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Marion Urvoy, Raphaël Lami, Catherine Dreanno, David Daudé, Alice M S Rodrigues, Michèle Gourmelon, Stéphane L'Helguen, Claire Labry

Urvoy Marion (Orcid ID: 0000-0002-7095-1835)

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Marion Urvoy[1,2,*], Raphaël Lami[3], Catherine Dreanno[5], David Daudé[4], Alice M.S. Rodrigues[3], Michèle Gourmelon[1], Stéphane L'Helguen[2], Claire Labry[1]

[1]Ifremer, DYNECO, F-29280 Plouzané, France

[2]Université de Bretagne Occidentale, CNRS, IRD, Ifremer, UMR 6539, Laboratoire des Sciences de l'Environnement Marin (LEMAR), F-29280 Plouzané, France

[3]Sorbonne Université, CNRS, Laboratoire de Biodiversité et Biotechnologies Microbiennes (LBBM), 66650 Banyuls-sur-Mer, France

[4]Gene&GreenTK, 19-21 Boulevard Jean Moulin, 13005 Marseille, France

[5]Ifremer, RDT, F-29280 Plouzané, France

[*]Correspondence: marion.urvoy@outlook.fr

Running title

Quorum sensing regulations in estuarine strains

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Significance statement

In this study, we isolated estuarine bacteria and evaluated their *N*-acylhomoserine lactone (AHL)-based quorum sensing and quorum quenching abilities using various techniques, which has never been done in an estuary ecosystem before. Thus, our work contributes to the depiction of communication pathways among estuarine bacteria, from genes to metabolites. We then studied the impact of quorum sensing-mediated microbial interactions on biofilm production, dissolved and cell-bound leucine aminopeptidases and β-glucosidases. To this extent, the AHL communication pathways were disrupted using a lactonase assay among 28 phylogenetically diverse estuarine bacterial isolates. To the best of our knowledge, this approach was only used on two environmental strains before. Furthermore, quorum sensing regulation of both dissolved and cell-bound enzymes has never been investigated simultaneously in environmental strains. This experiment evidenced the broad involvement of quorum sensing in the regulation of biofilm as well as cell-bound and dissolved leucine-aminopeptidases and β-glucosidases. As such, this study provides evidence that bacterial cell-cell communication can potentially influence global biogeochemical cycles through the modulation of prokaryotic functions and lifestyle.

Summary

Biofilms of heterotrophic bacteria cover organic matter aggregates and constitute hotspots of mineralization, primarily acting through extracellular hydrolytic enzyme production. Nevertheless, regulation of both biofilm and hydrolytic enzyme synthesis remains poorly investigated, especially in estuarine ecosystems. In this study, various bioassays, mass spectrometry and genomics approaches were combined to test the possible involvement of quorum sensing (QS) in these mechanisms. QS is a bacterial cell-cell communication system that relies notably on the emission of *N*-acylhomoserine lactones (AHLs). In our estuarine bacterial collection, we found that 28 strains (9%), mainly *Vibrio*, *Pseudomonas* and *Acinetobacter* isolates, produced at least 14 different types of AHLs encoded by various *luxI* genes. We then inhibited the AHL QS circuits of those 28 strains using a broad-spectrum lactonase preparation and tested whether biofilm production as well as β-glucosidase and leucine-aminopeptidase activities were impacted. Interestingly, we recorded contrasted responses, as biofilm production, dissolved and cell-bound β-glucosidase and leucine-aminopeptidase activities significantly increased in 4%-68% of strains but decreased in 0%-21% of strains. These findings highlight the key role of AHL-based QS in estuarine bacterial physiology and ultimately on biogeochemical cycles. They also point out the complexity of QS regulations within natural microbial assemblages.

Introduction

Heterotrophic microbial communities play a central role in biogeochemical carbon (C) and nutrients cycling in the oceans (Azam and Malfatti, 2007). As organic matter mostly consists of unavailable polymeric substrates, heterotrophic prokaryotes need to cleave them into smaller molecules that can be transported across their cell membranes (Arnosti, 2011). These activities rely on the expression of a diverse array of dissolved (cell-free) and cell-bound extracellular hydrolytic enzymes. Those enzymes initiate organic matter mineralization through the 'microbial loop' (Pomeroy, 1974; Azam *et al.*, 1983), ultimately affecting C export, nutrient cycling and energy flow through the food web (Chróst, 1990; Azam and Malfatti, 2007).

Estuaries are important transition zones where organic matter is heavily transformed before reaching coastal areas. Those mineralization processes are particularly intense in the maximum turbidity zone, where aggregate concentrations may reach several grams per liter. Marine bacteria living on such aggregates frequently reside in complex multi-layered environments called biofilms, where they are embedded in a matrix of extracellular polymers (Dang and Lovell, 2016; Flemming *et al.*, 2016). Those particle-attached microorganisms are often more active than their free-living counterparts in terms of bacterial production and enzymatic activity (Crump *et al.*, 1999; Millar *et al.*, 2015). These enzymes actively solubilize particulate organic matter into dissolved compounds, impacting both local organic matter composition and C export to coastal waters (Azam and Malfatti, 2007; Dang and Lovell, 2016). Dissolved enzymes, which may be actively secreted or result from viral lysis and grazing (Baltar, 2018), are also involved in the mineralization of organic matter. They often account for a large proportion of total enzymatic activities (Baltar, 2018; Thomson *et al.*, 2019).

Despite their important ecological implications, the mechanisms that regulate bacterial colonization, cell-bound and dissolved hydrolytic enzyme expression among these microbial consortia have been poorly characterized, especially in estuarine environments. *In situ* enzymatic activity of bacterial communities likely results from the communities' genetic potential (determined by the bacterial community composition), as well as the differential expression of those genes, driven

by environmental factors (Arnosti, 2011). Indeed, distinct microbial communities with specific taxonomic and functional compositions may colonize organic matter particles and express various hydrolytic enzymes (Delong *et al.*, 1993; Middelboe *et al.*, 1995; Rieck *et al.*, 2015). Hydrolytic enzyme production is also tightly regulated at the transcriptional level (Chróst, 1990; Arnosti, 2011). In particular, the concentration and composition of organic matter are key factors driving hydrolytic enzyme production through substrate induction, end-product repression or catabolic repression (Chróst and Siuda, 2002; Arnosti, 2011).

Quorum sensing (QS) is a widespread communication system allowing bacterial assemblages to synchronize their gene expression at the population level (Miller and Bassler, 2001; Waters and Bassler, 2005). This mechanism relies on the production, diffusion and sensing of small diffusible molecules called autoinducers (AIs). When the concentration of AIs increases under favorable conditions (*i.e*. high cellular density, low mass-transfer properties), they bind their cognate receptors and regulate the transcription of their target genes (Waters and Bassler, 2005; Papenfort and Bassler, 2016). These target genes are involved in diverse functions such as bioluminescence, motility, biofilm formation, virulence and hydrolytic enzyme production (Miller and Bassler, 2001; Hmelo, 2017). In Gram-negative bacteria, the most well-characterized QS system is based on the synthesis of *N*-acylhomoserine lactones (AHLs) (Papenfort and Bassler, 2016; Hmelo, 2017). These cell-cell QS-based interactions can be disrupted by quorum quenching (QQ) mechanisms (Kalia, 2013; Grandclément *et al.*, 2015). They rely on the enzymatic degradation of signal molecules or on the production of QS inhibitors that block the signal production or reception, among other mechanisms (Kalia, 2013; Grandclément *et al.*, 2015).

QS and QQ are widespread mechanisms among aquatic microbial communities. A large diversity of QS and QQ genes have been identified in metagenomics datasets (Doberva *et al.*, 2015; Muras *et al.*, 2018). AHLs have been detected in various types of aquatic environments such as marine aggregates (Hmelo *et al.*, 2011; Jatt, Tang, Liu, Zhang, and X. H. Zhang, 2015), corals and sponges (Tait *et al.*, 2010) and bacterial mats (Decho *et al.*, 2009). Similarly, numerous bacteria isolated from

aquatic environments exhibit AI production or quenching capacities in culture (Gram *et al.*, 2002; Romero *et al.*, 2011; Tourneroche *et al.*, 2019). To our knowledge, no QS study has been conducted in estuarine ecosystems.

In aquatic environments, biofilm production, emission of QS compounds and hydrolytic activities are inherently linked. Densely packed biofilm favors both high enzymatic activities and the accumulation of QS compounds (Dang and Lovell, 2016; Flemming *et al.*, 2016; Hmelo, 2017). QS mechanisms are also known to regulate the biofilm formation, maturation and dispersion (Lami, 2019). In this vein, it was shown that the addition of QS compounds to both natural bacterial communities and bacterial cultures modified the activity of extracellular hydrolytic enzymes (Hmelo *et al.*, 2011; Jatt, Tang, Liu, Zhang, and X. Zhang, 2015; Krupke *et al.*, 2016; Su *et al.*, 2019), as well as their biofilm production (Liu *et al.*, 2017; Su *et al.*, 2019). Nevertheless, the role of QS as a regulatory mechanism of those bacterial functions involved in crucial biogeochemical processes remains underexplored, especially in estuaries.

In this study, we investigated the prevalence of AHL-based QS and QQ circuits among bacterial strains isolated in the Aulne estuary (Bay of Brest, France), combining bioassays, liquid chromatography coupled to mass spectrometry (UHPLC-HRMS/MS) and genomics approaches. We then explored whether AHL-based QS regulates biofilm production as well as dissolved and cell-bound leucine aminopeptidase (LAM) and β-glucosidase (β-glc) synthesis among the identified AHL-producing bacteria. An original approach of our work is the use of enzyme-driven QQ to disrupt the expression of QS circuits in several strains. Taken together, our results revealed that the studied estuarine bacteria synthesize a diverse array of AHLs. The inhibition of AHL signals led to contrasted effects on biofilm production and hydrolytic enzyme activities in several of the targeted strains, highlighting the importance of QS in the regulation of estuarine bacteria physiology and ultimately in biogeochemical cycles.

Results

Production of QS metabolites among estuarine bacteria

A total of 299 bacterial strains were isolated from the Aulne estuary (Table S1) and screened for their ability to produce AHLs, using the *Pseudomonas putida* F117 and *Escherichia coli* MT102 biosensors. Among them, only a few strains (19 strains, 6%) clearly induced at least one biosensor compared with the negative control (ratio > 1.5, $p < 0.05$, Table S2). In addition, 56 strains (19%) induced either a questionable induction ($1.2 < \text{ratio} < 1.5$, $p < 0.05$) or an inhibition (ratio < 0.8, $p < 0.05$) of the green fluorescent protein (GFP) production. To increase the probability of finding AHL-producing bacteria, we selected all 75 strains (25% of total isolated strains) for the analytical assessment of AHL production. Among those 75 bacteria, AHLs were detected in the supernatant of 28 strains using a UHPLC-HRMS/MS approach. Among those 28 strains, 26 (9% of total isolated strains) showed an AHL production that was quantifiable using commercially available standards (Table 1, see the Experimental procedures for the full AHL names). The two additional strains (*Pseudomonas* sp. AF02-5 and *Sphingobacterium* sp. AF02-2) seemed to produce an unsaturated C16-HSL (C16:1-HSL) given its mass, molecular formula and the presence of its characteristic fragments. Unfortunately, this AHL could not be confirmed nor quantified because the corresponding standard is not commercially available. Such AHLs are qualified as “putative” in this study (Table 1).

Overall, the AHLs produced by the isolated estuarine strains were diverse. A total of 14 different AHLs (Table 1) were identified, with a side-chain length ranging from 4 to 14 C atoms. Both hydroxyl and carbonyl substitutions were found at the C3 position among the identified AHLs. We also found several other putative AHLs (C6:1-HSL, C12:1-HSL, C16:1- and 3-OH-C7-HSL), which also could not be confirmed nor quantified. It is worth noting that the χ^2 test and Fischer's exact test showed no significant impact of the salinity or the sampling fraction (*i.e.* attached or free-living) on the number of isolated AHL-producing strains (data not shown).

Taxonomic identification of AHL-producing isolates

The 28 AHL-producing bacteria were either Gammaproteobacteria (27 strains) or Bacteroidetes (1 strain). More precisely, they belonged to the genera *Vibrio* (10 strains), *Pseudomonas* (10 strains), *Acinetobacter* (5 strains), *Obesumbacterium* (1 strain), *Morganella* (1 strain) and *Sphingobacterium* (1 strain, Fig. 1, Table 1, Table S3). For samples at salinity 0, only *Pseudomonas*-affiliated strains (6 strains) were detected as AHL producers, except one strain affiliated with *Sphingobacterium faecium*. There was a greater diversity of AHL-producing isolates retrieved at salinity 5, corresponding to the maximum turbidity zone. Those strains were affiliated with the *Pseudomonas*, *Acinetobacter*, *Morganella* and *Obesumbacterium* genera. At salinity 25, all AHL-producing strains were affiliated with *Vibrio* species (2 strains affiliated with *V. echinoideorum* in April and 8 strains affiliated with *V. alginolyticus* in July). Interestingly, all the *Vibrio* strains were found to produce 3-OH-C6-HSL, while none of the other strains produced this type of AHL. The AHL production among the other *Pseudomonas* and *Acinetobacter* species was much more diverse as they produce both long and short-chain AHLs.

Identification of strains capable of QQ

AHL-producing strains were further assessed for their capacities to produce anti-AHL compounds (Fig. 1, Fig. S1), a common feature in environmental bacteria (Romero *et al.*, 2012). None of the 28 tested strains showed significant quenching of C6-HSL. Indeed, only *Vibrio* sp. CFL-9 and *Vibrio* sp. CFL-25 significantly inhibited the biosensor-specific GFP production (by 13 and 12%, respectively, $p < 0.05$). However, they also presented a cytotoxic effect as the biosensor viability was reduced by 18% and 12%, respectively, compared with the control ($p < 0.01$, data not shown). As viability reduction accounted for the GFP reduction, those strains were not counted as AHL quenchers.

A total of 10 strains (36% of the tested strains) significantly quenched C14-HSL ($p < 0.05$). They were affiliated with the *Pseudomonas* genus (7 strains), with the addition of *Acinetobacter* sp. DF3-8, *Vibrio* sp. EF02-7 and *Morganella* sp. DF3-16. All these strains exhibited low quenching abilities, with

a reduction of GFP production between 6% and 20%, except for *Pseudomonas* sp. AF3-2 and AF3-9, which decreased GFP production by 46% and 29%, respectively. In addition, *Vibrio* sp. CFL-9 and *Vibrio* sp. CFL-25 reduced the biosensor-specific GFP production, but with a similar reduction in the biosensor viability (data not shown), so those strains were not counted as AHL quenchers.

Effects of lactonase on AHL production

We then tested whether QS circuits regulated biofilm production and hydrolytic enzyme synthesis. To this end, QS mechanisms were inhibited using a lactonase preparation active over a broad range of AHLs, including among others C4-HSL, C6-HSL, C8-HSL, C9-HSL, C10-HSL, C11-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-OH-C10-HSL and 3-OH-C11-HSL. (Hiblot *et al.*, 2013; Rémy *et al.*, 2020; Mion *et al.*, 2021). We first evaluated whether the AHL production of the 28 AHL-producing strains was affected by the lactonase treatment (Table 2). Among those 28 strains, 14 were found to significantly induce at least one of the biosensors (specific fold induction > 1.5, $p < 0.05$) at the end of the lactonase assay, allowing the assessment of the lactonase efficiency. Interestingly, the AHL production of 11 of them was significantly reduced by the lactonase treatment ($ratio_{lac/ctr}$ ranged from 0.0 to 0.6, $p < 0.05$), confirming the broad spectrum of the lactonase treatment. Three strains (*Pseudomonas* sp. AF02-5 and *Vibrio* sp. CFL-9 and E14) activated one biosensor in the control condition but were not significantly impacted by the lactonase treatment.

The 14 other strains did not sufficiently induce the biosensors in the control condition at the end of the assay, preventing the assessment of the lactonase efficiency. Indeed, 5 *Vibrio strains* (CFL-25, EF3-1, EFL-3, EFL-16 and EF02-15) induced a questionable GFP fold induction between 1.21-1.33, which is too low to demonstrate a significant effect of the lactonases. The remaining 9 strains did not induce the biosensors (ratio < 1.2). Among them, 5 strains were in the “questionable induction” during the initial screening. The difference in the growth stage at which the screening was performed could explain the absence of induction since the AHL production and degradation is growth phase-dependent.

Effects of lactonase treatment on biofilm production

Among the 28 tested strains, 25 were found to produce biofilm in the tested set-up. Two of those strains had their biofilm production significantly impacted by the lactonase treatment (Fig. 1, Table 3): a decrease in biofilm production was seen in *Pseudomonas* sp. BF02-28 ($ratio_{lac/ctr}$ = 0.48, $p < 0.05$) while an increase was observed in *Vibrio* sp. E14 ($ratio_{lac/ctr}$ = 2.29, $p < 0.01$). Interestingly, among the 8 other *Vibrio* strains tested, biofilm production seemed reduced in 5 strains (*Vibrio* sp. CFL-9, CFL-25, EFL-3, EFL-16 and EF3-1) but increased in 3 strains (*Vibrio* sp. E15, EF02-7 and EF02-14), although not significantly (Table 3). In addition, biofilm production seemed to decrease in *Acinetobacter* sp. DF3-15 and DF02-2 ($ratio_{lac/ctr}$ = 0.6 and 0.8 respectively, $p < 0.1$).

Effect of lactonase treatment on LAM activity

Dissolved LAM production was found in 61% of the strains, especially in *Vibrio* and *Acinetobacter* species. However, they were mostly negligible compared with cell-bound LAM, except for *Vibrio* sp. CFL-9 and CFL-25, for which dissolved LAM represented most of the total activity (Table 3). Upon lactonase addition, dissolved LAM production was affected in 19 out of 28 strains ($p < 0.05$, Fig. 1, Table 3, Fig. S2). Dissolved LAM production increased ($ratio_{lac/ctr}$ between 1.25 and 7.47, $p < 0.05$) in 18 strains (7 *Pseudomonas*-, 1 *Sphingobacterium*-, 1 *Acinetobacter*-, 1 *Obesumbacterium*-, 1 *Morganella*- and 7 *Vibrio*-affiliated strains). By contrast, LAM production decreased in *Pseudomonas* sp. AF3-9 ($ratio_{lac/ctr}$ = 0.19, $p < 0.01$). Interestingly, among the 12 tested strains that did not produce dissolved LAM in the absence of AHLs, 8 strains showed a detectable production with the lactonase treatment.

The lactonase treatment affected cell-bound LAM production in 11 out of 28 strains ($p < 0.05$, Fig. 1, Table 3, Fig. S2). Among them, 5 *Pseudomonas*-affiliated strains (A14, A20, AF3-2, AF3-9, AF02-5) had their cell-bound LAM production increased ($ratio_{lac/ctr}$ between 1.30 and 2.44, $p < 0.05$). By contrast, 6 strains (*Vibrio* sp. CFL-9, E15, EFL-16, EF02-7; *Obesumbacterium* sp. DF3-3; and

Acinetobacter sp. DF3-8) exhibited a decreased cell-bound LAM ($ratio_{lac/ctr}$ between 0.59 and 0.89, $p < 0.05$).

Effect of lactonase treatment on β-glc activity

Production of dissolved β-glc was not frequent, with only 3 strains exhibiting a detectable hydrolysis of MUF-glc in the absence of lactonase (*Sphingobacterium* sp. AF02-2, *Pseudomonas* sp. B20 and *Pseudomonas* sp. BF02-28, Table 3). Among them, dissolved β-glc represented an important fraction of total β-glc in *Pseudomonas* sp. B20 and BF02-28 (respectively 57 and 72%, Table 3). *Pseudomonas* sp. B20 was significantly impacted by the lactonase treatment ($ratio_{lac/ctr} = 1.33$, $p < 0.01$, Fig. 1, Table 3). The strains that did not exhibit a detectable amount of dissolved β-glc in the absence of lactonase also did not produce a detectable amount of dissolved β-glc upon lactonase treatment.

Overall, 22 strains out of 28 produced cell-bound β-glc and most of them exhibited low activity, with the exception of *Sphingobacterium* sp. AF02-2, *Obesumbacterium* sp. DF3-3 and *Vibrio* sp. CFL-9 and CFL-25. Among the 28 strains, 7 strains had their cell-bound β-glc significantly impacted by the lactonase treatment ($p < 0.05$, Fig. 1, Table 3, Fig. S2). *Acinetobacter* sp. DF3-8 ($ratio_{lac/ctr} = 1.32$, $p < 0.05$) and *Pseudomonas* sp. A20 (production not detected in control treatment, $p < 0.01$) exhibited an increase in cell-bound β-glc with the lactonase treatment while 5 *Vibrio* sp. (CFL-9, E14, EFL-3, EFL-16 and EF3-1) exhibited decreased activity ($ratio_{lac/ctr}$ between 0.83 and 0.63, $p < 0.05$).

Identification of QS, QQ, hydrolase and biofilm-related genes in estuarine bacteria genomes

The genomes of four strains of *Pseudomonas* sp. (AF3-9 and B20), *Vibrio* sp. E14 and *Acinetobacter* sp. DF3-8 were sequenced to find AHL-related QS and QQ genes, carbohydrate-active enzymes (CAZymes), proteases and biofilm-related genes (pili, flagella, fimbriae and chemotaxis) (Table 4). Those strains were chosen because they covered the taxonomic diversity of the AHL-producing strains and exhibited marked responses to lactonase addition. The genome assembly

metrics are given in Table S5. *Pseudomonas* sp. AF3-9 and B20 possessed complex AHL-based QS circuits, with each having two putative AHL synthases (*luxI*-type and *hdtS*-type), several AHL receptors and three AHL acylases. Interestingly, the AHL receptors included several *luxR* solos, that is to say, without an accompanying synthase (Fuqua, 2006). The *hdtS* synthases were not located near an AHL receptor. *Acinetobacter* sp. DF3-8 and *Vibrio* sp. E14 encoded, respectively, one *luxI*/*luxR* and one *luxM*/*luxN* tandem and no QQ enzymes. The phylogenetic trees of the QS and QQ genes are presented in Fig. S3.

All the strains contained numerous genes coding for hydrolases, including metallopeptidases (which include LAM) and glycoside hydrolases (which include β-glc). Interestingly, one of the *Pseudomonas* sp. AF3-9 solo *luxR* genes was located directly next to two serine peptidases (Fig. S4). They also all possessed numerous genes involved in motility and adhesion (pili, flagella, fimbriae and chemotaxis), which are linked to biofilm development (Wolska *et al.*, 2016; Berne *et al.*, 2018; Rossi *et al.*, 2018).

Discussion

Although QS-dependent phenotypes have been extensively studied in strains of medical interest, much less work has been conducted on environmental strains. In particular, the question of a possible implication of QS mechanisms in the regulation of bacterial phenotypes involved in key biogeochemical processes remains open (Hmelo, 2017). In this study, several strains were isolated from the Aulne estuary (Bay of Brest, France), which is known to host high hydrolytic activities (Labry *et al.*, 2016). We then inhibited their AHL-based QS communication to test whether this mechanism regulated biofilm and extracellular hydrolytic enzyme production (β-glc and LAM), which are known to play a major role in organic matter biodegradation (Chróst and Siuda, 2002; Arnosti, 2011). One limitation to this approach is that it is restricted to the cultivable bacterial fraction.

Estuarine bacteria produce diverse QS signals

Several estuarine bacterial strains were found to produce AHLs quantifiable by UHPLC-HRMS/MS. They were affiliated with the genera *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Obesumbacterium*, *Morganella* and *Sphingobacterium* based on 16S rRNA gene sequencing. *Pseudomonas*, *Vibrio* and *Acinetobacter* species are frequently isolated in coastal and marine environments (Elliot and Colwell, 1985; Baffone *et al.*, 2006; Abd-Elnaby *et al.*, 2019) and have been well studied for their QS properties (Eberl, 1999; Miller and Bassler, 2001). The production of AHLs has also previously been reported in *Obesumbacterium* (Priha *et al.*, 2014) and *Morganella morganii* (Guzman *et al.*, 2020). To our knowledge, AHL production has not previously been reported in *Sphingobacterium* isolates, although AHL synthase sequences matching the *Sphingomonadaceae* family have been reported in environmental metagenomics databases (Doberva *et al.*, 2015). The AHL-producing isolates were phylogenetically different depending on the salinity at which they were isolated, suggesting that the bacterial community members engaged in QS communication may vary along the estuary.

The identified bacterial estuarine strains involved in QS communication produced a diverse array of QS molecules, with 14 AHLs detected and quantified in total. This finding is consistent with

previous studies showing that a single marine strain can produce a large, diverse array of QS signals (Doberva *et al.*, 2017). This broad spectrum of emitted AHLs suggests that estuarine bacterial communities produce and respond to multiple AHL signals emitted in the environment. Most strains produced short- and medium-length AHLs (C6-HSL to C12-HSL), and a majority of them were substituted at the C3 position, two structural properties that could increase AHL solubility in brackish water and seawater (Hmelo *et al.*, 2011). Interestingly, all the *Vibrio* strains were found to produce 3-OH-C6-HSL, while none of the other type of strains produced this type of AHL, suggesting a particular signal specificity within those strains. This is in contrast with previous studies that reported no clear pattern of QS activity in *Vibrionaceae* strains, including *Vibrio* sp. (Tait *et al.*, 2010; Freckelton *et al.*, 2018). The AHL production among *Pseudomonas* and *Acinetobacter* was much more diverse, with no clear production pattern. Interestingly, the sequencing data showed that the two *Pseudomonas*-affiliated strains had complex QS circuits, with several AHL synthases and receptors, including *luxR* solos. As QS compounds have been less studied in *Obesumbacterium*, *Morganella* and *Sphingobacterium*, our study contributes to describe their QS circuits, revealing the production of 3-oxo-C6-HSL and 3-oxo-C8-HSL in *Obesumbacterium*, C4-HSL in *Morganella* and the possible production of C16:1-HSL in *Sphingobacterium*.

QS and QQ circuits co-occur in estuarine bacteria

Interestingly, based on our bioassays, 10 out of 28 AHL-producing strains (36%) also showed the QQ ability to interfere with long-chain AHLs, while none of them interfered with short-chain AHLs. The sequencing data suggested that *Pseudomonas* sp. AF3-9 and B20 quenching abilities result from the degradation of AHLs based on the emission of acylases, a QQ enzyme that cleaves the acyl side chain of AHLs. No QQ enzymes (*i.e.* acylase or lactonase) were found in *Acinetobacter* sp. DF3-8 and *Vibrio* sp. E14 genomes, although *Acinetobacter* sp. DF3-8 presented a QQ activity during the bioassay. Such an observation concerning *Acinetobacter* suggests that estuarine bacteria could be a source of new QQ enzymes for biotechnological applications. The observation of bacteria that simultaneously emit pro- and anti-QS compounds has been evidenced in a few strains, for example,

in *Agrobacterium tumefaciens* (Zhang *et al.*, 2004), a marine *Shewanella* sp. (Tait *et al.*, 2009), in two isolates related to *Endozoicomonas* (Freckelton *et al.*, 2018) or *Acinetobacter* and *Burkholderia* strains isolated from the rhizosphere (Chan *et al.* 2011). Such co-occurrence of QS and QQ features in our collection of estuarine strains raises the question of the co-expression of these two phenotypes, especially within the natural environment. In any case, our data highlight the complexity of QS regulation within the natural estuarine communities. They also point out the importance of QS-based cell-cell cooperation and QQ-based competition within these communities.

QS regulates biofilm formation and hydrolytic enzyme production in estuarine bacteria

We then investigated the role of QS in biofilm formation and hydrolytic enzyme synthesis in the 28 AHL-producing estuarine strains, two important physiological features when considering the role of bacteria in the marine C cycle (Arnosti, 2011; Dang and Lovell, 2016). To this extent, we used a lactonase-based QS disruption approach. Lactonases have been shown to catalyze the opening of the lactone ring of AHLs, thus preventing their use as a signal molecule. *Sso*Pox lactonase has been shown to inhibit a range of behaviors under QS control, including biofilm and protease production, in *P. aeruginosa* (Hraiech *et al.*, 2014; Guendouze *et al.*, 2017; Mion *et al.*, 2019; Rémy *et al.*, 2020) and *Chromobacterium violaceum* (Mion *et al.*, 2021). They also modulated *in vitro* (Schwab *et al.*, 2019) and *in situ* (Huang *et al.*, 2019) bacterial community composition. Contrary to some other well-known QS inhibitors, such as 5-fluoro-uracil or the brominated furanone C-30, lactonases do not induce cytotoxic effects that may bias the experiments (Mahan *et al.*, 2020).

Most of the AHL-producing strains (89%) were also biofilm producers. The lactonase treatment significantly impacted the biofilm production in two of them, with contrasting effects: *Pseudomonas* sp. BF02-28 exhibited a significant biofilm decrease (-52%), while *Vibrio* sp. E14 biofilm production was increased (+128%). By contrast, the lactonase treatment did not significantly modify biofilm production in other *Vibrio* strains. Previous studies have mostly reported the inhibition of biofilm production in the presence of lactonases (Muras *et al.*, 2020; Paluch *et al.*, 2020; Rémy *et al.*, 2020), although biofilm upregulation has also been described in such conditions (Su *et al.*, 2019; Mahan *et*

al., 2020). Those contrasting effects probably result from the fact that biofilm production is under complex and interconnected regulatory mechanisms, including AHL-based QS, furanosyl diester borate (AI-2)-based QS and c-diGMP production, among others (Wolska *et al.*, 2016; Hmelo, 2017; Lami, 2019). In another report, Liu *et al.* (2017) noticed that the addition of 3-oxo-C10-HSL differentially affected the biofilm formation of *V. alginolyticus* isolates with apparent strain specificity and a dose-dependent response. Clearly, our results revealed that biofilm production is under the control of QS in some estuarine microbial communities, but they also suggest the complexity of QS-dependent regulations of biofilm production in such ecosystems. Biofilm formation among estuarine bacteria may drive their capacities to colonize particulate organic matter (Simon *et al.*, 2002; Dang and Lovell, 2016; Sivadon *et al.*, 2019). Thus, QS involvement in biofilm formation possibly shapes the diversity of particle-attached communities. However, we evaluated QS involvement in monospecific cultures while biofilm formation is a complex multi-species phenomenon in natural environments. As such, it will be necessary to evaluate QS involvement on complex community assemblages in the future to obtain a better picture of the QS-based interactions at play, as has been done in a few studies (Huang *et al.*, 2019; Schwab *et al.*, 2019)

We then assessed whether the expression of AHL-based QS regulates the activity of hydrolytic enzymes. Most studies to date that have examined the possible regulation of hydrolytic enzyme activity by QS in isolated strains have focused on dissolved extracellular enzymes (Gram *et al.*, 2002; Jatt, Tang, Liu, Zhang, and X. H. Zhang, 2015; Guendouze *et al.*, 2017; Li *et al.*, 2019; Su *et al.*, 2019; Mahan *et al.*, 2020). By contrast, most studies have been carried out on natural communities focused on cell-bound (Hmelo *et al.*, 2011) or total activities (Krupke *et al.*, 2016). Thus, in this study we decided to bridge this gap by assaying both dissolved and cell-bound activities of two hydrolytic enzymes: LAM, which are involved in N cycling and considered to be a broader proxy for proteases activity (Steen *et al.*, 2015), and β-glc, which are broad-specificity enzymes involved in C cycling and carbohydrate dynamics (Chróst, 1989). Interestingly, the lactonase treatment significantly impacted the activity of dissolved and cell-bound LAM in, respectively, 71% and 39% of the tested strains,

suggesting that QS might regulate LAM production in numerous estuarine bacteria. The influence of QS on β-glc production appeared less pronounced, with dissolved and cell-bound activities being significantly modified by the lactonase treatment in 4% and 26% of the tested strain, respectively. However, β-glc production was not as widespread as LAM production: only 3 and 17 strains produced dissolved and cell-bound β-glc, respectively, versus 18 and 28 for LAM, respectively. Clearly, our study evidenced that hydrolytic enzyme activities are under QS regulation in many strains. To our knowledge, only Jatt *et al.* (2015) and Su *et al.* (2019) have described such an effect after QS inhibition in *Pantoea* and *Ruegeria* strains isolated from marine snow, respectively. Our study demonstrated a broader effect because more phylogenetically diversified strains were tested. This clearly points out the need to investigate at a much larger scale this question in aquatic environments, as QS communication may represent an underestimated mechanism of N and C recycling by marine bacteria.

The effect of AHL-based QS inhibition on the activity of hydrolytic enzymes was contrasted as both increase and decrease in dissolved and cell-bound activities were observed. Indeed, the cell-bound LAM activity of several *Pseudomonas* strains increased, while those of other genera (*Acinetobacter*, *Obesumbacterium*, *Sphingobacterium* and *Vibrio*) decreased. Similar results were observed for β-glc activity, which decreased in some *Vibrio* strains but increased in one *Pseudomonas* strain and one *Acinetobacter* strain. Such complex effects have been reported after AHL addition to marine snow: Krupke *et al.* (2016) found that 3-oxo-C8-HSL induced phosphatase activity but decreased aminopeptidase and lipase activities after 6 h of incubation with communities from the Atlantic Ocean. Su *et al.* (2019) also highlighted that 3-oxo-C8-HSL increased galactosaminidase and β-xylosidase activities but decreased β-glucosidase and mannosidase activities in marine particles collected in the Yellow Sea of China. A few strains exhibited interesting behavior, where dissolved and cell-bound activities were differentially affected by the lactonase treatment. For example, the inhibition of QS circuits induced a decrease in *Pseudomonas* sp. AF3-9 dissolved LAM, but an increase in cell-bound LAM. The opposite phenomenon was observed for *Acinetobacter* sp. DF3-8 and *Vibrio*

sp. CFL-9. This overall variability is probably linked to the complexity of hydrolase regulation, which are under tight control given their metabolic cost (Chróst and Siuda, 2002).

Collectively, our findings revealed that estuarine bacteria can synthesize a large array of AHLs and present a wide range of quenching activities. Our results also clearly demonstrated the importance of AHL-based QS regulation on a range of phylogenetically different estuarine strains, suggesting an underestimated role of QS in microbial N and C cycling in aquatic environments. In the particular case of the Aulne estuary, for which high enzymatic activities have been reported (Labry *et al.*, 2016), QS involvement in hydrolase synthesis may have important consequences on biogeochemical fluxes of nutrients to coastal waters. The effects of QS inhibition were contrasted, illustrating the complexity of those cell-cell regulations. Thus, our study paves the way for future studies, highlighting the need to introduce concepts and tools of chemical ecology in marine microbial ecology to address broader questions relative to ecosystem functioning.

Experimental procedures

Standard *N*-acylhomoserine lactones

All AHLs used in this study were purchased from Sigma-Aldrich (Darmstadt, Germany). The following abbreviations are used: *N*-butyryl-DL-homoserine lactone (C4-HSL), *N*-hexanoyl-DL-homoserine lactone (C6-HSL), *N*-(3-hydroxyhexanoyl)-DL-homoserine lactone (3-OH-C6-HSL), *N*-(3-oxohexanoyl)-DL-homoserine lactone (3-oxo-C6-HSL), *N*-octanoyl-DL-homoserine lactone (C8-HSL), *N*-(3-hydroxyoctanoyl)-DL-homoserine lactone (3-OH-C8-HSL), *N*-(3-oxooctanoyl)-DL-homoserine lactone (3-oxo-C8-HSL), *N*-decanoyl-DL-homoserine lactone (C10-HSL), *N*-(3-hydroxydecanoyl)-DL-homoserine lactone (3-OH-C10-HSL), *N*-(3-oxodecanoyl)-DL-homoserine lactone (3-oxo-C10-HSL), *N*-dodecanoyl-DL-homoserine lactone (C12-HSL), *N*-(3-hydroxydodecanoyl)-DL-homoserine lactone (3-OH-C12-HSL), *N*-(3-oxododecanoyl)-DL-homoserine lactone (3-oxo-C12-HSL) and *N*-tetradecanoyl-DL-homoserine lactone (C14-HSL). All AHLs were dissolved in dimethyl sulfoxide (DMSO).

Isolation of estuarine bacterial strains

Bacteria were isolated from the surface water of the Aulne estuary (Bay of Brest) in April and July 2019. Three type of waters were sampled along the salinity gradient in order to cover a large range of suspended matter concentrations: riverine waters (salinity 0), brackish waters in the maximum turbidity zone (salinity 5) and brackish waters under greater marine influence (salinity 25, Table S1).The salinity was measured *in situ* with a thermosalinometer (WTW Cond 330i) using the practical salinity scale and as such, is reported with no unit (UNESCO, 1985). All culturing steps were performed at the *in situ* water temperature (*i.e*. 12 and 22°C for April and July, respectively) using modified Luria Broth medium (mLB) containing 1% (w/v) peptone and 0.5% (w/v) yeast extract and adjusted to the salinity at which the strains were isolated (0, 5 or 25) with artificial seawater (54 mM $MgCl_2$, 10 mM $CaCl_2$, 0.16 mM $SrCl_2$, 9 mM KCl, 2.4 mM $NaHCO_3$, 840 mM KBr, 440 mM H_3BO_3, 71 mM NaF, 0.40 M NaCl and 30 mM Na_2SO_4, pH 8.2). For the 0 salinity mLB, 1% (v/v) of artificial seawater was added to ensure the presence of essential ions.

For each sample, four different isolation strategies were conducted to isolate bacteria from the total, particle-attached (> 3 µm) and free-living (0.2-3 µm) fractions of sampled water. Thus, mLB agar plates (mLB amended with 1.5% w/v agar) were inoculated by (i) spreading 100 µL of total water, (ii) spreading 100 µL of < 3 µm-filtered water (Whatman Nuclepore™ PC membrane, GE Healthcare Life Sciences, Little Chalfont, UK), (iii) depositing > 3 µm filters or (iv) depositing 0.2 µm filters (Whatman Nuclepore™ PC membrane) used with 3 µm-filtered water. Single colonies were isolated, purified, grown in mLB and stored at -80°C using 10% (v/v) DMSO.

Identification of AHL-producing strains

We then determined whether the isolated bacterial strains could produce AHLs, following previously published protocols. Briefly, bacterial supernatants were screened with bacterial biosensors, *P. putida* F117 (pRK-C12) (Andersen *et al.*, 2001) and *E. coli* MT102 (pJBA132) (Riedel *et al.*, 2001). Those biosensors produce GFP in the presence of AHL. *P. putida* F117 allows a good detection of long-chain AHLs while *E. coli* MT102 preferentially detects short-chain AHLs (Andersen *et al.*, 2001; Riedel *et al.*, 2001).

Both biosensors were grown at 30°C (150 rpm) in LB (0.5% w/v yeast extract, 1% w/v peptone, 1% w/v sodium chloride) using, respectively, 20 µg mL^{-1} of gentamicin or 25 µg mL^{-1} of tetracycline. An overnight culture of each biosensor was inoculated at a 600 nm optical density (OD_{600}, measured using a NanoDrop 2000c with a 1 cm cuvette, Thermo Scientific) of 0.02 into fresh LB medium and dispensed into a 96-well black microplate (150 µL per well). Bacterial strains to be tested for AHL production were grown in mLB (in the salinity at which they were isolated) until an OD_{600} of 0.6-1.2 was reached (typically 12 to 48 h). Cultures were centrifuged at 12,000 *g* for 10 min and filtered through a 0.2 µm filter (Corning® PES syringe filter). The resulting supernatants were stored at -20°C until screening (1 week maximum) and then were added to the biosensors microplate in triplicate (50 µL/well) after thawing at ambient temperature.

Blanks consisted of sterile LB medium amended with mLB at the corresponding salinity. Negative controls for the test samples consisted of biosensor strains with sterile mLB amendments, as the mLB

was found to modify growth and fluorescence production of the biosensors depending on the salt concentration. Positive controls consisted of biosensor strains amended with 10 µM commercial AHL (C6-HSL for *E. coli* MT102 and 3-oxo-C10-HSL for *P. putida* F117) with a final DMSO concentration of 1% (v/v). In consequence, a second negative control was prepared with DMSO (1% v/v). GFP fluorescence (excitation: 485 nm, emission: 535 nm) and OD_{600} were read on a Spark Tecan Infinite M200PRO microplate reader (Tecan, Switzerland) after an overnight incubation (14-16 h) at 30°C under low agitation (100 rpm).

Specific fold induction of fluorescence was calculated by dividing the specific GFP fluorescence ($FLUO_{GFP}/OD_{600}$) of the test sample by the specific GFP fluorescence of the negative control (each containing mLB with the same salinity). Specific fold inductions were arbitrarily classified into inhibition (< 0.8), no modification (0.8-1.2), questionable induction (1.2-1.5), induction (1.5-3) and strong induction (> 3, see Table S1). All fold inductions that were not significantly different from the control were also counted in the 0.8-1.2 category.

Quantification of AHLs produced by isolated strains

Bacterial strains that induced a response with AHL biosensors were further investigated to confirm AHL production using non-targeted UHPLC-HRMS/MS (MS). For each strain, 50 mL of supernatant was obtained as described above. Supernatants were extracted with 50 mL of ethyl acetate (EtOAc, overnight incubation at room temperature). Aqueous and organic phases were separated, and the aqueous phase was extracted again with 50 mL of EtOAc. The 2 × 50 mL EtOAc extracts were pooled and evaporated using a rotary evaporator. Crude extracts were dissolved in 500 µL of EtOAc, evaporated to dryness using a GeneVac HT-4X and stored at -20°C until analysis. Extracts were then dissolved in 500 µL of methanol for UHPLC-HRMS/MS analysis, which was performed using a Q Exactive Focus Orbitrap System coupled to an Ultimate 3000™ UHPLC system (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The column was a Phenomenex Luna Omega Polar C18 (150 × 2.1 mm, 1.6 µm particle size). The mobile phase was composed of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The elution gradient started with 99% (v/v) of

A, keeping this composition constant for 4 min. The proportion of B was linearly increased to 100% (v/v) over 10 min and left at 100% (v/v) for 5 min.

The analytical method includes full-scan MS (50 to 750 *m/z*) with successive data-dependent MS/MS (dd-MS^2) scan, allowing the acquisition of high-resolution data of the parent ion and daughter ion masses in a single chromatographic run. The selected parent ions were fragmented using 15, 30 and 40 eV. AHL fragmentation spectra are characterized by the systematic presence of one abundant daughter ion (102.055 *m/z*), which corresponds to the lactone ring. Three other fragments are usually observed (56.050, 74.061 and 84.045 *m/z*). AHLs were confirmed and quantified using commercially available standards.

Identification of strains capable of QQ

AHL-producing strains were tested for both long- and short-chain AHL quenching. QQ identification relied on the use of the biosensors *E. coli* MT102 and *P. putida* F117 and followed previously published protocols (Blanchet *et al.*, 2017). Briefly, biosensors were grown as described above, except that 1 µM of a commercial AHL was added to the LB medium before dispatching into a 96-well plate (C6-HSL for *E. coli* MT102 and C14-HSL for *P. putida* F117). Supernatants exhibiting QQ capacities reduced GFP production compared with the control. Supernatants were produced as described above but were stored at 4°C until screening (4 h maximum) because QQ enzymes may be denatured upon freezing. Controls were realized as described previously for the screening of AHL-producing strains, except that positive controls contained 1 µM of AHLs and negative controls contained the equivalent quantity of DMSO (1% v/v). The fold reduction of fluorescence was calculated as described previously. To check that loss of fluorescence was not caused by cytotoxicity, 100 µL of each well of the biosensor assay was reacted with 30 µL of 0.01% (w/v) resazurin and incubated for 4 h at 30°C under low agitation (Tourneroche *et al.*, 2019). The resulting fluorescence (excitation: 530 nm, emission: 590 nm) corresponds to the reduction of resazurin to resorufin by viable cells with active metabolism.

Finally, the pH was checked at the end of separate cultures as it can affect AHLs degradation. The two biosensors and three representative strains (*Pseudomonas* sp. AF3-9, *Acinetobacter* sp. DF3-8 and *Vibrio* sp. E14) were grown in the same conditions as during the QQ assays, in 4 mL. The mean pH at the end of the incubation was 7.1 (range 6.5-7.9), which is near neutral pH.

Taxonomic identification of AHL-producing strains

AHL-producing bacteria were identified using partial 16S rRNA gene sequencing. For each bacterial strain, a single colony grown on an mLB agar plate was resuspended in 50 µL of MilliQ water and incubated at 95°C for 15 min. The polymerase chain reaction (PCR) mix contained 1 µL of this lysate, 21 µL of GoTaq Green Master Mix (Promega) and 1.5 µL of each universal primer 27F (10 µM, 5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (10 µM, 5'-ACGGYTACCTTGTTACGACTT-3'). One cycle of denaturation (95°C for 5 min) was followed by 35 cycles at 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. The final extension was realized at 72°C for 5 min. The PCR products were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). The final products were sequenced using the Sanger technique by Eurofins Genomics (Köln, Germany) using the same primers. Each sequence was manually checked using Bioedit and blasted against the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net, Kim *et al.*, 2012), from which reference sequences were retrieved. The pairwise sequence similarity between the isolates and their closest relatives was also calculated using the EzTaxon-e server (Table S3). All sequences were aligned in Muscle (MEGA 7.0), and a phylogenetic tree was constructed by maximum likelihood using the Tamura-Nei model. *Geobacter sulfurreducens* (NR 132673.1) and *Geobacter soli* (NR 134039.1) sequences were used as an outgroup. The reliability of each node in the tree was assessed by bootstrapping over 500 replicates and nodes with boostrap values inferior to 50 were collapsed. The resulting tree was displayed using the ggtree package (version 2.2.4) in R.

Biofilm production in isolated strains

Biofilm production was quantified using a crystal violet (CV) assay as described previously (Blanchet *et al.*, 2017) with slight modifications. Briefly, 200 µL of culture was grown in mLB medium (at the isolation salinity) in a 96-well microplate (Greiner Bio-One, Cat.-No 655 160) for 24 h (at 22°C) or 48 h (at 12°C) under low agitation (100 rpm). Each subsequent step was performed gently to preserve the biofilm. The supernatant of each culture was removed and non-adherent cells were washed with 200 µL of phosphate-buffered saline (PBS, pH 7.4, VWR). Microplates were dried at 60°C for 1 h. Biofilms were stained with 0.2% (w/v) CV for 15 min. Unbound CV was then removed and the wells were washed 3 times with sterile water. CV in biofilm was dissolved with 200 µL of a destaining solution (50% ethanol, 10% acetic acid in MilliQ water, v/v) and the resulting OD was measured at 540 nm (OD_{540}). Strains with $OD_{540} < 0.1$ were considered non-adherent to the plate surface.

Enzymatic activity assay on isolated strains

The potential LAM and β-glc activities were quantified using model fluorogenic substrates: L-leucine-7-amido-4-methylcoumarin (LLMCA) and 4-methylumbelliferyl-β-D-glucopyranoside (MUF-glc), respectively. Those substrates release a fluorescent moiety upon hydrolysis, 7-amido-4-methylcoumarin (MCA) and 4-methylumbelliferone (MUF), respectively (Chróst, 1990). LAM and β-glc were assayed in the supernatant (which corresponds to dissolved cell-free enzymes) and in total culture (which includes dissolved and cell-bound extracellular enzymes). To perform the assay, 100 µL of either supernatant or total culture was transferred into a 96-well microplate and amended with 1000 µM of LLMCA or 150 µM of MUF-glc, which were saturating concentrations (not shown). Fluorescence was monitored for 3-4 h using a Spark Tecan Infinite M200PRO with excitation/emission wavelengths of 364/460 nm for MUF and 380/440 nm for MCA. Activities were determined as the slope of the linear part of the curve (AU min^{-1}). Cell-bound activity was determined by subtracting the mean dissolved fraction activity from total fraction activity. All reported activities are specific (normalized using OD_{600}).

Effect of lactonases on enzymatic activities and biofilm production

QS involvement in biofilm formation and expression of β-glc and LAM was assessed by comparing their production with and without lactonase, which degrade AHLs and thus inhibit QS circuits (Grandclément *et al.*, 2015). A broad-spectrum lactonase preparation based on *Sso*Pox lactonase from the archaeon *Saccharolobus solfataricus* was obtained from Gene&GreenTK (Marseille, France), which is specialized in the development of enzymes, including quorum quenching lactonases. The commercial preparation obtained from Gene&GreenTK was produced heterogeneously in *E. coli*, purified to homogeneity by size-exclusion chromatography (Hiblot *et al.*, 2013; Guendouze *et al.*, 2017; Rémy *et al.*, 2020) and eluted in HEPES buffer (50 mM HEPES, 150 mM NaCl and pH 8.0).

Bacterial isolates that were shown to produce AHLs were pre-cultured overnight in mLB (in the salinity at which they were isolated) at the appropriate temperature (12 or 22°C). The pre-cultures OD_{600} was measured using a NanoDrop 2000c with 1 cm cuvette. They were diluted to a theoretical initial OD_{600} of 0.001 and amended with the lactonase preparation (at a final concentration of 0.5 mg L^{-1}) or the equivalent amount of HEPES buffer. All cultures were done in triplicate, starting from the same pre-culture. Two hundred microliters of each culture were immediately transferred into a microplate for biofilm production assay (see above procedure). Cultures were then incubated for 24 h (at 22°C) or 48 h (at 12°C) under low agitation (100 rpm). At the end of the incubation, OD_{600} and enzymatic activities (see above procedure) were quantified (one assay per replicate). The resulting supernatants were also tested with *P. putida* F117 and *E. coli* MT102 as described above to ensure the proper AHL reduction upon lactonase treatment. For each parameter (biofilm production, specific enzymatic activity and specific GFP fold induction), the effect of the lactonase treatment was assessed by calculating the ratio of control to test sample value ($ratio_{lac/ctr}$).

Whole-genome sequencing of representative strains

The genome of four strains (*Pseudomonas* sp. AF3-9, *Pseudomonas* sp. B20, *Acinetobacter* sp. DF3-8 and *Vibrio* sp. E14) was sequenced to identify the genes involved in QS, QQ, hydrolytic

enzymes and biofilm production. Those strains were chosen because they covered the taxonomic diversity of the AHL-producing strains and exhibited marked responses to lactonases addition. The four selected strains were grown to stationary phase in mLB (at the salinity at which they were isolated) at 12°C (*Pseudomonas* sp. AF3-9 and *Pseudomonas* sp. B20) or 22°C (*Acinetobacter* sp. DF3-8 and *Vibrio* sp. E14). Cells were harvested by centrifugation and genomic DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. DNA was sequenced using an Illumina MiSeq. Data quality was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and reads that met the following criteria were kept: (1) read length ≥ 50 base pairs (bp) and (2) phred score per base ≥ 30x. Adapters were filtered away based on an internal Illumina adapter database. *De novo* assembly of genomes from raw reads was performed using Unicycler (v0.4.8) with a minimal contig length of 500 bp (Wick *et al.*, 2017). Contigs were then searched for similarities against the UniVec database (03-20-2017) using BLASTN (Altschul *et al.*, 1990) to remove potential contaminants from the genome assemblies. Sequencing coverage was estimated by first mapping paired reads to the genome with Bowtie2 (v2.3.5) (Langmead and Salzberg, 2012) in 'sensitive' mode and then computing the coverage with Mosdepth (v0.2.7) (Pedersen and Quinlan, 2018). The completeness of the genome assembly was assessed using BUSCO (v4.0.0) (Simão *et al.*, 2015) in 'genome' mode specifying the profile as appropriate (i.e., *Pseudomonadales* or *Vibrionales*, release April 2019, Table S5). The obtained drafts were checked for the number of contigs, %GC content, total assembly size, N50 values and percentage of coverage. Automatic gene prediction was done with the Prokka pipeline (v1.14.5) (Seemann, 2014) using default parameters, specifying the corresponding genus.

The following analyses were done using the predicted protein sequence output from Prokka. To search for AHL synthases, AHL receptors, AHL acylases and AHL lactonases, reference databases were built using the reviewed UniProt/Swiss-Prot database (with the respective keywords: "acyl-homoserine-lactone synthase", "IPR036693", "AHL acylase" and "AHL lactonases") and manually inspected. In addition, three sequences from UniProt/ TrEMBL were added to represent the HdtS and

AinS AHL synthase families and the AinR AHL receptors. Predicted proteins were blasted against the custom reference databases using a cut-off E-value of 10^{-15}. Shortlisted proteins were then blasted against the complete UniProt/Swiss-Prot database and proteins with a better match in the UniProt/Swiss-Prot database were removed from the hit list. Finally, the protein domains of those hits were annotated using InterProScan (v5.36.75) (Jones *et al.*, 2014). Hits containing the appropriate domains were considered putative QS genes (see Table S4 for domain list). They were aligned to their custom reference databases using MUSCLE as implemented in Geneious Prime (v2021.0.3) and consensus neighbor-joining trees were built using 500 bootstraps.

Genes related to motility and adherence likely to be involved in biofilm production (flagella, pili, fimbriae, chemotaxis) were predicted using VFanalyzer (Liu *et al.*, 2019), specifying the appropriate genus. Proteases and protease inhibitors were predicted using BLASTN against the MEROPS protease database (v12.1) (Rawlings *et al.*, 2018) with a cut-off E-value of 1e-50 and 70% of identity. CAZymes were predicted against the CAZy database (Lombard *et al.*, 2014) using dbCAN2 (HMMdb release v9.0) (Zhang *et al.*, 2018). Only CAZymes that were predicted by HMMER, Diamond and Hotpep tools were selected.

Statistical analysis

All statistical analyses were implemented in R software (R Studio version 1.1.463). T-tests (package ggpubr, v0.4.0) were used to determine the statistical significance of the means between control and test samples (fold induction or inhibition of biosensors, hydrolytic enzymes and biofilm production), with a significance level of 0.05. Plots display the mean and associated standard error of triplicate values, unless specified otherwise. Statistical analysis for the effect of the origin of the sample (salinity, fraction and month of isolation) on the abundance of AHL-producing strains was carried out using the χ^2 square test and Fisher's exact test (chisq.test and fischer.test functions, stats package v4.1.0) with a significance level of 0.05.

Data availability

The genome assemblies of *Pseudomonas* sp. AF3-9, *Pseudomonas* sp. B20, *Acinetobacter* sp. DF3-8 and *Vibrio* sp. E14 are available under the DDBJ/ENA/GenBank accession numbers JAHPJF000000000, JAHPJG000000000, JAHPJH000000000 and JAHPJI000000000, respectively. The AHL-related genes databases are available upon request.

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Competing interest

D.D. has filled the patent FR3093894. D.D. is CEO and shareholder of Gene&GreenTK and received personal fees during the conduct of the study. The other authors declare that the research was

conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table and Figure legends

Fig 1. A) The phylogenetic tree of the strains for which AHLs were detected using MS. The tree was constructed by maximum likelihood using the Tamura-Nei model as implemented in MEGA 7.0. Nodes with bootstrap value inferior to 50 were collapsed. **B)** The ability of each strain to: quench C6-HSL and C14-HSL, produce hydrolytic enzymes (dissolved glucosidases, cell-bound glucosidases, dissolved proteases and cell-bound proteases) and produce biofilm. Red: producer, white: non-producer, grey: not tested. **C)** The impact of lactonase treatment on the production of hydrolytic enzymes and biofilm for each strain. White: non-significant effect of lactonase treatment, red: significant downregulation, green: significant upregulation, grey: not tested, black cross: no production in control treatment without lactonase. Significance threshold = 0.05.

Table 1. AHLs quantified in the supernatants of the bacterial strains isolated from the Aulne estuary. AHL concentrations are in nM. Putative AHLs are molecules that possess the characteristic fragmentation spectrum of AHLs but for which we do not have commercial standards.

Table 2. Mean value of specific GFP fold induction of the biosensors in the control condition (no lactonase) and the specific GFP ratio of lactonase treatment to control at the end of the lactonase assay, averaged over triplicate measurements. Only strains that were considered to activate the biosensors in the control condition at the end of the lactonase assay are reported for readability, that is to say strains with a specific fold induction > 1.5 that is significantly different from negative control ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Lac: lactonase treatment. ctr: control (buffer amendment).

Table 3. Mean values of biofilm production, dissolved LAM, cell-bound LAM, dissolved β-glc and cell-bound β-glc measured at the end of the lactonase assays for control (ctr) and lactonase treatment (lac) conditions with lactonases (lac) averaged over triplicated measurements. The ratio corresponds to the control over test condition. Significance was assessed using the t-test (° $p < 0.1$, * $p < 0.05$, **

p < 0.01, *** p < 0.001). Significant results are highlighted in red and bolded when p < 0.05. nd.: not detected. nt.: not tested. -: ratio not calculable.

Table 4. Number of functionally annotated genes related to QS, QQ, hydrolases and biofilm production in each draft genome.

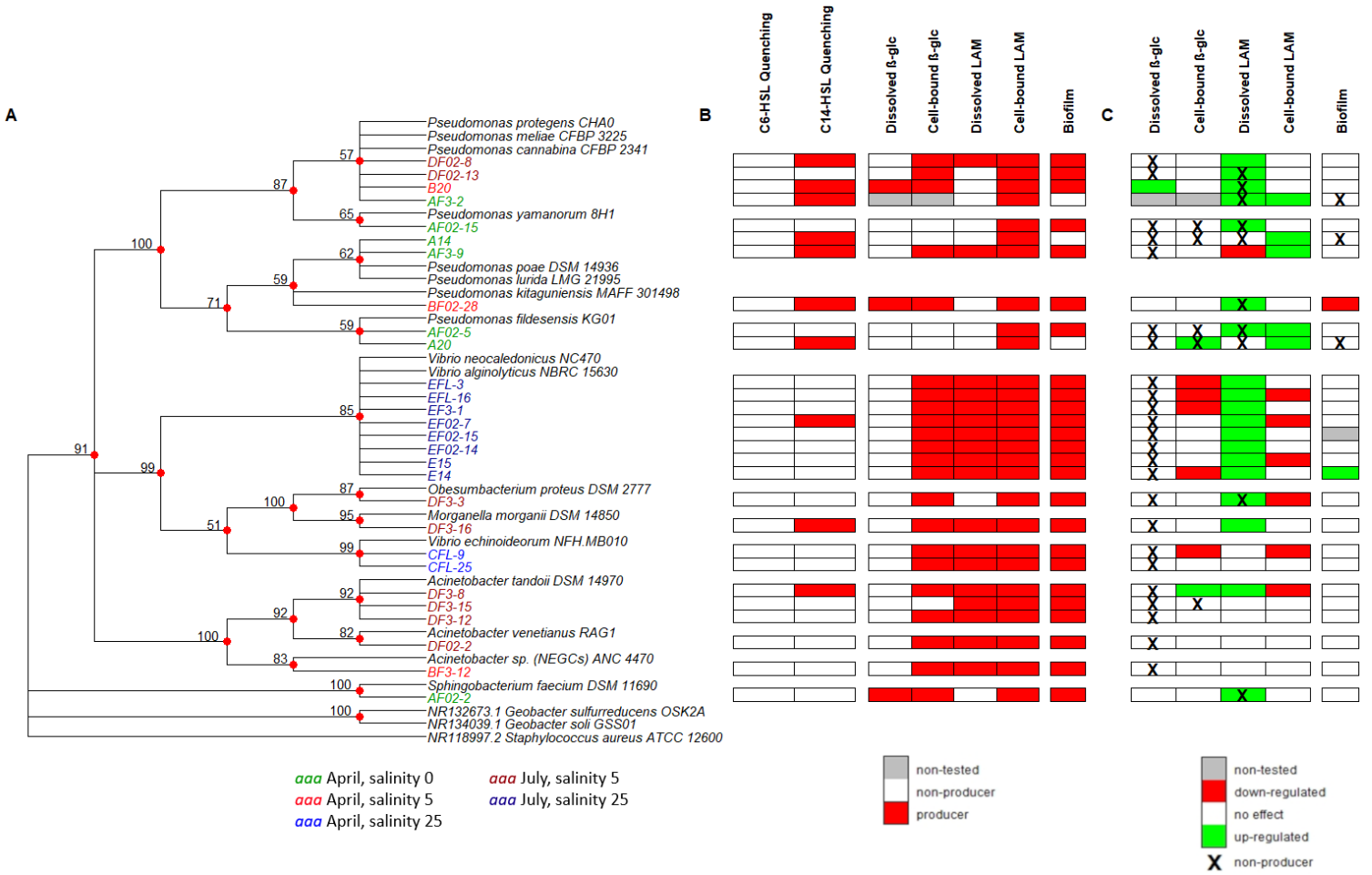

Fig. 1. **A)** The phylogenetic tree of the strains for which AHLs were detected using MS. The tree was constructed by maximum likelihood using the Tamura-Nei model as implemented in MEGA 7.0. Nodes with bootstrap value inferior to 50 were collapsed. **B)** The ability of each strain to: quench C6-HSL and C14-HSL, produce hydrolytic enzymes (dissolved glucosidases, cell-bound glucosidases, dissolved proteases and cell-bound proteases) and produce biofilm. Red: producer, white: non-producer, grey: not tested. **C)** The impact of lactonase-treatment on the production of hydrolytic enzymes and biofilm for each strain. White: non-significant effect of lactonase-treatment, red: significant down-regulation, green: significant up-regulation, grey: not tested, black cross: no production in control treatment without lactonase. Significance threshold = 0.05.

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Strain	Salinity	Month	Isolation fraction	C4	C6	3-OH-C6	3-oxo-C6	C8	3-OH-C8	3-oxo-C8	C10	3-OH-C10	3-oxo-C10	3-OH-C12	C12	3-oxo-C12	C14	Putative AHLs
Pseudomonas sp. A14	0	April	total	-	-	-	-	6	-	-	71	1	-	-	21	-	-	C12:1
Pseudomonas sp. A20	0	April	total	-	30	-	73	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp. AF3-2	0	April	attached	-	37	-	229	-	-	4	12	-	-	-	1	-	0.1	-
Pseudomonas sp. AF3-9	0	April	attached	-	-	-	-	5	-	-	66	6	-	-	8	-	-	C12:1
Sphingobacterium sp. AF02-2	0	April	free	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C16:1
Pseudomonas sp. AF02-5	0	April	free	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C16:1
Pseudomonas sp. AF02-15	0	April	free	-	-	-	-	-	-	-	-	-	108	-	-	24	-	-
Pseudomonas sp. B20	5	April	total	-	11	-	23	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp. BF3-12	5	April	attached	-	-	-	-	-	-	-	16	-	-	-	4	-	-	C12:1
Pseudomonas sp. BF02-28	5	April	free	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
Vibrio sp. CFL-9	25	April	free	-	-	19	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio sp. CFL-25	25	April	free	-	-	18	-	-	-	-	-	-	-	-	-	-	-	-
Obesumbacterium sp. DF3-3	5	July	attached	-	-	-	172	-	-	1	-	-	-	-	-	-	-	3-oxo-C7
Acinetobacter sp. DF3-8	5	July	attached	-	-	-	22	-	-	0.2	-	-	-	-	-	-	-	-
Acinetobacter sp. DF3-12	5	July	attached	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Acinetobacter sp. DF3-15	5	July	attached	311	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Morganella sp. DF3-16	5	July	attached	356	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp. DF02-2	5	July	free	84	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp. DF02-8	5	July	free	116	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp. DF02-13	5	July	free	-	-	-	-	-	-	-	-	-	-	14	-	-	-	
Vibrio sp. E14	25	July	total	-	-	234	-	-	44	-	-	-	-	-	-	-	-	C6:1
Vibrio sp. E15	25	July	total	-	-	233	-	-	40	-	-	-	-	-	-	-	-	C6:1
Vibrio sp. EFL-3	25	July	free	-	-	179	-	-	40	-	-	-	-	-	-	-	-	C6:1
Vibrio sp. EFL-16	25	July	free	-	-	16	-	-	-	-	-	-	-	-	-	-	-	-
Vibrio sp. EF3-1	25	July	attached	-	-	194	-	-	38	-	-	-	-	-	-	-	-	C6:1, 3-OH-C7
Vibrio sp. EF02-14	25	July	free	-	-	75	-	-	17	-	-	-	-	-	-	-	-	-
Vibrio sp. EF02-15	25	July	free	-	-	57	-	-	13	-	-	-	-	-	-	-	-	C6:1
Vibrio sp. EF02-7	25	July	free	-	-	53	-	-	7	-	-	-	-	-	-	-	-	-

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	Specific fold induction of *E. coli* MT102 biosensor in control treatment	**$Ratio_{lac/ctr}$ of *E. coli* MT102 specific GFP production**	**Specific fold induction of *P. putida* F117 biosensor in control treatment**	**$Ratio_{lac/ctr}$ of *P. putida* F117 specific GFP production**
Lactonase-treatment decreases the biosensors GFP production				
Pseudomonas sp. AF3-9	4.8 ***	0.0 ***		
Pseudomonas sp. AF02-15	50 **	0.0 **		
Sphingobacterium sp. AF02-2	2.1 **	0.0 **		
Pseudomonas sp. B20	126 ***	0.0 ***		
Acinetobacter sp. BF3-12	98 ***	0.0 ***	1.7 **	0.7
Pseudomonas sp. BF02-28	7.7 **	0.1 **	2.7 ***	0.3 **
Acinetobacter sp. DF3-8	2.0 *[a]	0.0 ***		
Pseudomonas sp. DF02-13	1.7 **	0.5 *	2.5 ***	0.1 ***
Vibrio sp. EF02-7	1.5 **	0.5 *		
Vibrio sp. EF02-14	1.6 **	0.6 **		
Vibrio sp. E15	1.6 **	1.1	2.4 ***	0.4 *
Lactonase-treatment does not decrease the biosensors GFP production				
Pseudomonas sp. AF02-5			2.1 ***	0.9
Vibrio sp. CFL-9			1.8 *	1.5
Vibrio sp. E14			1.6 *	0.6

Table 3. Mean values of biofilm production, dissolved LAM, cell-bound LAM, dissolved β-glc and cell-bound β-glc measured at the end of the lactonase assays for control (ctr) and lactonase-treatment (lac) conditions averaged over triplicated measurements. The ratio corresponds to the control over test condition. Significance was assessed using the t-test (° $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Significant results are highlighted in red and bolded when $p < 0.05$. nd.: not detected. nt.: not tested. -: ratio not calculable.

		Biofilm (OD_{540})	Dissolved LAM (AU min^{-1} OD_{600}^{-1})	Cell-bound LAM (AU min^{-1} OD_{600}^{-1})	Dissolved β-glc (AU min^{-1} OD_{600}^{-1})	Cell-bound β-glc (AU min^{-1} OD_{600}^{-1})
***Pseudomonas* sp. A14**	**ctr**	0.1	nd.	256	nd.	nd.
	lac	0.1	nd.	626	nd.	nd.
	ratio	1.0	**- ****	**2,4 ****	-	-
***Pseudomonas* sp. A20**	**ctr**	0.1	nd.	371	nd.	nd.
	lac	0.1	nd.	724	nd.	0.04
	ratio	1.0	**- ***	**2.0 ***	-	**- ****
***Pseudomonas* sp. AF3-2**	**ctr**	0.1	nd.	5	*nt.*	*nt.*
	lac	0.1	1.7	10	*nt.*	*nt.*
	ratio	1.0	**- ***	**2.08 ***	-	-
***Pseudomonas* sp. AF3-9**	**ctr**	2.6	10.3	304	nd.	0.9
	lac	2.0	2.0	397	nd.	0.6
	ratio	0.8	**0,2 ****	**1.3 ****	-	0.7
***Sphingobacterium* sp. AF02-2**	**ctr**	1.9	nd.	1029	6	183
	lac	1.6	0.9	907	7	168
	ratio	0.8	**- ***	0.9 °	1.1	0.9
***Pseudomonas* sp. AF02-5**	**ctr**	1.2	nd.	378	nd.	nd.
	lac	2.0	0.8	536	nd.	1.6
	ratio	1.7	**- *****	**1.4 ****	-	-
***Pseudomonas* sp. AF02-15**	**ctr**	2.5	nd.	686	nd.	0.1
	lac	2.0	2.0	644	nd.	0.1
	ratio	0.8	**- *****	0.9	-	1.1
***Pseudomonas* sp. B20**	**ctr**	0.5	nd.	601	4.9	3.5
	lac	0.4	6.9	584	7.4	3.8
	ratio	0.9	**- *****	1.0	**1,5 ****	1.1
***Acinetobacter* sp. BF3-12**	**ctr**	2.6	2.1	263	nd.	0.3
	lac	2.1	9.9	247	nd.	1.0
	ratio	0.8	4,8 °	0.9	-	3.1
***Pseudomonas* sp. BF02-28**	**ctr**	2.8	nd.	454	1.8	0.7
	lac	1.3	5.4	431	1.8	0.8
	ratio	**0,5 ***	**- *****	1.0	1.1	1.2
***Vibrio* sp. CFL-9**	**ctr**	2.1	313	166	nd.	35
	lac	1.4	392	101	nd.	30
	ratio	0,6 °	1,2 °	**0.6 ***	-	**0.9 *****
***Vibrio* sp. CFL-25**	**ctr**	2.3	377	262	nd.	37
	lac	1.6	436	270	nd.	38
	ratio	0,7 °	1.2	1.0	-	1.0
***Obesumbacterium* sp. DF3-3**	**ctr**	1.9	nd.	2424	nd.	49
	lac	1.7	20.4	2146	nd.	46
	ratio	0.9	**- ***	**0.9 ***	-	0.9
***Acinetobacter* sp. DF3-8**	**ctr**	1.4	13	533	nd.	4
	lac	1.8	42	430	nd.	5
	ratio	1.2	**3,2 ****	**0.8 ****	-	**1.3 ***
***Acinetobacter* sp. DF3-12**	**ctr**	3.6	nd.	303	nd.	1.1
	lac	2.6	nd.	277	nd.	1.2
	ratio	0.7	-	0.9 °	-	1.0
***Acinetobacter* sp. DF3-15**	**ctr**	5.0	33	756	nd.	nd.
	lac	3.2	30	763	nd.	0.3
	ratio	0,6 °	0.9	1.0	-	-

***Morganella* sp. DF3-16**	**ctr**	7.7	1.0	526	nd.	0.5
	lac	8.3	3.3	536	nd.	0.4
	ratio	1.1	**3,5 *****	1.0	-	0.9
***Acinetobacter* sp. DF02-2**	**ctr**	2.9	1.2	597	nd.	0.1
	lac	2.5	1.8	661	nd.	0.3
	ratio	0,8 °	1,5 °	1.1	-	4.0
***Pseudomonas* sp. DF02-8**	**ctr**	4.9	2.3	492	nd.	0.4
	lac	4.0	8.2	469	nd.	0.5
	ratio	0.8	**3,6 ***	1.0	-	1.1
***Pseudomonas* sp. DF02-13**	**ctr**	4.1	nd.	275	nd.	1
	lac	5.5	2.4	287	nd.	1
	ratio	1.4	**- ****	1.0	-	1.0
***Vibrio* sp. E14**	**ctr**	2.4	0.6	260	nd.	10
	lac	5.4	2.9	297	nd.	8
	ratio	**2,3 ****	**4,6 ****	1.2	-	**0.8 ***
***Vibrio* sp. E15**	**ctr**	1.5	0.4	340	nd.	6
	lac	2.3	2.7	303	nd.	6
	ratio	1.5	**7.4 ****	**0.9 ***	-	0.9 °
***Vibrio* sp. EFL-3**	**ctr**	2.6	0.5	328	nd.	9
	lac	2.1	4.1	277	nd.	7
	ratio	0.8 °	**7.4 ****	0.8 °	-	**0.8 ****
***Vibrio* sp. EFL-16**	**ctr**	2.5	1.3	465	nd.	12
	lac	1.8	5.4	276	nd.	9
	ratio	0.7	**4.0 ****	0.6	-	**0.7 ***
***Vibrio* sp. EF3-1**	**ctr**	4.7	0.5	387	nd.	19
	lac	2.4	2.2	309	nd.	12
	ratio	0.5	**4,3 *****	0.8	-	**0.6 ***
***Vibrio* sp. EF02-7**	**ctr**	1.6	1.0	321	nd.	12
	lac	2.9	5.0	266	nd.	11
	ratio	1.8	**5.0 ****	**0.8 ****	-	1.0
***Vibrio* sp. EF02-14**	**ctr**	2.0	1.2	305	nd.	8
	lac	3.0	5.3	289	nd.	9
	ratio	1.5	**4.4 ****	1.0	-	1.2
***Vibrio* sp. EF02-15**	**ctr**	*nt.*	1.5	501	nd.	16
	lac	*nt.*	8.8	524	nd.	17
	ratio	-	**5,9 ***	1.1	-	1.0

Table 4. Number of functionally annotated genes related to QS, QQ, hydrolases and biofilm production in each draft genome.

	Gene	*Pseudomonas* sp. AF3-9	*Pseudomonas* sp. B20	*Acinetobacter* sp. DF3-8	*Vibrio* sp. E14
QS and QQ	*LuxI-type AHL-synthase*	1	1	1	0
	LuxR-type AHL-receptor	3	4	1	0
	LuxM-type AHL-synthase	0	0	0	1
	LuxN-type AHL-receptor	0	0	0	1
	HdtS-type AHL synthase	1	1	0	0
	AinS-type AHL synthase	0	0	0	0
	AinR-type AHL receptor	0	0	0	0
	AHL-acylase	3	3	0	0
	AHL-lactonase	0	0	0	0
Protease	*Aspartic peptidase*	7	6	7	4
	Cysteine peptidase	40	32	42	28
	Glutamic peptidase	0	0	0	0
	Metallo peptidase	65	68	66	70
	Asparagine peptidase	9	10	4	5
	Mixed peptidase	1	0	0	0
	Serine peptidase	83	90	55	67
	Threonine peptidase	6	6	5	5
	Unknown peptidase	4	1	4	4
	Peptidase inhibitor	12	10	5	13
CAZyme	*Glycoside hydrolase*	27	31	35	43
	Glycosyl transferase	28	34	28	14
	Polysaccharide lyase	2	6	0	1
	Carbohydrate esterase	4	2	3	2
	Auxiliary activity	1	2	0	3
Motility and adherence	*Flagella*	45	46	48	48
	Pili	24	32	2	16
	Fimbriae	1	0	9	0
	Chemotaxis	2	2	6	7