrast to the genomes of eukaryotes [13]. Previous studies carried out on the identification of *P. ulvae* TC14 based on phylogenetic analysis of the rRNA16S gene, showed that the closest species was *P. tunicata* [14]. We have therefore confirmed this phylogenetic analysis through this work, showing that *Pseudoalteromonas ulvae* TC14 does indeed belong to the ulvae species and that the closest relative is *P. tunicata*. On the other hand, the number of new isolates has complicated interpretation and identification of the species that would be their closest relatives. Some strains are grouped together in taxonomic units despite having different affiliations. On the other hand, some bootstraps are less than 70%, demonstrating a degree of uncertainty as to their true positioning. For example, the various members of the piscida species are not grouped together in a single taxonomic unit. This suggests that the 16S rRNA gene is not sufficiently

discriminating in this part of the tree and that other phylogenetic markers should be used. This is not the case for the ulvae species. Since it is grouped with *P. ulvae* UL12, the bootstrap separating *P. ulvae* from *P. tunicata* is 93%.

3.3. Genome Analysis and Comparison Based on Nucleotide Percentage

This study could only be carried out on strains whose genome is sequenced and available at https://img.jgi.doe.gov. For example, the strains *Pseudoalteromonas xiamenenis* Y2 and *Pseudoalteromonas fenneropenaei* rzy34, which are the closest strains to *P. ulvae* and *Pseudoalteromonas tunicata* (Table 2), do not have their genomes available in this database. We wanted to verify and refine these results with the help of genomic data by performing a pairwise analysis of the average nucleotide identity percentage (ANI), corresponding to a measure of genomic similarities between the coding regions of the two genomes. Using the tools available at https://img.jgi.doe.gov, we can see that *P. tunicata* D2 has the highest ANI (75.7913%), followed by *P. flavipulchra* NCIMB2033 (72.2736%) and *P. phenolica* KCTC 12086 (71.6685%). It should be pointed out here that a number of strains identified by 16s rRNA gene analysis did not have their genome sequenced, as is the case for *P. ulvae* UL12 for example, so it was only possible to carry out the ANI analysis on the basis of the sequenced genomes available on IMG JGI. This comparison method was used by [15]. In the same comparative study, these authors demonstrated the functional similarity of the strains. However, without describing the functional similarity of the bacterial strains in this study, this method reinforced the phylogenetic data noted in the previous section. In addition, there are two methods for comparative genome studies involving ANI, which are BLAST and NUMMER [16] [17]. In this study, the BLAST method was used to determine the similarity between the *P. ulvae* TC14 genome and the other strains cited above. In addition, this comparison technique was used for taxonomic purposes, hence the choice of BLAST [16]-[19].

Table 2. Average percentage nucleotide identity analysis result.

Genome1 ID	Genome1 Name	Genome2 ID	Genome2 Name	ANI1->2	ANI2->1	AF1->2	AF2->1
2718218507	*P. ulvae TC14*	2519899641	*P. piscicida* ATCC 15057	71.1341	71.1350	33.876	29.248
2718218507	*P. ulvae TC14*	638341157	*P. tunicata* D2	75.7913	75.7993	64.726	59.834
2718218507	*P ulvae TC14*	2630968884	*P. flavipulchra* NCIMB 2033 = ATCC BAA-314	72.2728	72.2736	36.223	37.019
2718218507	*P. ulvae TC14*	2684623173	*P. phenolica* KCTC 12086	71.6685	71.6826	35.168	33.138
2718218507	*P. ulvae TC14*	2728369620	*P. luteoviolacea* DSM6061	71.0796	71.0802	32.462	25.309
2718218507	*P. ulvae TC14*	2541047006	*P. rubra ATCC* 29570	70.5949	70.5936	32.371	25.072

P: *Pseudoalteromonas*; ANI calculation formula: $\text{gANI}_{(G_1 \to G_2)} = \frac{\sum \text{percent Identity} \times \text{Alignement length}}{\text{lengths of BBH genes}}$, ($G_1$: Genome number 1; G_2: Genome number 2; BBH: Bidirectional Best Hits).

3.4. Characterization of the Quorum Sensing System in *P. ulvae* TC14

3.4.1. Search for Genes Involved in QS in *P. ulvae* TC14

The bacterial communication system called quorum sensing (QS) previously described in this study, relies on different types of small diffusible molecules that are synthesized by different genes: luxI/luxR and luxS/luxP/luxQ, which are specific to the AI-1 and AI-2 systems [20] [21]. Thus, we consulted the protein repertoire provided by *P. ulvae* TC14 genomic sequencing data. Genes with annotations identical to those of the research genes found in the *P. ulvae* TC14 genome were the focus of our biocomputational analyses. Nucleic and protein sequences of QS genes previously characterized in other Vibrio, Shewanella, *Pseudoalteromonas* and *Chromobacterium* bacteria were also used in this study. BLASTN (nucleic sequence comparison) and BLASTP (protein sequence comparison) searches were carried out on the *P. ulvae* TC14 genome to verify identity and detect homologous sequences.

3.4.2. Search for the LuxI/LuxR pair in *P. ulvae* TC14

Of the 4110 coding genes contained in the TC14 genome according to sequencing data, no gene has been annotated with LuxI. A search for the presence of pfam00765-type domains (InterPro001690) associated with genes homologous to luxI from *Vibrio fisheri* (lasI, or rhlI from *Pseudomonas aeruginosa*) yields no results when looking specifically at the *P. ulvae* TC14 genome. A search based on pfam00765 associated with the luxI-type autoinducer synthase domain found in *V. fisheri*, lasI and rhlI from *P. aeruginosa* on the *P. ulvae* TC14 genome and all the other sequenced strains belonging to the *Pseudoalteromonas* bacteria (in total representing 33 different species) available on https://img.jgi.doe.gov/, only the genomes of *P. luteoviolacea* strains contain this pfam and would therefore have a luxI. These 8 luxI genes (among the 16 *P. luteoviolecea* species) do not appear to be associated with a luxR gene. The results therefore showed that no protein family played the role of AHL synthase in *P. ulvae* TC14. Interestingly, with pfam17327 corresponding to homologs of luxM, also an AHLs synthase, out of 65 selected *Pseudoalteromonas* genomes representing 33 different species), none yielded results. Our research also took into account another protein family capable of synthesizing AHLs in Gram-negative bacteria. This is the AinS/LuxM complex (belonging respectively to *Vibrio harveyi* [11] [22]. These proteins bear no similarity to the LuxI protein family. However, they are known to produce AHLs in these bacteria [23]. In the case of *P. ulvae* TC14, our investigations revealed the absence of the AinS/LuxM complex, synonymous with a lack of AHL production. However, searches carried out on the same genome revealed the presence of 8 gene sequences annotated "LuxR" (**Table 3**) at https://img.jgi.doe.gov, none of which possess pfam03472 (InterPro IPR005143). However, other *Pseudoalteromonas* strains such as *P. luteoviolacea*, *P. piscida* or *P. rubra* possess this pfam (among the 33 different species sequenced and referenced on the JGI IMG website). On the other hand, these do not appear to be next to a luxI. Consequently, protein analyses of the pfam and

COG groups of the various luxR sequences found in *P. ulvae* TC14 revealed that most of them belong to the luxR transcriptional regulatory gene families. The desired result would have been the detection of at least one LuxI sequence in addition to the 8 LuxR homologs detected presented in Table 3, as in the work carried out by [24]. Indeed, these authors detected at least one luxI and 5 luxR sequences in a *Pseudoalteromonas* strain. Their results showed that this strain was capable of synthesizing AHLs. However, the absence of LuxI in our case shows that *P. ulvae* TC14 has very different molecular characteristics. The 8 LuxR homologs found in this bacterium (Table 3) could be LuxR solos sequences. In AHL-producing bacteria, LuxR solo (R solo) can bind endogenous AHLs (produced by the bacterium) and/or exogenous AHLs (produced by other microorganisms). In non-AHL-producing bacteria, AHLs solos (R solo) can bind not only to AHLs supplied by other microorganisms, but also to other AHLs from eukaryotic organisms.

Table 3. Bioinformatics data from the evaluation of the presence of sequences from the LuxR gene family in TC14.

N˚	Locus tag	Numéro d'identification	Famille de gènes	Séquences de primers (5'→3')	Pourcentage G-C (%)	TM (˚C)	Gènes homologues	Organisme hôte et pourcentage d'identité
1	B1199_03300	2721734215	LUX R	5'-GCATGCCGAACCTAGATGGA-3'	55/50	62/62	DNA-binding response regulator diguanylate cyclase	*Pseudoalteromonas* tunicate (94%) Vibrio fischeri (36%)
2	B1199_02065	2721733968	LUX R	5'-AGTCGACACCAACCTACCTG-3'	55/55	62/62	DNA-binding response regulator LuxR family transcriptional regulator	*Pseudoalteromonas* tunicate (78%) *Pseudoalteromonas* sp. H103 (42%)
3	B1199_01515	2721733858	LUX R	5'-GAAGGGTTAGCACTGGCGAT-3'	55/55	62/62	Transcriptionnel regulator LuxR family system response regulator NarL	Vibrio fischeri (33%) *Pseudoalteromonas* sp. T1lg23B (93%)
4	B1199_05620	2721736060	LUX R	5'-TATTGGACGATGTTCGTGGCT-3'	47.6/55	47.3/62	Lux R family system response regulator UvrY	Vibrio fischeri (72%) *Pseudoalteromonas* piscicida (92%)
5	B1199_01760	2721733907	LUX R	5'-CCGCATCCGAGAGGATTACC-3'	60/50	64/60	Lux R family DNA-binding response regulateur	Vibrio fischeri (47%) *Pseudoalteromonas* sp (91%)
6	B1199_00340	2721733623	LUX R	5'-CACTGTGCCTTCGGCTAAGA-3'	55/50	62/60	Transcriptionnel regulator LuxR family Transcriptionnel regulator LuxR family	*Pseudoalteromonas luteoviolacea* B = ATCC 29581 (63%) Alkalimonas amylolytica (47%)
7	B1199_10560	2721735666	LUX R	5'-CAAAGAAGCCCCGAGTGAGT-3'	55/55	62/62	DNA-binding response regulator	Shewanella fidelis (60%) *Pseudoalteromonas luteoviolacea* (67%)
8	B1199_07575	2721735070	LUX R	5'-ATGCTATCACTTGGCGCTGA-3'	50/55	60/62	DNA-binding response regulator	*Pseudoalteromonas tunicate* (54%)

3.4.3. Evaluation of the Expression of LuxR Gene Sequences Detected on the Genome of *P. ulvae* TC14

Apart from the bioinformatics data, a molecular characterization of the presumptive LuxR sequences was carried out in order to evaluate their gene expression. For this, a q-PCR analysis was used to evaluate the expression of these LuxR sequences. The results are presented in Figure 1 below.

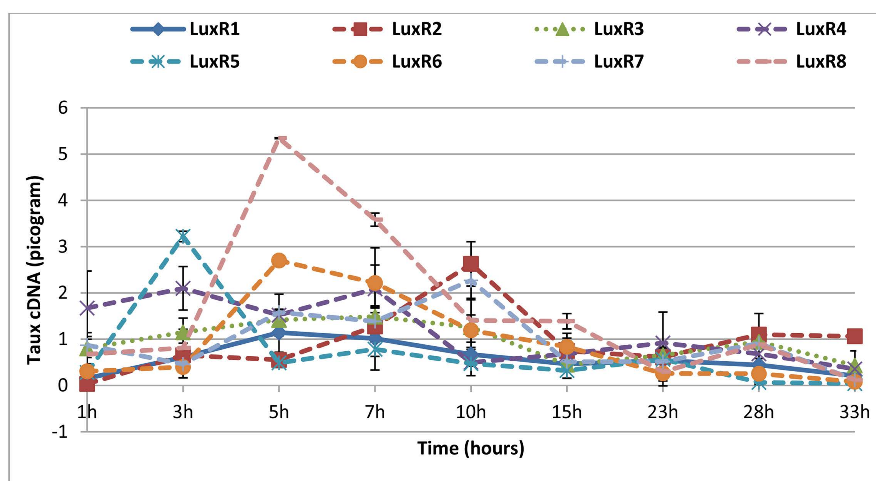

Figure 1. Evaluation curves of the quantities of cDNA from the presumptive LuxR sequences Found on the genome of *P. ulvae* TC14.

Primers previously selected on NCBI from the presumptive LuxR sequences found on the TC14 genome were used to amplify single-stranded DNA from total RNAs collected at times between 1 h and 33 h. The experiment was carried out several times using q-PCR equipment (Bio Rad). Labeling was carried out using SsoAdvanced™ Universal SYBR® Green Supermix from Bio-Rad. To better evaluate the expression of the presumptive LuxR sequences found in TC14, cDNA production was monitored using the q-PCR technique. The results obtained above (**Figure 1**) show a relatively high production of cDNA (5.3438 pg) from the LuxR8 sequences around 5 hours of incubation. The LuxR5 and LuxR6 sequences admit peaks respectively at 3 h (3.22 pg) and 5 h (2.70 pg), which are below that of sequence number 8. Still in decreasing order of the rate of cDNA production by the presumptive LuxR sequences tested in **Figure 1**, we find sequences number 2, 4 and 7. These admit peaks which are above 2 pg but low compared to the cDNA rates from sequences 5 and 6. As for the last two sequences, *i.e.* LuxR1 and 3, the cDNA production rates for each of them remaining below 2 pg throughout the monitoring period (from 1 h to 33 h), it turns out that these sequences admit the lowest cDNA rates.

3.4.4. Approach between LuxR Gene Expression and Cell Density in *P. ulvae* TC14

The expression of presumptive LuxR sequences was evaluated through the cDNA production rate in **Figure 1**. Monitoring of this production can theoretically be divided into two phases: a first phase between 1h and 15h and a second between 15h and 33h. The first phase has an overall cDNA rate that is higher than that of the second phase. These results could be linked to the growth phases of TC14 described in the work of Brian-Jaisson et al, 2014. Indeed, this work showed that TC14 has an exponential phase that would be around 10h and a stationary phase that would begin around 15h. That is to say, bacterial cells tend to produce most of the LuxR genes before the stationary phase.

3.4.5. The Hypothesis of QS Regulation by Solo LuxRs in TC14

QS detection based on AHL signaling molecules generally marks the presence of a protein pair consisting of a LuxI family synthase and a LuxR transcriptional regulator. However, recent studies on a large number of proteobacteria have shown that they generally possess a LuxR family protein for which there is no related LuxI synthase [25]. These LuxRs acting alone without resorting to a related signal generator (LuxI) have been proposed to be called "solo LuxRs". This type of receptor has also been described in several studies, to have diverse roles in bacterial inter-species and inter-domain communication. The mode of operation of solo LuxR has been widely described by some authors [26].

The QS system of TC14 as described in this study, presents similarities with a QS system based on solo LuxRs. Indeed, our research showed the presence of functional Lux R in TC14. However, this QS system based on AHLs, is devoid of the Lux I synthase protein. In addition, no LuxI analogue such as Ains or LuxM could be detected on the TC14 genome.

These results are similar to those of [9] who showed by chromatographic techniques the absence of endogenous AHLs in TC14. On the other hand, these authors suggested that the absence of AHL production in this bacterium was, it seems, filled by exogenous AHLs. This would allow the bacteria to regulate its biofilm and the production of violacein.

Beyond the non-production of AHLs by TC14 described by Aye and colleagues, we have shown through this study that this bacterium possesses a transcriptional regulator LuxR which controls the different expressions of target genes by its fixation to exogenous AHLs. Thus the mode of operation of luxR described in TC14 is likely to be close to that of the solo LuxRs previously mentioned. Indeed, according to the work carried out by [26], proteobacteria studied by them possessed a protein of the LuxR family for which there was no obvious related LuxI synthase; these proteins were found in bacteria that possess one or more complete AHL QS systems as well as in bacteria that do not have them. These authors therefore proposed to call these LuxR homologues, solo luxRs. These allow the bacterium to respond to the dogenic and exogenous signals of their neighbors. In addition, these solo LuxRs are able to act autonomously without requiring an associated signal generator. This could be the case for the *P. ulvae* TC14 strain described in this study.

3.5. AI-2 Gene Search in TC14

LuxR, a gene characteristic of type I autoinduction, was thus characterized in TC14 in the previous part of our study. It is then appropriate to verify the presence of other types of autoinduction in this bacterium in order to better understand the functioning of its QS system. For this, the specific genes of AI-2 were taken into account in our research on the TC14 genome. As previously described, the AI-2 system has LuxS as its synthase and LuxPQ as its receptors. These genes have been described in *Vibrio harveyi* and Salmonella typhimurium [27]. However, the BLASTP and BLASTN performed on TC14 during this study did not reveal any

homologous sequence of AI-2 genes. The different families of COG and Pfam proteins consulted in TC14 do not mention any functional similarity with AI-2. This therefore marks the absence of this auto-induction system in TC14.

3.6. Characterization of the violacein operon in TC14

The results of sequencing the TC14 genome reveal the presence of the Vio gene group comprising five ORFs (vioABCDE). In terms of number of ORFs (5) these results are similar to those of Sanchez and colleagues in 2006 who found the same number in the vio operon in *C. violaceum*. Furthermore, the vio operon can comprise 4 ORFs (vioABCD) in certain bacteria [28]. A schematic representation of the vio operon in TC14 is depicted in Figure 2 below.

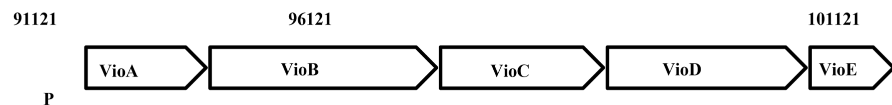

Figure 2. Schematic representation of the Vio operon in TC14 (The Vio operon consisting of five VioABCDE ORFs is between positions 91121 and 101121. The promoter (P) is located at the beginning of the VioA gene).

3.7. Evaluation of the Expression of the Vio Operon in TC14

The expression of the vio operon was studied under the same conditions as that of the presumptive LuxR sequences (Figure 3). Thus, primers from the VioA, VioD and VioE genes were used for q-PCR amplification of the cDNAs. These three genes were studied as representatives of the Vio operon. These single-stranded DNAs were previously produced from total RNA extracted from TC14 at times between 1h and 33h. The results presented in Figure 3 below show the production of a high level of cDNA (4.86 pg) from the Vio A sequence at 10 a.m. The Vio D and E sequences each have a peak at 10 a.m. but remain relatively low.

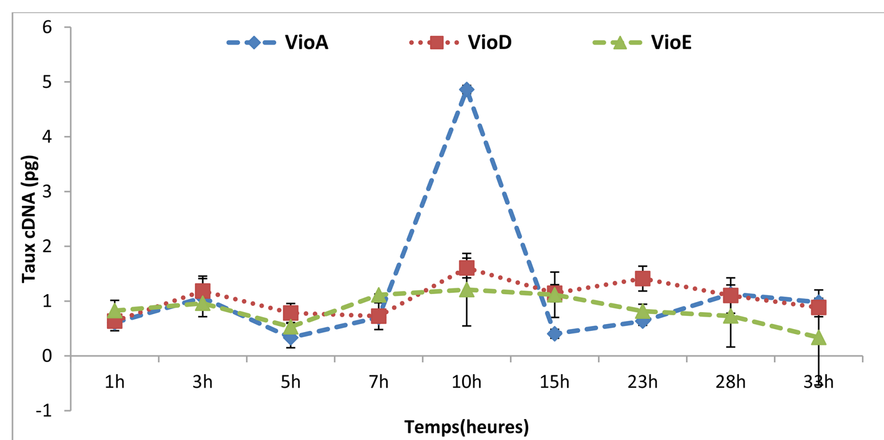

Figure 3. Expression rate of the Vio operon in TC14.

4. Conclusion

Until now, hypotheses concerning the absence of LuxI in the *P. ulvae* TC14

bacterium led to some confusion as to the possibility of regulating certain functions such as biofilm formation and biofilm production. This study not only verified this hypothesis, but also provided a relatively broad view of QS function in this particular model strain. Thus, the complete sequencing of the TC14 genome was the basic step in this work. Using genomic comparison methods, the genomic identity of the bacterium was demonstrated. The bioinformatics techniques subsequently employed showed that of the LuxR/LuxI regulator pair present in other bacteria harboring the QS system, there was only one element, LuxR. Moreover, no AI-2 auto-induction system regulator was found on the genome. Nevertheless, an approach was made between LuxR homologs and solos R sequences that would act autonomously to regulate exogenous AHLs. However, more detailed studies at genomic sequence level will be necessary to obtain more complete information on QS function in this bacterium.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Lee, H.Y. (2006) *Étude phylogénétique des γ-protéobactéries basée sur les gènes 16S ARNr et des gènes codant pour des protéines*. Ph.D. Thesis, Université du Québec.

[2] Zhao, X., Yu, Z. and Ding, T. (2020) Quorum-Sensing Regulation of Antimicrobial Resistance in Bacteria. *Microorganisms*, **8**, Article 425. https://doi.org/10.3390/microorganisms8030425

[3] Liaqat, I. (2019) Biofilm Formation, Maturation and Prevention: A Review. *Journal of Bacteriology and Mycology*, **6**, 1092. https://doi.org/10.26420/jbacteriolmycol.2019.1092

[4] Williams, P. and Cámara, M. (2009) Quorum Sensing and Environmental Adaptation in *Pseudomonas Aeruginosa*: A Tale of Regulatory Networks and Multifunctional Signal Molecules. *Current Opinion in Microbiology*, **12**, 182-191. https://doi.org/10.1016/j.mib.2009.01.005

[5] Azimi, S., Klementiev, A.D., Whiteley, M. and Diggle, S.P. (2020) Bacterial Quorum Sensing during Infection. *Annual Review of Microbiology*, **74**, 201-219. https://doi.org/10.1146/annurev-micro-032020-093845

[6] Alfinito, E., Beccaria, M. and Cesaria, M. (2023) Quorum Sensing as a Long-Range Interaction for Bacteria Growth and Bioluminescence. arXiv: 2304.11133.

[7] Mayer, C., Muras, A., Parga, A., Romero, M., Rumbo-Feal, S., Poza, M., *et al.* (2020) Quorum Sensing as a Target for Controlling Surface Associated Motility and Biofilm Formation in Acinetobacter Baumannii ATCC® 17978tm. *Frontiers in Microbiology*, **11**, Article 565548. https://doi.org/10.3389/fmicb.2020.565548

[8] Kumar, L., Patel, S.K.S., Kharga, K., Kumar, R., Kumar, P., Pandohee, J., *et al.* (2022) Molecular Mechanisms and Applications of N-Acyl Homoserine Lactone-Mediated Quorum Sensing in Bacteria. *Molecules*, **27**, Article 7584. https://doi.org/10.3390/molecules27217584

[9] Mireille Ayé, A., Bonnin-Jusserand, M., Brian-Jaisson, F., Ortalo-Magné, A., Culioli, G., Koffi Nevry, R., *et al.* (2015) Modulation of Violacein Production and Phenotypes Associated with Biofilm by Exogenous Quorum Sensing N-Acylhomoserine Lactones

in the Marine Bacterium *Pseudoalteromonas Ulvae* TC14. *Microbiology*, **161**, 2039-2051. https://doi.org/10.1099/mic.0.000147

[10] Lingeswaran, A. (2020) Rôle clé du transporteur PptAB dans le quorum sensing chez Streptococcus thermophilus. Master's Thesis, Université Paris-Saclay.

[11] Fuqua, C. and Greenberg, E.P. (2002) Listening in on Bacteria: Acyl-Homoserine Lactone Signalling. *Nature Reviews Molecular Cell Biology*, **3**, 685-695. https://doi.org/10.1038/nrm907

[12] Gilson, L., Kuo, A. and Dunlap, P.V. (1995) AinS and a New Family of Autoinducer Synthesis Proteins. *Journal of Bacteriology*, **177**, 6946-6951. https://doi.org/10.1128/jb.177.23.6946-6951.1995

[13] Wen, Z., He, M., Peng, C., Rao, Y., Li, J., Li, Z., *et al.* (2019) Metabolomics and 16S Rrna Gene Sequencing Analyses of Changes in the Intestinal Flora and Biomarkers Induced by Gastrodia-Uncaria Treatment in a Rat Model of Chronic Migraine. *Frontiers in Pharmacology*, **10**, Article 1425. https://doi.org/10.3389/fphar.2019.01425

[14] Brian-Jaisson, F., Ortalo-Magné, A., Guentas-Dombrowsky, L., Armougom, F., Blache, Y. and Molmeret, M. (2014) Identification of Bacterial Strains Isolated from the Mediterranean Sea Exhibiting Different Abilities of Biofilm Formation. *Microbial Ecology*, **68**, 94-110. https://doi.org/10.1007/s00248-013-0342-9

[15] Thomas, J.W., Touchman, J.W., Blakesley, R.W., Bouffard, G.G., Beckstrom-Sternberg, S.M., Margulies, E.H., *et al.* (2003) Comparative Analyses of Multi-Species Sequences from Targeted Genomic Regions. *Nature*, **424**, 788-793. https://doi.org/10.1038/nature01858

[16] Rosselló-Móra, R. and Amann, R. (2015) Past and Future Species Definitions for Bacteria and Archaea. *Systematic and Applied Microbiology*, **38**, 209-216. https://doi.org/10.1016/j.syapm.2015.02.001

[17] Kim, S., Kim, D., Cho, S.W., Kim, J. and Kim, J. (2014) Highly Efficient RNA-Guided Genome Editing in Human Cells via Delivery of Purified Cas9 Ribonucleoproteins. *Genome Research*, **24**, 1012-1019. https://doi.org/10.1101/gr.171322.113

[18] Stropko, S.J., Pipes, S.E. and Newman, J.D. (2014) Genome-based Reclassification of *Bacillus cibi* as a Later Heterotypic Synonym of *Bacillus indicus* and Emended Description of *Bacillus indicus*. *International Journal of Systematic and Evolutionary Microbiology*, **64**, 3804-3809. https://doi.org/10.1099/ijs.0.068205-0

[19] Yu, Z., Shi, X., Zhang, Z., Gou, Y., Miao, X. and Kalipi, I. (2022) Numerical Investigation of Blast-Induced Rock Movement Characteristics in Open-Pit Bench Blasting Using Bonded-Particle Method. *Rock Mechanics and Rock Engineering*, **55**, 3599-3619. https://doi.org/10.1007/s00603-022-02831-w

[20] Tan, J., Zhao, H., Li, J., Gong, Y. and Li, X. (2023) The Devastating Rice Blast Airborne Pathogen Magnaporthe Oryzae—A Review on Genes Studied with Mutant Analysis. *Pathogens*, **12**, Article 379. https://doi.org/10.3390/pathogens12030379

[21] Verma, S. and Miyashiro, T. (2013) Quorum Sensing in the Squid-Vibrio Symbiosis. *International Journal of Molecular Sciences*, **14**, 16386-16401. https://doi.org/10.3390/ijms140816386

[22] Kimbrough, J.H. and Stabb, E.V. (2013) Substrate Specificity and Function of the Pheromone Receptor AinR in *Vibrio fischeri* Es114. *Journal of Bacteriology*, **195**, 5223-5232. https://doi.org/10.1128/jb.00913-13

[23] Colton, D.M., Stabb, E.V. and Hagen, S.J. (2015) Modeling Analysis of Signal Sensitivity and Specificity by *Vibrio fischeri* LuxR Variants. *PLOS ONE*, **10**, e0126474. https://doi.org/10.1371/journal.pone.0126474

[24] Kuo, A., Callahan, S.M. and Dunlap, P.V. (1996) Modulation of Luminescence Operon Expression by N-Octanoyl-L-Homoserine Lactone in AinS Mutants of Vibrio Fischeri. *Journal of Bacteriology*, **178**, 971-976. https://doi.org/10.1128/jb.178.4.971-976.1996

[25] Dang, H.T., Komatsu, S., Masuda, H. and Enomoto, K. (2017) Characterization of Luxi and LuxR Protein Homologs of N-Acylhomoserine Lactone-Dependent Quorum Sensing System in *Pseudoalteromonas* sp. 520p1. *Marine Biotechnology*, **19**, 1-10. https://doi.org/10.1007/s10126-016-9726-4

[26] Subramoni, S. and Venturi, V. (2009) LuxR-family 'Solos': Bachelor Sensors/Regulators of Signalling Molecules. *Microbiology*, **155**, 1377-1385. https://doi.org/10.1099/mic.0.026849-0

[27] Taga, M.E., Semmelhack, J.L. and Bassler, B.L. (2001) The LuxS-Dependent Autoinducer AI-2 Controls the Expression of an ABC Transporter That Functions in AI-2 Uptake in *Salmonella typhimurium*. *Molecular Microbiology*, **42**, 777-793. https://doi.org/10.1046/j.1365-2958.2001.02669.x

[28] August, P.R., Grossman, T.H., Minor, C., Draper, M.P., MacNeil, I.A., Pemberton, J.M., Osburne, M.S., *et al*. (2000) Sequence Analysis and Functional Characterization of the Violacein Biosynthetic Pathway from *Chromobacterium violaceum*. *Journal of Molecular Microbiology and Biotechnology*, **2**, 513-519.