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Resistance of the oyster pathogen *Vibrio tasmaniensis* LGP32 against grazing by *Vannella* sp. marine amoeba involves Vsm and CopA virulence factors

Etienne Robino,^{1¶} Aurore C. Poirier,^{1‡,¶}
Hajar Amraoui,¹ Sandra Le Bissonnais,¹
Angélique Perret,¹ Carmen Lopez-Joven,^{1‡}
Jean-Christophe Auguet,² Tristan P. Rubio,^{1‡}
Chantal Cazevieille,³ Jean-Luc Rolland,¹
Yann Héchard,⁴ Delphine Destoumieux-Garzón¹ and
Guillaume M. Charrière ^{1*}

¹IHPE UMR 5244, CNRS, Ifremer, University of Montpellier, University of Perpignan Via Domitia, Montpellier, France.

²MARBEC, University of Montpellier, CNRS, Ifremer, IRD, Montpellier, France.

³INM, University of Montpellier, Montpellier, France.

⁴EBI UMR CNRS 7267, University of Poitiers, Poitiers, France.

Summary

Vibrios are ubiquitous in marine environments and opportunistically colonize a broad range of hosts. Strains of *Vibrio tasmaniensis* present in oyster farms can thrive in oysters during juvenile mortality events and behave as facultative intracellular pathogen of oyster haemocytes. Herein, we wondered whether *V. tasmaniensis* LGP32 resistance to phagocytosis is specific to oyster immune cells or contributes to resistance to other phagocytes, like marine amoebae. To address this question, we developed an integrative study, from the first description of amoeba diversity in oyster farms to the characterization of LGP32 interactions with amoebae. An isolate of the *Vannella* genus, *Vannella* sp. AP1411, which was collected from oyster farms, is ubiquitous, and belongs to one clade of *Vannella* that could be found associated with *Vibrionaceae*. LGP32 was shown to be resistant to grazing by *Vannella* sp. AP1411 and this phenotype depends on some

previously identified virulence factors: secreted metalloprotease Vsm and copper efflux p-ATPase CopA, which act at different steps during amoeba–vibrio interactions, whereas some other virulence factors were not involved. Altogether, our work indicates that some virulence factors can be involved in multi-host interactions of *V. tasmaniensis* ranging from protozoans to metazoans, potentially favouring their opportunistic behaviour.

Introduction

Vibrios are γ -proteobacteria living in aquatic environments ranging from saline to freshwater. They are ubiquitous in marine coastal environments and have evolved the capacity to colonize a broad range of hosts from protozoans to metazoans (Austin, 2010). Vibrios belong to the microbiota of healthy oysters but some species that behave as opportunistic pathogens can thrive in host tissues and cause disease (Lemire *et al.*, 2014; Bruto *et al.*, 2017; Rubio *et al.*, 2019). This occurs mostly as a result of environmental changes such as shifts in water temperature, exposure to high animal densities and stressful farming practices or upon immune suppression of host defences by other microorganisms such as the OsHV-1 virus (de Lorgeril *et al.*, 2018). Currently, vibrioses are recognized as a major factor limiting the development of aquaculture (Le Roux *et al.*, 2015). In addition, vibrios can cause severe disease outbreaks in human populations, the best-known example being cholera. As they are multi-host pathogens, the ecology of vibrios depends on a series of biotic interactions in the environment that influence their ecology, their evolution and their transmission to animal and human hosts (Constantin de Magny *et al.*, 2008; Takemura *et al.*, 2014).

Bacteriivorous protists, such as free-living amoebae (FLA), are bacterial predators present in natural environments. They can be found in most aquatic environments and their predation through grazing activity shapes bacterial communities (Pernthaler *et al.*, 2005). However, it has been shown that some bacteria are able to resist grazing, persist and even grow within amoebae (Greub and

Received 20 May, 2019; accepted 4 August, 2019. *For correspondence. E-mail guillaume.charriere@umontpellier.fr; Tel. +33(0) 4-67-14-46-25. Present addresses: [†]Department of School of Veterinary Medicine, University of Surrey, Guildford, UK; [‡]Universidad Austral de Chile, Valdivia, Chile; [§]MMSB UMR 5086, University of Lyon, Lyon, France. [¶]The authors contributed equally to this work.

Raoult, 2004). The best-known and described example is the facultative intracellular pathogen *Legionella pneumophila* (Rowbotham, 1980). Predation activity by protists shares cellular and molecular processes with the antibacterial responses of metazoan immune cells, in particular phagocytosis (Boulais *et al.*, 2010). Hence, amoebae have been hypothesized to act as 'evolutionary training ground' for grazing-resistant bacteria, especially for intracellular bacterial pathogens, by favouring the selection of virulence factors (Molmeret *et al.*, 2005).

A number of studies have demonstrated that some *Vibrio* species adopt intracellular stages (Rosenberg and Falkovitz, 2004; Ma *et al.*, 2009; Vidal-Dupiol *et al.*, 2011; Ritchie *et al.*, 2012; de Souza Santos and Orth, 2014). In particular, intracellular stages are required for strains of *Vibrio tasmaniensis* to express virulence in oysters (Duperthuy *et al.*, 2011; Rubio *et al.*, 2019). Virulent strains of this species have been isolated from moribund oysters during juvenile mortality events on the French Atlantic coast (Gay *et al.*, 2004; Lemire *et al.*, 2014; Bruto *et al.*, 2017). This species was also found in oyster flesh during juvenile mortalities, as well as in the water column and the sediment of oyster farms in the Mediterranean Thau Lagoon, France (de Lorgeril *et al.*, 2018; Lopez-Joven *et al.*, 2018). Some of the *V. tasmaniensis* LGP32 virulence factors have been described in oysters. They include secreted proteases, such as Vsm (Binesse *et al.*, 2008) or Vsp (Vanhove *et al.*, 2014), antioxidants such as the superoxide dismutase SodA, efflux pumps to detoxify heavy metals such as the copper p-ATPase CopA (Vanhove *et al.*, 2016), and a type VI secretion system that mediates the intracellular secretion of toxic effectors (Rubio *et al.*, 2019).

Here, we wondered whether some virulence factors of the *Vibrio tasmaniensis* LGP32 playing a key role in its interaction with oysters could also be beneficial during other biotic interactions in the environment. With this objective, we first investigated the natural associations between vibrios and marine amoebae. Second, we did comparative cellular biology to study the interaction between LGP32 and an environmental free-living marine amoeba (*Vannella* sp. strain AP1411) ubiquitous in oyster farms, which belongs to a clade of *Vannella* sp. that establish interactions with vibrios. By performing a combination of flow cytometry, confocal and electron microscopy analyses, we show that LGP32 resists to the predation by *Vannella* sp. AP1411. Using mutant strains inactivated for previously characterized virulence factors in oysters, we found that the copper p-ATPase efflux pump CopA and the secreted metalloprotease Vsm are both involved. CopA appears to play a role in the intracellular survival of LGP32 whereas Vsm appears to play a role in the inhibition of amoebae motility during grazing.

Altogether, our results indicate that some of the previously identified virulence factors involved in *V. tasmaniensis* LGP32 interaction with oysters, are also involved in resistance to predation by marine amoeba *Vannella* sp. AP1411, thus potentially favouring opportunism.

Results

Marine amoebae belonging to the Vannellidae family and present in oyster farming area can interact with vibrios

Although amoebae represent an important category of benthic bacterial grazers, their diversity remains poorly explored in marine environments, particularly in Mediterranean lagoons exploited for oyster farming. Therefore, we first attempt to identify and isolate FLA in marine environments hosting oyster farms. Amoebae were isolated from the Thau lagoon at the vicinity of an oyster farming area. Samples were collected from the water column, oyster gills and sediment at all seasons between the year 2014 and 2015, and at least two cultures of amoebae per fraction and per season were analysed. Cloning-sequencing of the v7 hypervariable region of 18S rDNA gene was used to identify the isolated amoebae. A total of 110 sequences were analysed. From 24 different cultures, 103 amoebae sequences showed more than 90% sequence homology to the *Vannella* genus, which belongs to the *Vannellidae* family. The 103 sequences were classified into 11 different Operational Taxonomic Units (OTUs) (Fig. 1 and Table S1). In parallel, 16S barcoding of the different amoebae cultures confirmed that only species belonging to the *Vannella* genus were present in the samples (Fig. S1). One OTU of *Vannella* sp. was ubiquitous, as it was found in the three fractions during four seasons, and referred to the isolate AP1411 (arrow in Fig. 1). In an attempt to determine whether marine amoebae could be found associated with *Vibrionaceae* in the environment, a dedicated sampling was performed in 2017 and amoebae were clonally isolated from oyster gills and from water column in another site (Banyuls-sur-mer, France) as an out-group. Then 16s barcoding analyses were performed to characterize the bacteria that remained associated to these *Vannella* clones 4 weeks after culturing. From all the clones analysed, four were found associated with *Vibrionaceae* OTUs (Fig. 2A). In order to validate this association, the presence of *Vibrionaceae* associated with the four *Vannella* sp. clones was then assessed by FISH using a 16s probe specific for vibrios (Huggett *et al.*, 2008) (Fig. 2B). Among the four clones that were analysed, two showed an association with *Vibrionaceae* (0517-TOEc-2 and 0517-TOEc-4). The vibrios could be found inside amoebae as well as outside. These results suggest that amoebae of the *Vannella* genus can establish interactions with vibrios in the environment.

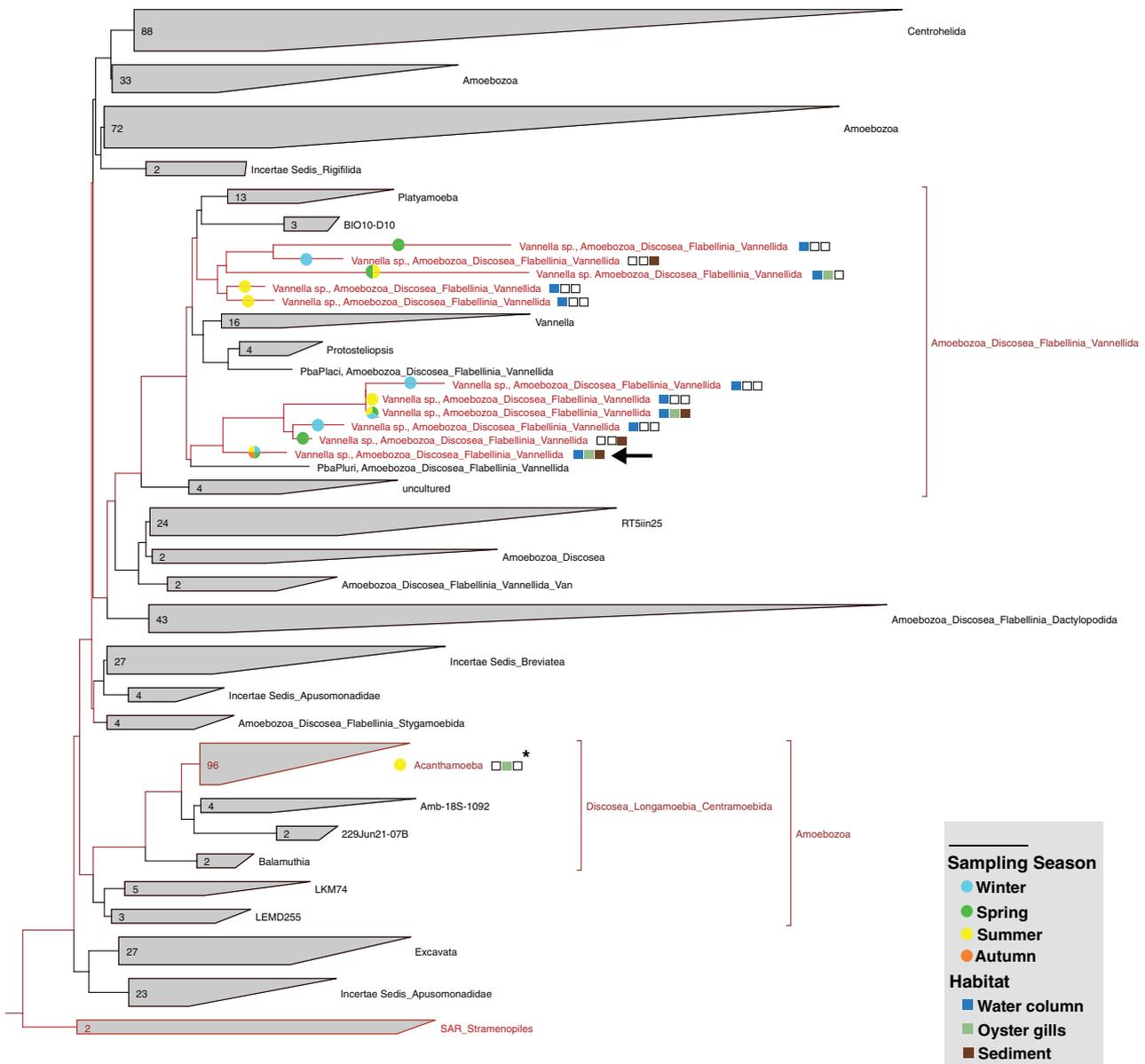


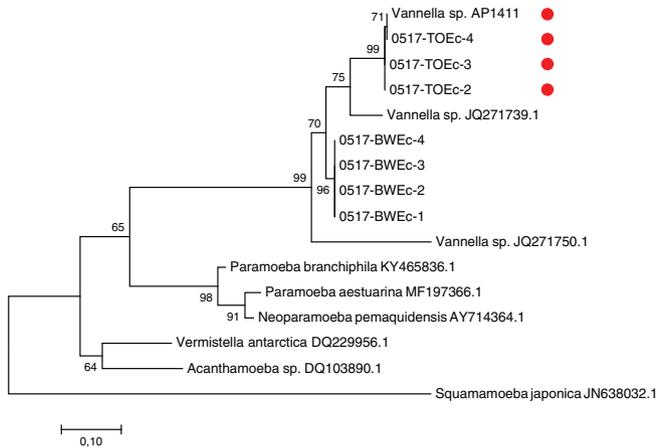
Fig. 1. Most of the isolated free-living amoebae from the Thau lagoon belong to *Vannellidae* family. Phylogenetic 18S rRNA-based trees of the Amoebozoa constructed with ARB software (Ludwig *et al.*, 2004; <http://www.arb-home.de>) loaded with the silva database (<http://www.arb-silva.de>). A base frequency filter was applied using the parsimony quick add marked tool implemented in ARB. Our isolates are written in red. Most of the isolated amoebae during the environmental survey from water column (blue squares) and/or the sediment (brown squares), and/or oyster gills (light green squares) during winter (light blue circles) and/or spring (green circles) and/or summer (yellow circles) and/or autumn (orange circles) belong to the *Vannellidae* family.

The pathogen V. tasmaniensis LGP32 resists grazing by *Vannella* sp. AP1411

To study further the interaction between vibrios and *Vannella* amoebae, we chose the *Vannella* sp. AP1411 clone, which belongs to the most ubiquitous OTU of marine amoebae and which is not already associated with other vibrios, as shown by FISH (Fig. 2B). The trophozoite, planktonic and cystic forms of *Vannella* sp. AP1411 were typical of the *Vannella* genera and very similar to *Vannella*

plurinucleolus (Smirnov *et al.*, 2007), which was also the closest 18S matching sequence for this isolate (98% BLASTn score). Transmission electron microscopy of trophozoites revealed a typical intracellular organization of *Vannella* with a well-defined, thick plasma membrane, one nucleus (sometimes two or three), numerous mitochondria and a rich vacuolar system with large digestive vacuoles containing remnants of degraded bacteria, and some phagosomes containing individual bacteria (Fig. S2).

A



B

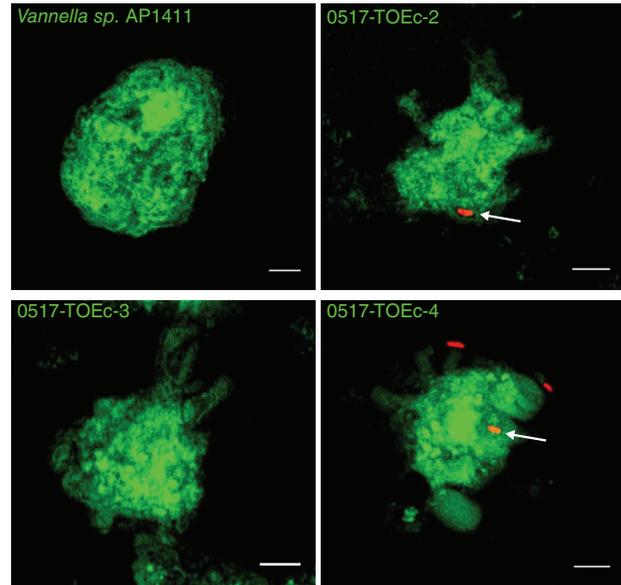


Fig. 2. Amoebae belonging to the *Vannella* genus can be found associated with *Vibrionaceae* bacteria in oyster farms. *Vannella* were clonally isolated from oyster gills from Thau lagoon (*Vannella* sp. AP1411, 0517-TOEc-2, 0517-TOEc-3, and 0517-TOEc-4) or from seawater in Banyuls-sur-Mer (0517-BWEc-1, 0517-BWEc-2, 0517-BWEc-3, and 0517-BWEc-4), then 16s barcoding sequencing was performed to identify potentially associated *Vibrionaceae*.

A, The 18S rRNA tree was constructed with maximum-likelihood by bootstrap method (1000 replications), isolates found associated with *Vibrionaceae* by 16s barcoding analyses are indicated with red dots.

B, *Vibrionaceae* were revealed by FISH using the VIB572a *Vibrionaceae* specific probe (Red) and amoebae cells were counterstained with FITC. *Vibrios* could be observed both outside and inside (arrows) amoebae by confocal microscopy. Scale bar: 5 μm.

To study LGP32 – *Vannella* sp. AP1411 interactions, grazing assays were performed on solid media (non-nutrient seawater agar). *Vibrio* strains (pathogenic or non-pathogenic to oysters) that constitutively express the Green Fluorescent Protein (GFP) were used to seed the plates and monitor the abundance of living vibrios by fluorescence quantification. In parallel, *Vannella* sp. AP1411 growth was monitored by counting the cell density at the surface of the grazing lawns. When the amoeba was cultured in the presence of the non-virulent *Vibrio tasmaniensis* LMG20012^T, a rapid decay of GFP-expressing bacteria was observed 4 days after the beginning of the experiment, whereas in the case of the pathogenic *V. tasmaniensis* LGP32, the amount of GFP-expressing bacteria remained stable for up to 7 days (Fig. 3A). Amoeba growth (cells count) on the vibrio lawns counter mirrored the kinetics of the decay of GFP-expressing bacteria. Indeed, amoeba density increased much faster in the presence of LMG20012^T than in the presence of LGP32 (Fig. 3B). These data indicate that *Vannella* sp. AP1411 do not prey efficiently on the virulent LGP32, whereas they can feed on the non-virulent LMG20012^T.

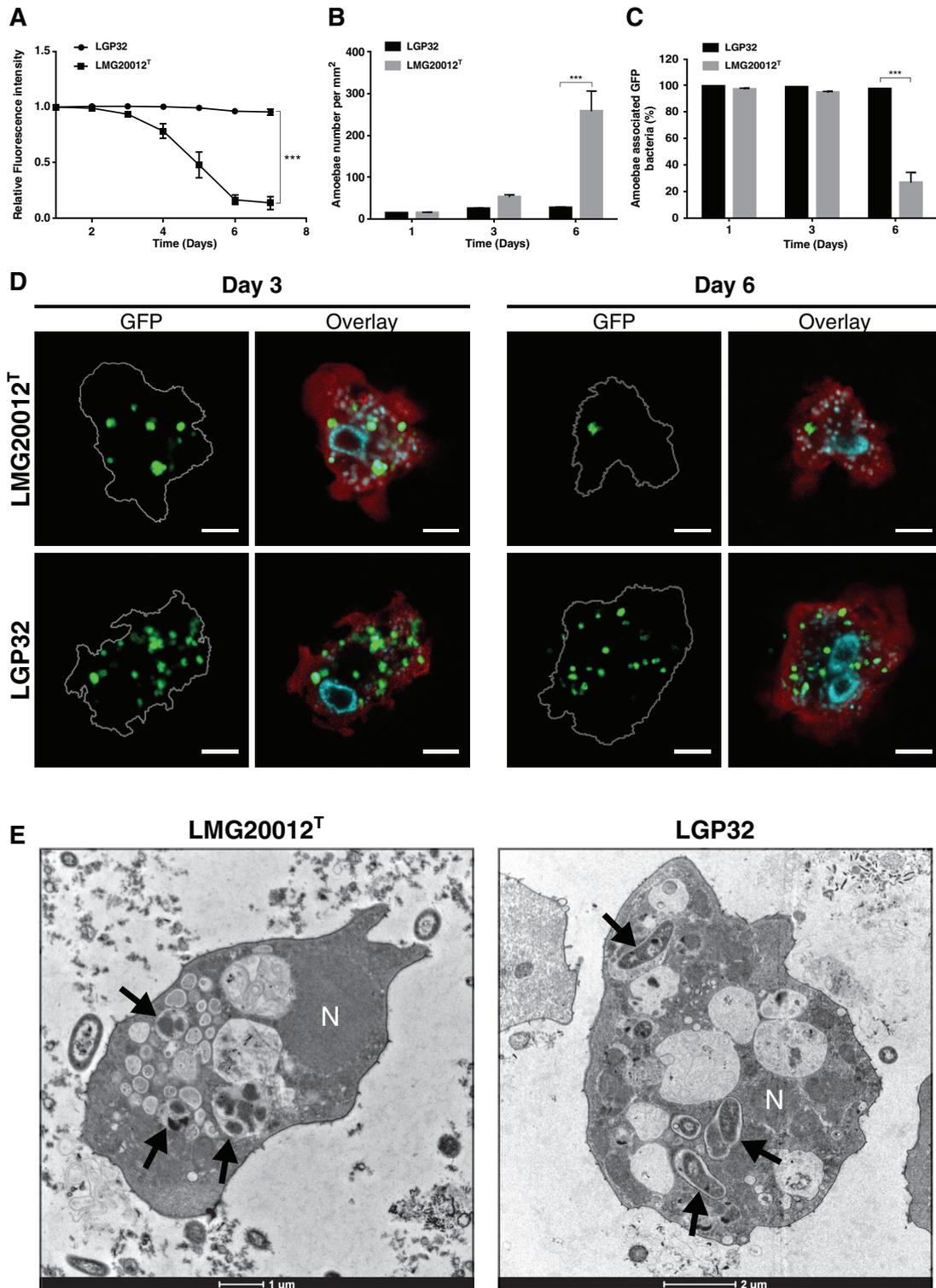
To determine whether the amoebal growth defect observed in the presence of LGP32 was due to inefficient phagocytosis and/or resistance to intracellular degradation by LGP32, we next quantified the capacity of *Vannella*

sp. AP1411 to phagocytose LGP32 and LMG20012^T, using flow cytometry (Fig. S3). The amount of amoebae carrying GFP-vibrios was quantified 3 and 6 days after grazing, at 3 days after the beginning of the interaction, 100% of amoebae were found to carry GFP-LMG20012^T or GFP-LGP32 (Fig. 3C). However, at day 6, the percentage of amoebae carrying GFP-LMG20012^T decreased down to 20% whereas it remained stable with GFP-LGP32 (Fig. 3C) suggesting that LGP32 could resist intracellular degradation. Confocal microscopy investigations confirmed that the amoebae were able to phagocytose both vibrios (Fig. 3D). These observations also confirmed that 6 days after the onset of the grazing assay, 80% of amoebae were devoid of any GFP-LMG20012^T as quantified by flow cytometry, with only 20% of amoebae that were still carrying intracellular GFP-LMG20012^T, whereas 100% of the amoebae were still carrying numerous GFP-LGP32 (Fig. 3D). This suggests that GFP-LGP32 is able to resist intracellular degradation after engulfment in contrary to the non-virulent control strain GFP-LMG20012^T that seemed to be digested by the amoeba.

To investigate further the intracellular fate of the vibrios inside amoebae, transmission electron microscopy was performed 3 days after the onset of grazing (Fig. 3E). Most LMG20012^T cells were found clustered inside vacuoles and showed altered morphologies whereas LGP32 remained intact inside amoebae and were mostly found in

individualized phagosomes confirming that LGP32 is resistant to intracellular degradation compared with LMG20012^T. Altogether, these results show that LGP32 is resistant to

grazing by *Vannella* sp. AP1411 as opposed to the non-virulent LMG20012^T, and that LGP32 is able to resist to intracellular degradation by the amoeba.



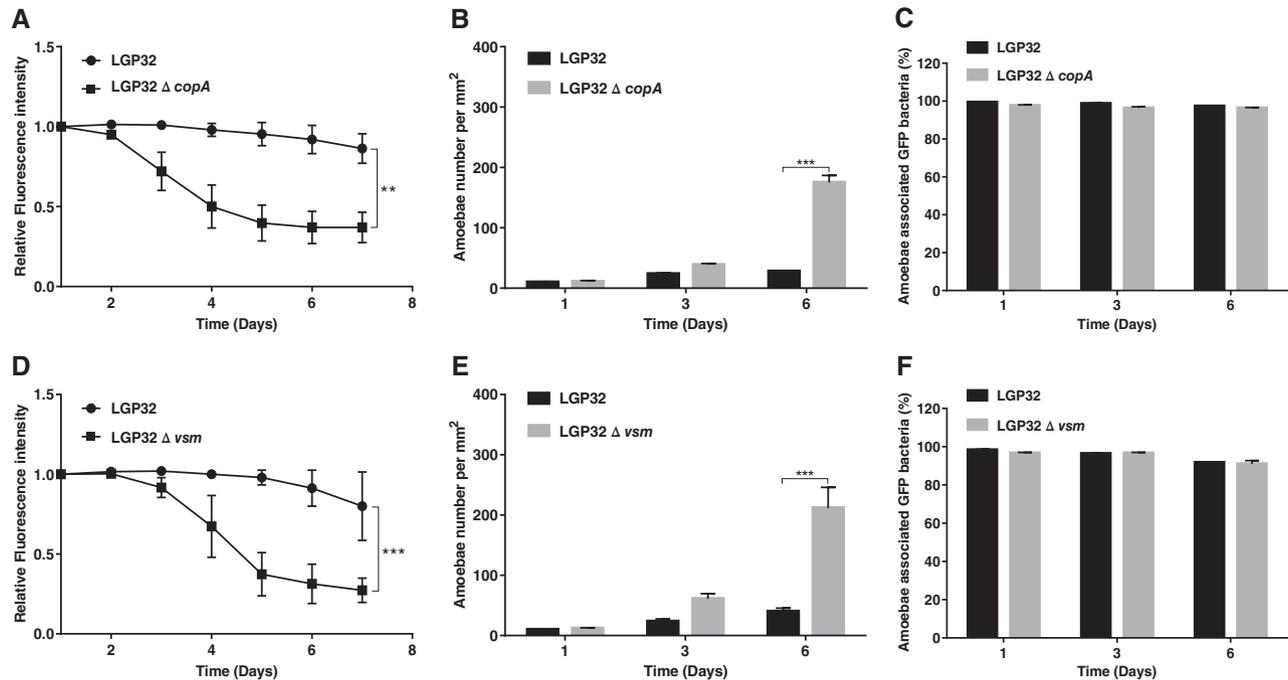


Fig. 4. Copper p-ATPase efflux pump CopA and metalloprotease Vsm are involved in LGP32 resistance to grazing by *Vannella* sp. 1411. A–D, Bacterial resistance of $\Delta copA$ and Δvsm strains of LGP32 was assessed by measurement the fluorescence of the GFP-expressing bacteria, after contact with amoebae. Each condition was performed in triplicate and the results shown are the average of three independent experiments. Values are presented \pm SEM, $^{**}P < 0.01$, $^{***}P < 0.001$ (RM-ANOVA). B–E, Amoeba growth was monitored by manual counting under phase light microscopy. Each condition was counted in three technical replicates. The results shown are representative of two independent experiments \pm SEM. Data were analysed by two-way ANOVA with Holm-Sidak's multiple comparisons test, $^{***}P < 0.001$. C–F, In two independent experiments, percentage of amoebae associated with GFP bacteria was measured by flow cytometry. Each condition was performed in three technical replicates. The results shown are representative of two independent experiments \pm SEM. Data were analysed by two-way ANOVA with Holm-Sidak's multiple comparisons test.

The Vsm and CopA virulence factors participate in LGP32 resistance to grazing by marine amoeba

To investigate further the molecular basis of LGP32 resistance to predation by *Vannella* sp. AP1411, we studied the phenotype of mutant strains of LGP32 deleted for virulence factors previously identified to be involved in its interaction with oysters. On the one hand, we examined stress resistance systems, that are, the antioxidant SodA, and the copper resistance systems CopA and CusAB, involved in intraphagosomal resistance to antimicrobial activities of

oyster immune cells (Vanhove *et al.*, 2016) and on the other hand, we examined secreted virulence factors, that are, the metalloprotease Vsm and the serine protease Vsp that both play a role in oyster pathogenesis (Binesse *et al.*, 2008; Vanhove *et al.*, 2014); as well as the metalloprotease InhA (the orthologue of PrtV) that has been involved in multiple biotic interactions of vibrios (Vaitkevicius *et al.*, 2006), and finally the T6SS1 secretion system required for cytotoxicity towards oyster immune cells (Rubio *et al.*, 2019). Among the eight mutant strains of LGP32 tested here, only

Fig. 3. LGP32 is resistant to grazing by marine amoebae *Vannella* sp. AP1411. Grazing assays were performed with *Vannella* sp. AP1411 with GFP-expressing strains of LGP32 or of the avirulent LMG20012^T. A, Bacteria survival was assessed by monitoring the intensity of the GFP fluorescence. Each condition was performed in triplicate and the results represent the average of three independent experiments. Values are presented \pm SEM $^{***}P < 0.001$ (RM-ANOVA). B, Amoebae growth was monitored by manual counting under phase light microscopy. Each condition was performed in three technical replicates and depicted results are representative of two independent experiments \pm SEM. Data were analysed by two-way ANOVA with Holm-Sidak's multiple comparisons test, $^{***}P < 0.001$. C, The percentage of amoebae carrying GFP-expressing bacteria was quantified by flow cytometry. Each condition was performed in three technical replicates and depicted results are representative of two independent experiments \pm SEM. Data were analysed by two-way ANOVA with Holm-Sidak's multiple comparisons test, $^{***}P < 0.001$. D, LMG20012^T and LGP32 were observed inside amoebae by confocal microscopy 3 days and 6 days after contact. Nuclei were stained with DAPI (Blue) and amoeba proteins with Blue Evans (Red). Scale bar: 5 μ m. E, Transmission electron microscopy was performed on amoebae, 3 days after grazing on LGP32 or LMG20012^T lawns. Avirulent LMG20012^T vibrios were found clustered in intracellular vacuoles with an altered shape (arrows), compared with extracellular vibrios around the amoeba. Virulent LGP32 vibrios were found mostly in individualized phagosomes, and appeared intact (arrows). Scale bar: 1 and 2 μ m.

the $\Delta copA$ and Δvsm deletion mutants were significantly less resistant to grazing by *Vannella* sp. AP1411 than the wild-type strain, as shown by a faster clearance of GFP-

vibrios on grazing lawns (Fig. 4A and D; and Fig. S4). Accordingly, on lawns of both $\Delta copA$ and Δvsm , rapid bacterial clearance correlated with faster amoebal growth as

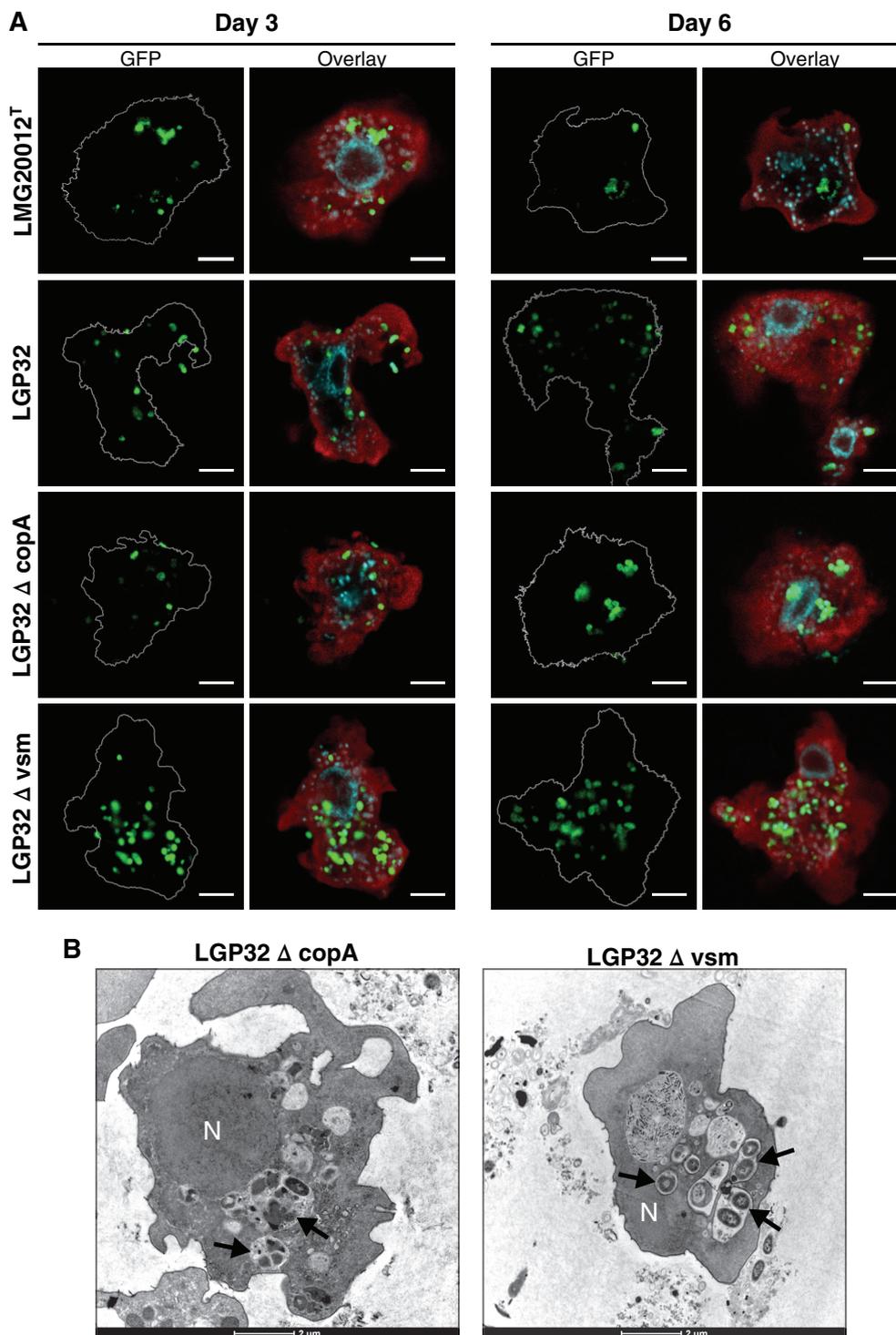


Fig. 5. Copper p-ATPase efflux pump CopA is necessary to resist to intracellular degradation in amoeba whereas the metalloprotease Vsm is not. A, Intracellular vibrios were observed 3 days and 6 days after infection, by confocal microscopy. Nuclei were stained with DAPI and amoeba proteins with Blue Evans. Scale bar: 5 μ m. B, Transmission electron microscopy was performed on amoeba 3 days after grazing on $\Delta copA$ or Δvsm mutant strains. Mutant $\Delta copA$ vibrios were found clustered in intracellular vacuoles with an altered shape and appearance (arrows). Vibrios Δvsm LGP32 were found mostly in individualized phagosomes, and appeared intact (arrows), scale bar: 2 μ m.

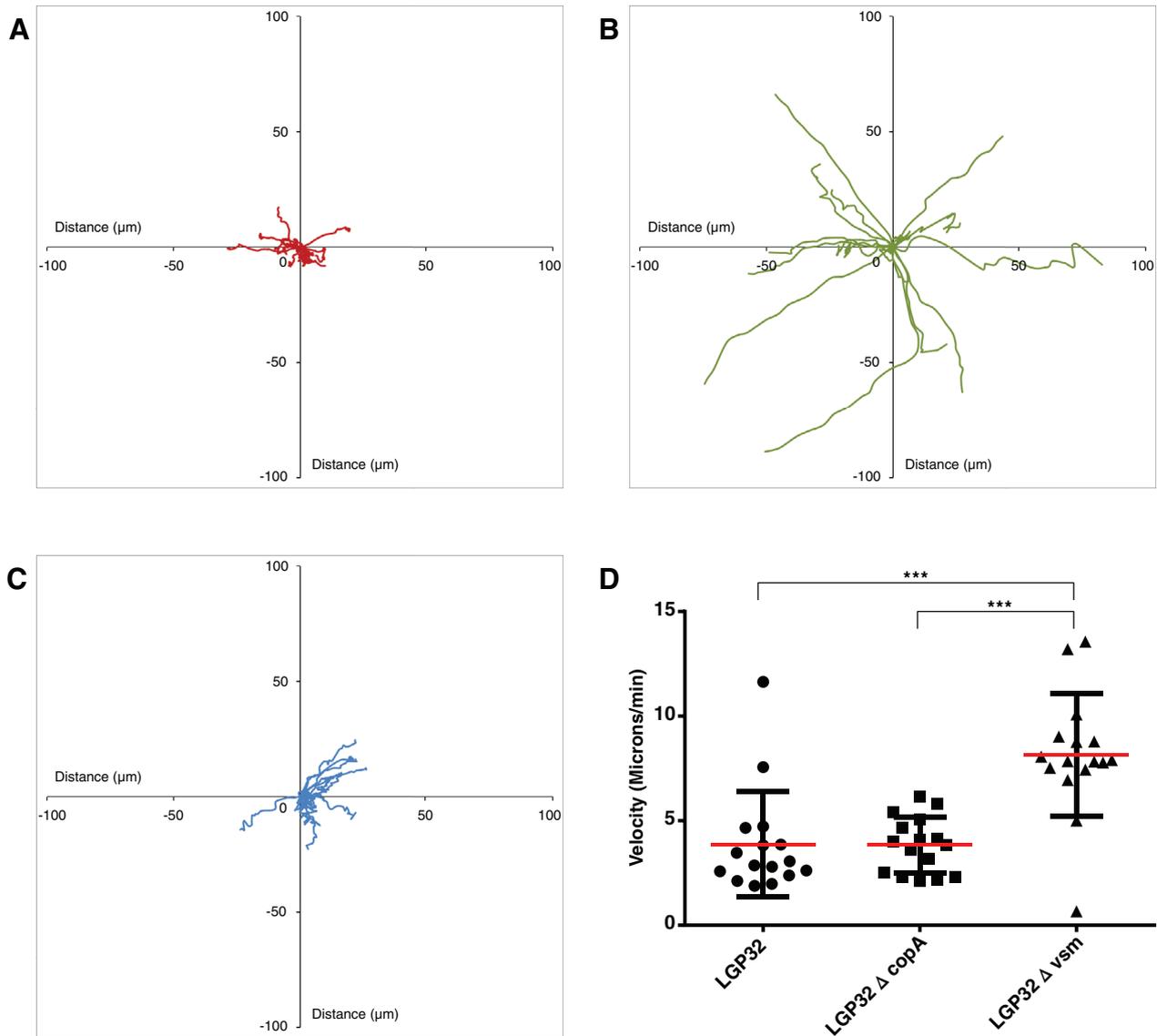


Fig. 6. Extracellular metalloprotease Vsm decreases the velocity of the amoebae *Vannella* sp. AP1411 and alters amoeba grazing efficiency. Amoebae migration on LGP32wt (A), Δvsm (B) or $\Delta copA$ (C) mutant strains lawns was recorded by time-lapse phase light microscopy during 10 min. Each presented track corresponds to the migration of one amoeba. The results shown are representative of at least two independent experiments. (D) Average velocity of amoebae on LGP32wt, $\Delta copA$ or Δvsm mutant strains. The results are representative of at least two independent experiments \pm SD. Data were analysed by Kruskal–Wallis test with Dunn’s multiple comparisons test, *** $P < 0.001$.

shown by the higher amoeba density than on wild-type LGP32 lawns (Fig. 4B and E). These results show that both the CopA resistance factor and the Vsm virulence factor play a role in the resistance of LGP32 to grazing by *Vannella* sp. AP1411 whereas the other tested virulence factors did not seem to be involved.

The p-ATPase CopA confers resistance to intracellular killing and the extracellular protease Vsm inhibits amoebae motility during grazing

To investigate further the role of LGP32 virulence factors in resistance to amoeba grazing, we analysed different

steps of the grazing process, from phagocytosis to intracellular degradation. Flow cytometry analyses showed that the percentage of phagocytosis (Fig. 4C and F) and the phagocytosis index (Fig. S5) were not significantly different between the mutant strains $\Delta copA$ and Δvsm and the wild-type LGP32, which suggested that the efficiency of the phagocytosis process was not disturbed by CopA or Vsm. Confocal and TEM microscopy confirmed that both mutant strains were readily phagocytosed by the amoeba, and revealed that $\Delta copA$ LGP32 vibrios were found mostly clustered in vacuoles and the bacteria appeared to have an altered morphology after 3 days of grazing. These observations suggest that $\Delta copA$ LGP32

was more sensitive to intracellular degradation, indicating a role for *copA* in resistance of LGP32 to intracellular degradation. In contrast, Δvsm LGP32 appeared intact inside vacuoles and individual phagosomes in a similar manner than the wild-type strain of LGP32, suggesting that Vsm is not involved in LGP32 resistance to intracellular degradation (Fig. 5A and B).

To investigate further the role of Vsm in LGP32 resistance against grazing by *Vannella* sp. AP1411, amoeba grazing was recorded by time-lapse microscopy. Amoeba grazing behaviour on vibrio lawns was analysed using Δvsm LGP32 and $\Delta copA$ LGP32 lawns and compared with wild-type strain. Cell tracking was performed to measure amoeba migratory speed on the different lawns of vibrios (Fig. 6) and the most striking difference that could be observed was that amoebae migratory speed was two fold faster ($p < 0.001$) on lawns of Δvsm LGP32 compared with wild-type or $\Delta copA$ LGP32 lawns (Supplementary Movies 1, 2 and 3), with mean velocities of 8.5, 3.9 and 3.8 $\mu\text{m min}^{-1}$ for Δvsm LGP32, wild-type LGP32 and $\Delta copA$ LGP32 respectively (Fig. 6D). These results indicate that the metalloprotease Vsm acts extracellularly by inhibiting amoeba motility, which likely disturbs grazing efficiency of amoeba, whereas CopA participate to the intracellular survival capacity of LGP32.

Discussion

In this study, we uncovered that the oyster pathogen *V. tasmaniensis* LGP32 is resistant to grazing and intracellular degradation by free-living marine amoebae of the *Vannella* genus. Here we used *Vannella* sp. AP1411 as a representative amoeba isolate, which belongs to the genus of amoeba that is ubiquitous in the oyster farming environment and that interacts with vibrios in the environment. We also uncovered some of the molecular determinant that are involved in the resistance of LGP32 to *Vannella* sp. AP1411, the copper efflux p-ATPase CopA and the secreted metalloprotease Vsm. These results indicate that at least two different mechanisms contributing to *V. tasmaniensis* LGP32 pathogenicity in oysters are also involved in resistance to amoeba grazing.

Amoebae of the *Vannellidae* family were found to be ubiquitous in the Thau lagoon, a Mediterranean coastal lagoon used for oyster farming (20% of its total surface). Indeed, most of the isolated amoebae branched into the *Vannella* genus, independently of seasons. Together with the present study, the current literature suggests that *Vannellidae* amoebae are frequent in aqueous environments, mostly in saltwater but are also frequently observed in freshwater (Smirnov *et al.*, 2007). By performing 16s barcoding sequencing and FISH analysis, we were able to find that some *Vannella* sp. isolated from oyster farms were associated with *vibrionaceae* and that

this association can last for weeks *in vitro*; suggesting that these amoebae can interact with vibrios in this environment. Although the ecological dynamics of these *Vannella-Vibrionaceae* associations deserves to be investigated in greater details, our data suggest that the *Vannella* sp. isolated in the present study are an ecologically relevant host to investigate the biotic interactions between *Vibrio* species and marine amoebae.

By studying the interactions between *Vannella* sp. AP1411 and the vibrio LGP32, we found that *V. tasmaniensis* LGP32 is able to resist predation by the marine amoeba. Vibrios belonging to the *tasmaniensis* species have been shown to resist haemocyte phagocytosis and to behave as facultative intracellular pathogen, a behaviour that was shown to be central for its virulence in oysters (Duperthuy *et al.*, 2011; Vanhove *et al.*, 2016; Rubio *et al.*, 2019). The slow growth of amoebae on LGP32 was due, to some extent, to an inefficient intracellular killing of LGP32. Indeed, after phagocytosis most intracellular LGP32 were isolated in individual phagosomes inside the amoebae without any morphological alteration whereas the non-virulent control *V. tasmaniensis* LMG20012^T was progressively clustered in vacuoles and degraded. This strongly suggests that LGP32 interferes with phagosome maturation and intracellular trafficking and this is reminiscent of its capacity to inhibit phagosome acidification in oyster phagocytes (Duperthuy *et al.*, 2011).

The copper efflux p-ATPase CopA was found here to play a major role in the capacity of LGP32 to resist intracellular degradation within the amoeba. As CopA is involved in the resistance of LGP32 to high concentration of copper within oyster haemocytes (Vanhove *et al.*, 2016), our results indicate that LGP32 resistance to grazing by the amoeba depends on its capacity to tolerate high concentrations of copper inside phagosomes and thus strongly suggest that *Vannella* sp. AP1411 use intraphagosomal heavy metal poisoning in a similar manner than other professional phagocytes. Intraphagosomal copper accumulation appears to be an ancestral highly conserved antimicrobial mechanism from FLA, social amoebae (Hao *et al.*, 2016), invertebrates (Vanhove *et al.*, 2016) to vertebrate professional phagocytes (Soldati and Neyrolles, 2012). As this antimicrobial mechanism is used by a variety of phagocytes we can hypothesize that, like other bacterial species resisting intracellular killing (Espariz *et al.*, 2007; Rowland and Niederweis, 2012), for some vibrios, copper resistance confers them a fitness advantage in diverse host-vibrio interactions (Brooks *et al.*, 2014; Vanhove *et al.*, 2016).

Another determinant that was found here to be important for LGP32 resistance to grazing by *Vannella* sp. AP1411 was the secreted metalloprotease Vsm, a major secreted protease that plays a role as a virulence factor in oysters (Binesse *et al.*, 2008). In the present study, the Vsm deficient LGP32 strain showed an increased sensitivity to grazing by the amoeba, without

losing its ability to resist intracellular degradation. This suggested that Vsm acts extracellularly rather than intracellularly, during LGP32 interaction with amoeba, which was confirmed by studying amoeba grazing behaviour by time-lapse microscopy revealing an important increase of grazing migratory speed of amoebae on Δvsm LGP32 bacteria lawns compared with lawns with the wild-type strain. The involvement of Vsm metalloprotease in LGP32 resistance to grazing is reminiscent of the role played by other proteases secreted by vibrios during their interaction with eukaryotic cells. In particular, the involvement of the secreted protease PrtV, that participates in the resistance of *V. cholerae* to the grazing by the bacterivorous ciliate *Tetrahymena pyriformis* (Vaitkevicius *et al.*, 2006), the metalloprotease Vsm can also damage oyster haemocytes (Binesse *et al.*, 2008), and its homologue Vam, from *V. aestuarianus*, can inhibit oyster haemocyte phagocytosis (Labreuche *et al.*, 2010). Interestingly, recent work studying the interaction between clinical isolates of *V. cholerae* and the model amoebae *Acanthamoeba castellanii* (Neff strain) reported that minor virulence factors like secreted enzymes, including the HapA metalloprotease and the haemolysin, can be involved in fine tuning the fitness of *V. cholerae* during its interaction with grazers in the aquatic environment (Van der Henst *et al.*, 2018). Then the secretion of proteases by vibrios appears to be an important ancestral mechanism that may have been acquired to inhibit predation by grazers in aquatic environments but can also participate to their pathogenicity in animal hosts.

Hence, as copper resistance and secretion of proteases appear to be important virulence mechanisms for different vibrios and involved in interactions with diverse hosts, the presence of these virulence traits in vibrios against diverse hosts may represent evidences for the coincidental selection hypothesis (Adiba *et al.*, 2010).

As bacterivorous protists exert a selective pressure on bacterial communities, different bacteria species are thought to have developed a diversity of survival strategies against predation through either pre-ingestional or post-ingestional adaptations (Matz and Kjelleberg, 2005). Pre-ingestional adaptations including high motility, filamentation, surface masking or toxin release are commonly found for extracellular pathogens; whereas post-ingestional adaptations including digestional resistance through vacuolar trafficking inhibition or vacuolar escaping, and toxin release are commonly found for intracellular pathogens and tend to favour bacterial growth (Matz and Kjelleberg, 2005). By establishing a new ecologically relevant cellular model to study the Vibrio–phagocytes interactions in marine environment, we found that, in the case of the facultative intracellular pathogen LGP32, a combination of pre-ingestional and post-ingestional adaptations could be involved. The involvement of the

secreted Vsm represents a pre-ingestional adaptation, whereas the involvement of the CopA p-ATPase represents a post-ingestional adaptation. Thus, the predator–prey interactions between bacterial communities and bacterivorous protists may select for common virulence factors allowing bacteria to resist to a diversity of phagocytic cells from amoeba to immune cells in animals, favouring the emergence of opportunistic pathogens able to colonize a diversity of hosts, like many vibrios do.

However, not all virulence factors studied here were found to participate to LGP32 resistance to grazing by *Vannella* sp. AP1411. For instance, *V. tasmaniensis* T6SS1, Vsp, CusAB and SOD, which were found to play a role in oysters (Vanhove *et al.*, 2014, 2016; Rubio *et al.*, 2019), do not seem to play a role during interactions with *Vannella* sp. AP1411. These results strengthen the hypothesis that although some virulence factors could be involved in diverse biotic interactions, as observed here for CopA and Vsm, hence favouring opportunism, some other virulence factors could be more specifically involved in more specialized interactions. As for example, the metalloprotease InhA, the orthologue of PrtV in LGP32, does not appear to play a role during LGP32–Oyster interactions or LGP32–*Vannella* sp. AP1411 interaction, although it was shown to participate in the resistance of *V. cholerae* to the grazing by the bacterivorous ciliate *Tetrahymena pyriformis* (Vaitkevicius *et al.*, 2006). In a similar manner, T6SS, which was shown to be involved during interaction of *Vibrio cholerae* with the social amoeba *D. discoideum* (Pukatzki *et al.*, 2006), did not appear to be involved in LGP32 resistance to *Vannella* sp. AP1411 although it was shown recently to be a major determinant of LGP32 cytotoxic activity towards oyster haemocytes (Rubio *et al.*, 2019). Such host-specialization of some vibrios virulence factors was also suggested recently for *V. cholerae*, as some of the virulence factors involved in the resistance to grazing by *A. castellanii* are considered to have a minor role in pathogenesis in human (Van der Henst *et al.*, 2018). These subtleties in the involvement of different virulence factors during different biotic interactions are reminiscent of the subtleties that are being uncovered for the intracellular pathogen *L. pneumophila*. This well studied intracellular pathogen harbours a broad range of virulence factors and can interact with a diversity of hosts, but this does not imply that all virulence factors have a similar importance in each interactions with these diverse hosts (Boamah *et al.*, 2017; Ghosh and O'Connor, 2017). Vibrios being mostly opportunistic pathogens, they probably have acquired, on the one hand, some virulence factors that are involved in diverse host interactions and could represent core virulence factors, and on the other hand, some virulence factors that are involved in more restricted host interactions that could represent specialized virulence factors. Studying diverse host–vibrios interactions will help to decipher the diversity of their virulence factors and provide insights about opportunist pathogens co-evolutionary trajectories that are

more complex than the host–parasite co-evolutionary arm race observed for host specialized pathogens (Brown *et al.*, 2012).

In conclusion, by developing a new ecologically relevant cellular model of interaction, we showed here that the oyster pathogen *V. tasmaniensis* LGP32 can resist phagocytosis by environmental grazers *Vannella* spp., which are ubiquitous in oyster farming area and appear to interact with vibrios in the oyster farming environment. By performing comparative cellular biology, we also demonstrated that some of LGP32 virulence factors can be involved in different types of interactions with multiple hosts from protozoans, such as environmental marine amoeba, to metazoans, for example, oysters. This suggests that some virulence factors such as CopA and Vsm could play a central role in the interaction of *V. tasmaniensis* with diverse hosts whereas some other virulence factors appear to be involved in more specific host–vibrio interactions. Hence, the present study implies that studying diverse host–vibrio interactions will help to gain further insights about the ecology of vibrios and to uncover the relative roles of biotic interactions in pathogen emergence.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli strain SBS363 was grown in Luria–Bertani (LB) or LB-agar (LBA) at 37°C. Marine amoebae *Vannella* sp. AP1411 and isolates 0517-TOEc-2, 0517-TOEc-3, 0517-TOEc-4, 0517-BWEc-1, 0517-BWEc-2, 0517-BWEc-3, 0517-BWEc-4 (isolated in this study) were grown in 70% sterile seawater (SSW) with *E. coli* wild-type strain SBS363 at 18°C, for 3 days prior experiments. Vibrios strains used in this study are *V. tasmaniensis* LMG20012^T, *V. tasmaniensis* LGP32 and previously described LGP32 deletion mutants $\Delta copA$, $\Delta cusAB$; $\Delta sodA$; Δvsm , Δvsp , $\Delta inhA$ (Binesse *et al.*, 2008; Vanhove *et al.*, 2014, 2016) and insertion mutants $\Delta vipA1$, $\Delta vipA2$ (Rubio *et al.*, 2019). Vibrios strains carrying the pMRB-GFP or pD3-GFP-mut3 plasmids were grown in LB + NaCl 0.5 M supplemented with chloramphenicol (10 $\mu\text{g mL}^{-1}$) or kanamycin (20 $\mu\text{g mL}^{-1}$) at 20°C, for 24 h prior experiments.

Isolation of environmental amoebae from the Thau Lagoon and Banyuls-sur-mer

Between 2014 and 2015, seawater, oysters and sediment from the Thau lagoon (South France) were sampled at least two times per season. Seawater was collected next to oyster tables at the Bouzigues station Ifremer-REPHY (GPS: N 43°26.058' E 03°39'0.878') and filtered, on the boat, with a 180 μm pore size nylon filter. In the

lab, the water was re-filtered using an 8.0 μm pore size MF-Millipore membrane. The 8.0 μm pore size membrane was then cut in four pieces and each quarter was put upside down on a lawn of *E. coli* SBS363 seeded on SSW-agar. Oyster gills were cut in four pieces of roughly 0.5 cm^2 and each quarter was put upside down on a lawn of *E. coli* SBS363 seeded on non-nutrient SSW-agar. Sediment was collected under the oyster tables (depth of 9 m) by core sampling. One gram of sediment was then deposited in the centre of a lawn of *E. coli* SBS363 seeded on SSW-agar, in triplicate. After 1–3 weeks, depending of the sampling season, migrating amoebae were observed at the periphery of the plates. An agar square of 0.5 \times 0.5 cm containing a single amoeba was then cut and deposited upside down on a fresh lawn of *E. coli* SBS363 seeded on SSW-agar. This was repeated several times to isolate and replicate amoebae. DNA was extracted using the High Pure PCR Template Preparation Kit (Roche), according to the manufacturers' protocol. In May 2017, sampling was performed from oyster gills from Thau lagoon at Bouzigues station Ifremer-REPHY (GPS: N 43°26.058' E 03°39'0.878') and seawater from Banyuls-sur-mer at SOLA station (GPS: 42°29'300 N – 03°08'700 E). Samples were treated with the same protocol than previously and put on a lawn of *E. coli* SBS363 seeded on SSW-agar. After 2 weeks, amoebae were flushed by SSW and clonally isolated by dilutions within 96 well plates with statistically less than one amoeba per well. Amoebae clones were amplified by culturing in six well plates, and DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel), according to the manufacturer's protocol.

Identification of isolated marine amoebae from Thau Lagoon and Banyuls-sur-Mer and their associated bacteria

In order to infer the phylogenetic assignment of environmental amoebae, the v7 region of the 18s rDNA gene was sequenced using specific primers. The primers were designed using SILVA database dedicated tool (<http://www.arb-silva.de/search/testprime>). The selected primers, Amo_AP_1154_F: 5'GAGRAAATTAGAGTGTTYAAAG 3' and Amo_AP_1470_R: 5'TTATRGTTAAGACTA CGACGG 3', were used to amplify the hypervariable region V7 of 18S rDNA gene at an annealing temperature of 54°C. PCR amplicons were cloned using the TOPO TA Cloning kit (Invitrogen), according to the manufacturers' protocol. Nucleotide sequences were determined by Sanger sequencing (GenSeq platform, Labex CEMEB, Montpellier, France). Sequence homologies were searched using BLASTn available at the NCBI website (National Centre for Biotechnology Information) (Wheeler *et al.*, 2007) (Table S1). For phylogenetic analyses, sequences were aligned in MOTHUR

(Schloss, 2009) and imported into ARB software (Ludwig *et al.*, 2004) loaded with the silva database (<http://www.arb-silva.de>). A base frequency filter was applied to exclude highly variable positions before adding sequences to the maximum parsimony backbone tree using the parsimony quick add marked tool implemented in ARB, thereby maintaining the overall tree topology provided by default. In order to verify that the Sanger sequencing strategy used here allowed us to identify the entire diversity of amoebae that could be isolated through culturing techniques. The total DNA from our different amoebae cultures was analysed by performing barcoding on the v7 region of the 18S rDNA gene using generic primers that allowed amplifying more than 80% of all the eukaryote 18s rDNA sequences available in SILVA database (F-v7-1173: CCT GCG GCT TAA TTT GAC and R-v7-1438: CAT CAC AGA CCT GTT ATT GC). Sequencing was performed by Genome Quebec facility, Montreal, Canada.

Bacteria associated with amoeba clonally isolated in May 2017 sampling were investigated using barcoding sequencing on the V3/V4 region of the 16S rDNA gene with Illumina generic primers according to the manufacturer's protocol (16Sv3v4-ILLAFWD: 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG YRC CTA CGG GNG GCW GCAG and 16Sv3v4-ILLAREV: 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GYR GAC TAC HVG GGT ATC TAA TCC). Sequencing was performed by GenSeq platform, Labex CEMEB, Montpellier, France. OTUs taxonomic affiliation was performed using FROGS tool from galaxy software (Escudie *et al.*, 2018). To determine the taxonomic affiliation of clones isolated during the second sampling campaign, the sequence of the V4 hypervariable region of the 18S rDNA gene was analysed using TAReuk454FWD1: 5'-CCAGCA(G/C)C(C/T)GCGG-TAATTCC-3' and TAReukREV3: 5'-ACTTTCGTTCTTGAT(C/T)(A/G)A-3' primers (Stoeck *et al.*, 2010). Nucleotide sequences were determined by Sanger sequencing (GenSeq platform, Labex CEMEB, Montpellier, France) and aligned with MUSCLE using MEGA7 software (maximum-likelihood by bootstrap method, 1000 replications).

Grazing assay

To prepare the co-culture of vibrios and amoebae, 1 ml of vibrio overnight culture (3.10^9 bacteria. mL^{-1}) was mixed with 100 μl of 3 days old *Vannella* sp. AP1411 culture (5×10^5 cells mL^{-1}) or with 100 μl of 70% SSW for control condition. A volume of 50 μl per well of the mixed culture was seeded on top of 500 μl of 1% SSW-agar, in 24-well plates with transparent flat bottoms. Amoebae and bacteria lawns were carefully homogenized in the wells and let dry 4 h at room temperature in a laminar flow cabinet and then incubated at 18 °C in a humidified atmosphere. GFP fluorescence intensity was measured

every day over 7 days using a TECAN plate reader (λ_{exc} 480 nm/ λ_{em} 520 nm). To estimate the effect of the amoebae grazing activity on the abundance of living vibrios expressing GFP, the fluorescence intensity of the wells containing amoebae was compared with the fluorescence of vibrios lawn without amoebae, and expressed as a ratio. Each condition was performed in technical triplicates and the results shown are the average of three independent experiments. Error bars represent the standard error of the mean (\pm SEM). Statistical analysis was performed using RM-ANOVA over the independent experiments.

Monitoring amoeba growth and association with GFP-vibrios

To estimate the proliferation of *Vannella* sp. AP1411 at days 1, 3 and 6 in grazing assays, amoebae were directly imaged by phase-contrast microscopy and enumerated in triplicate for each condition. Cells were flushed from the SSW-agar surface with 1 ml of 70% SSW and fixed for 30 min with 2% paraformaldehyde at room temperature. Pellets were washed and suspended in 500 μl of PBS for flow cytometry analysis (BD FACS Canto). Data analyses were performed using Flowing Software by gating on amoeba cells using SSC/FSC parameters then quantifying the percentage of GFP fluorescence associated amoebae (as shown in Fig. S2). In order to estimate the phagocytosis index, which corresponds to the average number of phagocytosed bacteria per cell, the mean intensity of GFP fluorescence per amoeba was normalized to the mean GFP fluorescence per bacteria (as shown in Fig. S3). The experiments were performed with three technical replicates per condition in each experiment, and depicted results are representative of at least two independent experiments. Error bars represent the \pm SEM. Statistical analysis was performed using two ways ANOVA with Holm-Sidak's multiple comparisons test.

Fluorescence in situ hybridization

In situ hybridization for *Vibrionaceae* was performed on *Vannella* sp. AP141, 0517-TOEc-2, 0517-TOEc-3 and 0517-TOEc-4. After at least 2 weeks of culturing in flask in 70% SSW complemented with *E. coli* SBS363, amoebae were washed twice with 3 ml of 70% SSW and resuspended in 70% SSW using a cell scraper and fixed with 2% formaldehyde for 25 min at room temperature. After fixation, formaldehyde was removed by centrifugation of the cells by centrifugation 10 min at 6000g, pellets were washed and resuspended in 500 μl of PBS. Cell suspensions were cytospun on glass slides for 10 min at 500 g. Glass slides were then labelled with the

Vibrionaceae specific probe VIB572a at 10 ng mL⁻¹ (5'-ACCACCTGCATGCGCTTT-3') (Huggett *et al.*, 2008) using hybridization buffer (900 mM NaCl, 20 mM Tris-HCl, 30% Formamide, 0.01% SDS, dH₂O) for 90 min at 46°C in the dark. The samples were then washed for 25 min in washing buffer (0.112 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, 5 mM EDTA, dH₂O) at 48°C in the dark. Then, cells were counterstained using FITC at 0.5 ng mL⁻¹ for 30 min (Sigma). Confocal fluorescence imaging was performed using a 63× oil objective and images were captured using a Leica TCS SPE confocal scanning laser microscope, at 1 airy to ensure the focal plan.

Microscopy

The different amoebae shapes were observed on living amoebae using phase contrast (on an inverted wide-field microscope, Zeiss). The shape of the floating forms was recorded by scraping in the cell suspension followed by immediate observation. The cystic form was observed after amoebae were starved for a week. Intracellular organization of amoebae was observed using transmission electron microscopy. Briefly, amoebae cultured in flasks were washed twice using 70% SSW, and fixed in 2.5% glutaraldehyde for 2 h in the dark at room temperature, followed by overnight at 4°C. After centrifugation, the supernatant was replaced by 3% low melting-point agarose (Sigma) and the pellet was resuspended. Cells entrapped in 3% agar were immersed in a solution of 2.5% glutaraldehyde in PHEM buffer (1×, pH 7.4) overnight at 4°C. Then they were rinsed in PHEM buffer and post-fixed in a 0.5% osmic acid for 2 h in the dark at room temperature. After two rinses, samples were dehydrated in a graded series of ethanol solutions (30%–100%). Samples were embedded in EmBed 812 using an Automated Microwave Tissue Processor for Electronic Microscopy, Leica EM AMW. Ultrathin sections (70 nm; Leica-Reichert Ultracut E) were collected at different levels of each block, then counterstained with uranyl acetate 1.5% in 70% ethanol and lead citrate and observed using a Tecnai F20 transmission electron microscope at 120 kV in the CoMET MRI facilities, INM, Montpellier, France.

Confocal fluorescence microscopy was performed on amoebae suspensions after PFA fixation to observe the localization of intra-amoeba vibrios. Cell suspensions were cytospun on glass slides for 10 min at 800 g. Glass slides were then stained with 0.25 µg mL⁻¹ DAPI (Sigma) and 0.1 µg mL⁻¹ Blue Evans (Sigma). Confocal fluorescence imaging was performed using a 63× oil objective and images were captured using a Leica TCS SPE confocal scanning laser microscope, at 1 airy to ensure the focal plan.

Time-lapse imaging and cell tracking

Co-cultures of vibrio and amoebae were prepared following the same protocol as for grazing assays. Time-lapse imaging started 24 h after the beginning of the grazing assays. Acquisitions were obtained using an inverted wide-field epifluorescence microscope (Zeiss) and B&W coolsnap camera. Time-lapses were done during 30 min with one frame every 30 s. TrackMate Tools software (ImageJ) was used for manual tracking to determine velocity and migration distance by amoebae on different vibrios lawns (Supplementary Movies 1, 2 and 3). Migration distances were measured for 18 amoebae at the same time (i.e. 20 frames per amoebae trace) for each condition. Each track depicted corresponds to the migration of an amoeba during 10 min. The migration path was determined by the *x* and *y* positions of amoebae tracked at each frame. Depicted results are representative of at least two independent experiments. Velocities were measured for 16 amoebae tracks, only tracks made of at least five frames were considered. Each presented result corresponds to the average velocity of one amoeba per track. Depicted results are representative of at least two independent experiments. Error bars represent the standard deviation (±SD). Statistical analysis was performed using Kruskal–Wallis test with Dunn's multiple comparisons test.

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Conflict of Interest

The authors declare that there are no conflict of interests related to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Phylogeny of the diversity of amoebae OTUs found by barcoding of the v7 region of the 18 s rDNA gene. Phylogenetic 18S rRNA-based trees of the Amoebozoa constructed with ARB software ([http://www.arb-](http://www.arb-home.de)

<http://www.arb-silva.de>) loaded with the silva database (<http://www.arb-silva.de>). A base frequency filter was applied using the parsimony quick add marked tool implemented in ARB. All 18S rDNA gene sequences assigned to amoeba belonged to the *Vannellidae* family (written in red).

Fig. S2. *Vannella* sp. AP1411 isolated from Thau lagoon, France.

(A) Different forms of *Vannella* sp. AP1411 under phase light microscopy. Trophozoite (left), pelagic (centre) and cystic form (right). Scale bar: 10 μ m. **(B)** Transmission electron microscopy of trophozoite shape of the amoeba *Vannella* sp. AP1411. Whole cell overview showing nuclei (N) and several digestive vacuoles (DV) with or without *E. coli* SBS363 bacteria inside (B). Scale bar: 2 μ m.

Fig. S3. Quantification of *Vannella* sp. AP1411 phagocytosis by cytometry. **(A)** FSC/SSC Gate corresponding to amoebae **(B)** Determination of fluorescence background on amoebae alone. **(C)** Gate determination for GFP+ vibrios (Bacteria). **(D)** Measurement of the percentage of amoebae carrying GFP fluorescence after 3 days of co-culture with GFP-expressing vibrios.

Fig. S4 CusAB, SodA, Vsp, InhA, VipA1 and VipA2 are not involved in LGP32 resistance to grazing by *Vannella* sp. 1411. Bacterial resistance to grazing by *Vannella* sp. AP1411 of Δ cusAB, Δ sodA, Δ vsp, Δ inhA, Δ vipA1 and Δ vipA2 mutant strains of LGP32 was assessed by measuring the fluorescence of the GFP-expressing bacteria, after contact with amoebae. Each condition was performed in triplicate and the results shown are the average of three independent experiments. Values are presented \pm SEM.

Fig. S5. Vsm or CopA do not affect the phagocytosis rate of LGP32 by the amoeba *Vannella* sp. AP1411. Grazing amoebae on GFP-vibrio LGP32wt, LGP32 Δ copA or of LGP32 Δ vsm were monitored by flow cytometry to estimate phagocytosis index per amoeba, which correspond to the average number of phagocytosed bacteria per cell. The results shown are representative of two independent experiments. Data were analysed by one-way ANOVA with Friedman test.

Table S1. Sanger sequences of sampled marine amoebae **Supplementary Movie 1.** Time-lapse microscopy movie of *Vannella* sp. AP1411 and LGP32wt lawn in co-culture.

Supplementary Movie 2. Time-lapse microscopy of *Vannella* sp. AP1411 and LGP32 Δ copA lawn in co-culture.

Supplementary Movie 3. Time-lapse microscopy of *Vannella* sp. AP1411 and LGP32 Δ vsm lawn in co-culture.