Comparative Proteogenomics of Twelve Roseobacter Exoproteomes Reveals Different Adaptive Strategies Among These Marine Bacteria*

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Roseobacters are generalist bacteria abundantly found in the oceans. Because little is known on how marine microorganisms interact in association or competition, we focused our attention on the microbial exoproteome, a key component in their interaction with extracellular milieu. Here we present a comparative analysis of the theoretically encoded exoproteome of twelve members of the Roseobacter group validated by extensive comparative proteogenomics. In silico analysis revealed that 30% of the encoded proteome of these microorganisms could be exported. The ratio of the different protein categories varied in accordance to the ecological distinctness of each strain, a trait reinforced by quantitative proteomics data. Despite the interspecies variations found, the most abundantly detected proteins by shotgun proteomics were from transporter, adhesion, motility, and toxin-like protein categories, defining four different plausible adaptive strategies within the Roseobacter group. In some strains the toxin-secretion strategy was over-represented with repeats-in-toxin-like proteins. Our results show that exoproteomes strongly depend on bacterial trophic strategy and can slightly change because of culture conditions. Simulated natural conditions and the effect of the indigenous microbial community on the exoproteome of Ruegeria pomeroyi DSS-3 were also assayed. Interestingly, we observed a significant depletion of the toxin-like proteins usually secreted by R. pomeroyi DSS-3 when grown in presence of a natural community sampled from a Mediterranean Sea port. The significance of this specific fraction of the exoproteome is discussed. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.013110, 1–12, 2012.

The strong interaction with the environment, the competition with the biotic community, and the biological complexity

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sulfur-containing compound tropodithietic acid (17) or tryptanthrin (18). Antibiotic and biofilm formation properties have been assigned to tropodithietic acid converting the bacterial producer into an aggressive colonizer (19–21). Aligicidal agents are widely searched for biotechnological applications, as exemplified by Jeong et al. (22), which have shown that Hahella chejuensis from the Oceanospirillales clade is able to secrete a pigmented compound with algicidal properties. Roseobacter clade members have also been related to algal bloom decline (23). Recently, novel potent and selective algaeicides called roseobacticides have been characterized (24, 25).

Generally, only type III, IV, V, and VI protein secretion systems have been considered during the analysis of virulence factors in the genomes of marine microorganisms (26). In Roseobacters, type I secreted toxins have never been taken into account (16, 27) although they have recently proven to be a principal component in the exoproteome of Ruergeria pomeroiyi DSS-3 (13). Repeat-in-toxin (RTX)1 proteins are entities with a large number of short peptide sequence repetitions able to bind Ca2+ ions. They are secreted by a type I secretion system (28). The mechanism of action of these putative toxins relies on the transition from the water-soluble form into a channel protein after insertion in a targeted biomembrane (29). Some of these RTX proteins are well characterized as they are considered the main virulence factors in several well-known uropathogenic strains or different pathogenic Vibrio species (28). Nevertheless, RTX-like proteins are also found encoded in bacteria considered nonpathogenic such as members of the Roseobacter clade (27).

This study was focused on defining the interaction of different Roseobacter strains with their extracellular milieu. For this we carried out an extensive analysis by comparative proteogenomics (30) of the exoproteomes of 12 Roseobacter isolates establishing the putative pan-exoproteome of the clade by comparative genomics. The experimental exoproteome of the different Roseobacter representatives was identified by high-throughput proteomics revealing different trophic strategies. Secretion of RTX toxins resulted as a common feature among Roseobacters. The significance of these exoproteins is discussed in the light of experimental assays with seawater from port in the presence or absence of the natural indigenous community.

MATERIALS AND METHODS
Bacteria and Culture Conditions Used—Roseobacter clade strains Ruergeria pomeroiyi DSS-3, Oceanicola batsensis HTCC2597, Pelagibaca bermudensis HTCC2601, Roseobacter denitrificans OCh114, Oceanialucia granulosus HTCC2516, Oceanibulbus indolifex HEL45, Ruergeria lacuscaeruleus PM11157, Roseobacter littoralis OCh149, Roseobacter sp. MED193, Roseovarius nubinhibens ISM, Dinoroseobacter shibae DFL12, and Sagittula stellata E-37 used in this work were obtained from the DSMZ collection. Duplicated bacterial cultures were performed in Erlenmeyer flasks containing 50 ml of Marine Broth (MB) (Difco, Detroit, MI) incubated at 30 °C and agitated at 180 rpm until midexponential phase. The exoproteomes of R. pomeroiyi DSS-3 grown under different culture conditions was analyzed systematically in duplicates. Exoproteomes were collected from mid exponentially grown cultures from four conditions based on different media: (1) rich nutrient MB; (2) a 1:1 mix of MB with Luria-Bertani (LB) broth; (3) poor nutrient minimal marine medium (MMM) ((31); and (4) autoclaved seawater obtained from the open Mediterranean sea (Cap Croisette, Marseille, France). The two last media were supplemented with 0.5% succinate as a source of carbon and energy and 0.005% yeast extract as a source of vitamins. A fifth condition was included to test the exoproteomes when stationary phase was reached in MB rich broth. It is worth noting that drastic stresses or inadequate growth conditions could not be applied for such analysis as cell lysis should be avoided in order to not cross-contaminate the exoproteomes with abundant cytosolic proteins. The exoproteome samples from simulated natural conditions were obtained after incubating R. pomeroiyi DSS-3 in Mediterranean Sea coastal winter surface seawater (February 16, 2011) taken from Marseille (France). Sampling areas were Cap Croisette (cape seawater, 43.2146607N-5.33602952E) and Vieux Port (marina seawater, 43.29534234N-5.36682128E) (32). A volume of 750 ml of natural seawater (presence of the natural microbial community) or seawater filtered through a 0.22 μm diameter-pore filter (absence of the natural community) was introduced into 1l-Erlenmeyer flasks. Each flask was inoculated at a final concentration of 10^6 cells/ml with MB pregrown R. pomeroiyi DSS-3 cells, previously washed in the final medium. Flasks were incubated at 25 °C and 200 rpm for 20 h. These incubations were systematically carried out in duplicate. Bacterial maximal doubling times in MB medium were calculated from growth curves measured at OD600 nm.

Exoproteome Preparation and Trypsin In-gel Proteolysis—Exoproteomes were obtained as previously described (13). Briefly, duplicated cultures grown until early exponential phase (OD600 nm > 0.6), late stationary phase (3 days after reaching maximum of growth), or simulated natural conditions were centrifuged at 3000 x g for 10 min at 20 °C and supernatants were further filtered (0.22 μm) in order to remove residual cells. Supernatant proteins were concentrated by trichloroacetic acid precipitation. Precipitated exoproteins were dissolved in 40 μl of lithium dodecyl sulfate-β-mercaptoethanol SDS-PAGE sample buffer (Invitrogen, Carlsbad, CA) and incubated at 99 °C for 5 min prior to SDS-PAGE. The protein content from the equivalent of 10 ml of laboratory culture supernatant or 375 ml of liquid from simulated natural conditions was loaded on a 10% Tris-Bis NuPAGE gel (Invitrogen) for a 3-mm short migration. The bands containing the different exoproteomes were excised from the polyacrylamide gel and proteolyzed in-gel by trypsin using the ProteaseMax protocol (Promega, Charbonnières, France) as described previously (11).

Shotgun Nano-LC-MS/MS Analysis and Parameters for Peptide Identification—Nano-liquid chromatography-tandem MS (LC-MS/MS) experiments were performed using a LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher) coupled to an UltiMate 3000 LC system (Dionex-LC Packings) in similar conditions as those previously described (33, 34). For MS/MS peptide assignments, a protein sequence database containing all the annotated protein coding sequences (CDS) of each Roseobacter clade strain used in the present study was made in-house from FASTA files available from NCBI. As an example, R. pomeroiyi DSS-3 database comprises 4252 polypeptide sequences, totaling 1,373,960 amino acids. Peak lists were generated with the MASCOT DAEMON software (version 2.3.2) from Matrix Science using the extract_msn.exe data import filter from the

1 The abbreviations used are: RTX, repeat-in-toxin; MB, marine broth; MMM, minimal marine medium; CDS, coding sequence.
Xcalibur FT package (version 2.0.7) proposed by ThermoFisher. Data import filter options were set at: 400 (minimum mass), 5000 (maximum mass), 0 (grouping tolerance), 0 (intermediate scans), and 1000 (threshold). MS/MS spectra were searched against the corresponding in-house database with the MASCOT 2.2.04 software (Matrix Science). Search parameters were: tryptic peptides with a maximum of two miss-cleavages during proteolytic digestion, mass tolerances of 5 ppm on the parent ion and 0.5 Da on the MS/MS, fixed modification for carboxamidomethylated Cys and variable modification for oxidized Met. MASCOT results were parsed using the IRMa 1.28.0 tools: (1) SignalP 3.0 server (www.cbs.dtu.dk/services/SignalP/) for predicting N-terminal signal peptides for secretion (37); (2) SecretomeP 2.0 server (www.cbs.dtu.dk/services/SecretomeP/) for predicting proteins secreted by nonclassical systems (38); and (3) LipoP 2.0 server (www.cbs.dtu.dk/services/LipoP/) for predicting lipoproteins (39). In all cases we chose the settings for Gram-negative bacteria.

Local BLAST analyses were done with the BioEdit BLAST tool version 7.0.5 (40) using default parameters. On-line PSI-BLAST and protein domain searches were performed with the NCBI website facilities (blast.ncbi.nlm.nih.gov) using the nonredundant protein sequences database and default parameters.

Protein Statistical Abundance Variations—Protein semiquantization by spectral abundance was performed as previously described (41). Statistical comparison between nanoLC-MS/MS detected proteins in different conditions was carried out with the PatternLab program (42).

Data were normalized by the total number of spectral counts per replicate and analyzed using the ACFold method with a p value below 0.01 and a minimum fold of 2. A BH-FDR statistical test was calculated to evaluate the global false discovery rate for each comparison.

RESULTS

Putative Pan-exoproteome of Members of the Roseobacter Clade Established by Comparative Genomics—The prediction of the potential pan-exoproteome encoded by 12 Roseobacter clade reference genomes was carried out. A total of 14,845 proteins from the 12 genomes analyzed were predicted to be exported by at least one of the three predictor tools used (supplemental Table S1). This means that almost 30% of the total annotated CDS in each analyzed strain is potentially exported (Table I), generally exhibiting functions in accordance with a secreted status. Nevertheless, some listed proteins were probably badly predicted (e.g. several ribosomal proteins) because of overprediction of nonclassical secreted proteins by the SecretomeP algorithm stressing the need of further experimental evidence to train in-silico predictions. It is also important to note that the translation start codons used by the prediction tools are sometimes badly annotated, thus generating important biases in in silico analysis (43, 44). The listed proteins found in the predicted exoproteome of each reference genome were distributed into main functional categories (Table I). Over 40% of CDSs predicted as secreted proteins were annotated as “hypothetical proteins.” Such ratio was largely higher than that found for the whole genome, in which around 30% of CDSs belong to this category. This difference shows the relative lack of knowledge on bacterial exoproteomes.

Transport, Motility, and Adhesion Proteins—ABC and TRAP-like transporters represented a large fraction of secreted proteins. Remarkably, the strain with the smallest number of transporters was R. lacuscaerulensis ITI1157, the only one not isolated from a marine environment. A large number of proteins dedicated to adhesion, motility, or chemotaxis were also found in most genomes (Table I). Most of the proteins belonging to this functional category were involved in the flagellar motility system. The genes coding for flagella biosynthesis (i.e. basal body, flagellar hook, or the main flagellin protein) were generally found grouped. Most strains have one large genomic cluster comprising 10 to 27 genes and one or two other smaller clusters scattered over the genome and comp-
prising 2 to 7 genes. This was not the case for *P. bermudensis* HTCC2601 and *O. granulosus* HTCC2516, two isolates from 10 meter-deep Bermuda seawater that have two large clusters with flagellar functions comprising 27–21 and 26–20 genes, respectively. The exception to this general rule was *R. nubinhibens* ISM, isolated from Caribbean surface water, which comprises only three polypeptides involved in the flagellum complex and encoded by genes scattered onto the genome as previously reported (45). We confirmed this result by checking the nucleic acid sequence of the genome of this strain for other possible genes missed during annotation (tnt-blast, E-value $10^{-20}$).

**RTX-like Proteins**—Another important characteristic of the exoproteomes of *Roseobacters* is the abundance of RTX proteins (Table I). The presence of the known repeated motif (L/I/F)GxG(N/D)D (28) is a remarkable feature in these multidomain proteins (Fig. 1). This repeated motif is related to the conserved serralysin peptidase domain. Although this domain is generally found at the C terminus of the protein (28), this is not the case in RTX proteins of *Roseobacter* clade strains where the serralysin peptidase domain can be located in diverse positions within the polypeptide and can be also found in several copies. This serralysin peptidase domain binds Ca$^{2+}$ and has a plausible corkscrew function through cell walls. The other domains found in these proteins were diverse. The N-terminal Zn-metalloprotease domain found in some of these proteins may contribute to their putative pathogenic function (46). This specific domain is illustrated only once in the examples shown (Fig. 1) but was abundantly found. Apart from Zn-metalloproteases, we observed a large diversity of adhesion-related domains, comprising cadherin, integrin, and fasciclin-like domains (supplemental Table S2). Other domains like carboxypeptidase, invasion, or nuclease-like factors could also be associated with the serralysin peptidase domain as well as large amino acid sequences with no known conserved domains and without a known function. It is important to highlight the extremely high diversity and low degree of similarity among the RTX polypeptides found in the

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**Fig. 1.** Multidomain structure of representative RTX polypeptides from 12 *Roseobacter* clade isolates. Boxes represent structural domains predicted by the CDD algorithm (NCBI) after PSI-BLAST analysis. An example of each RTX-toxin detected in each of the 12 bacteria analyzed in this study is represented. Stand-alone RTX polypeptides with only the typical serralysin peptidase domain are not indicated although frequently found. Numbers on the schematic structure represent amino acid lengths. The NCBI references of each protein are indicated as well as the corresponding bacterial strains.
Roseobacter strains. Several RTX proteins present an intein structure in their sequence. Inteins are specific peptide sequences with the ability to auto-excite from the polypeptide after translation, being the flanking exteins rejoined by a peptide bond (47). The RTX proteins found in the Roseobacter clade strains were generally large, as exemplified by YP_167926 (830 kDa) of R. pomeroyi DSS-3 and ZP_01155761 (1,132 kDa) of O. granulosus HTCC2516. Furthermore, it is interesting to note that all identified RTX proteins presented a nonclassical secretion system predicted by SecretomeP 2.0. The common type I secretion export system typically seen in the RTX exoproteins (28) is among these nonclassical secretion systems as an N-terminal signal peptide is not required.

Comparative Proteomics of Exoproteomes of 11 Roseobacter Clade Strains Grown in Marine Broth Medium—Whether the listed predicted proteins were secreted by roseobacters and in which conditions were further investigated. We analyzed the exoproteome of the 11 different Roseobacter clade strains cited here above as previously performed for R. pomeroyi DSS-3 (13). The patterns of secreted proteins strongly differed among the strains (Fig. 2A). In any case, no strain presented a protein as abundant as PaxA found in the exoproteome of R. pomeroyi DSS-3 (Fig. 2, first lane in B). As previously reported, PaxA represented over 50% of the total exoproteome (13). We identified the most abundant proteins present in the exoproteomes of the 11 Roseobacter clade strains by a shotgun proteomic LC-MS/MS approach and estimated their relative quantities by spectral count. We validated on average 98 different proteins per exoproteome (supplemental Table S3), with mean sequence coverage of 23%. As expected from the SDS-PAGE shown in Fig. 2, the most diverse list of proteins was obtained for O. granulosus HTCC2516 (251 proteins) whereas the less diverse was found for R. nubinhibens ISM (24 proteins). We first compared our experimental list with the theoretical list of exported proteins established before. In most cases we found only a limited number of predicted cytosolic proteins in the exoproteome samples. However, for three strains (namely O. granulosus HTCC2516, R. lacuscaerulensis ITI1157, and Roseobacter sp. MED193), a higher number of predicted cytosolic proteins was found as expected by their exoproteome SDS-PAGE pattern (Fig. 2A). As the same precautions were taken for all strains and calculated maximal doubling times indicated no plausible growth stress because of the medium used (Fig. 3), we assumed these strains were simply more easily lysed than the other strains in our experimental set-up. Finally, only polypeptides both predicted secreted and experimentally detected were taken into consideration for the following comparative proteomic analyses.

Transport Proteins—Fig. 3 shows the ratio of MS/MS identified proteins per functional category. Extracellular components of transport systems were among the most abundant polypeptides detected in exoproteome samples in most
Roseobacters’ Exoproteomes

![Graph showing the relative abundance of MS/MS detected polypeptides in different Roseobacter clade exoproteomes in terms of functional categories.](image)

**Fig. 3.** Relative abundance of MS/MS detected polypeptides in the different Roseobacter clade exoproteomes in terms of functional categories. The percentages of the different categories are graphed by stacked bars. Relative numbers of the different polypeptides, spectral counts and protein abundance normalized by the corresponding molecular weight are labeled for each strain CDS, SC, and NSAF, respectively. Location and origin of the initial sampling for the bacterium isolation are indicated (see www.roseobase.org for additional details). Abbreviations used are: Med. sea for Mediterranean sea, Dinofl. for Dinoflagellate, and 10m for isolations done from 10-meter deep seawater. Maximal doubling times (D.T.) established in marine broth in this study are indicated in hours. Trophic strategies assigned to each Roseobacter clade isolate are indicated by A, B, C, and D indicating a large production of nutrient transporters, mobility proteins, adhesion-like proteins, and toxin secretion, respectively.

Cases. Integral membrane proteins were also detected in the milieu. Although the diversity of detected transport proteins was high in all cases (from 19% of total detected polypeptides in *R. litoralis* OCh149 to 60% in *S. stellata* E-37), we observed that their accumulation differed in terms of quantities. For example, porins and transporter systems such as ABC or TRAP related polypeptides represented ~70% of the total MS/MS spectra detected in exoproteomes from *R. lacuscaerulensis* IT1157, *Roseobacter* sp. MED193 and *S. stellata* E-37, whereas only ~10% for *O. batsensis* HTCC2597 and *R. litoralis* OCh149. The trophic strategy of secreting a higher abundance of transporter systems can suppose an advantage in rich nutrient milieu as seen by the fast growth values of the three former strains (Fig. 3). However, this was not the case for *O. indoliflex* HEL45.

**Adhesion and Motility Proteins**—Adhesion and motility proteins belong to an abundant protein fraction found in the exoproteomes of most Roseobacters. Although these proteins are usually anchored to the bacterial membrane, their presence in the milieu could be explained by their fragility. When shaking the culture or removing the cells by filtration or centrifugation, they can be too easily broken into small pieces and contribute to the exoproteome. Notably, the flagellin and the flagellar system proteins in *O. indoliflex* HEL45, *O. batsensis* HTCC2597, and *P. bermudensis* HTCC2601 exoproteomes were abundantly detected in terms of spectral counts as seen in supplemental Table S3 with 42, 27, and 22% of total MS/MS spectra, respectively. The number of different polypeptides dedicated to adhesion functions was generally lower than those seen for motility, although they were also highly present in terms of spectral counts, and thus abundant. *O. batsensis* HTCC2597 and *D. shibae* DFL12 showed elevated ratios of MS/MS detection for adhesion functions (36 and 37%, respectively) mainly because of the presence of the cadherin-like proteins ZP_00998260 and YP_001534926, which represented 22 and 28% of the total MS/MS spectra, respectively, in each strain. Cadherin-like proteins seemed to prevail over other adhesion systems such as hemagglutinin,
curlin, or pilus-like adhesion fimbria. Interestingly, we noted in *R. lacuscaerulensis* ITI1157, *Roseobacter* sp. MED193, and *S. stellata* E-37 an anticorrelation between transporter-like proteins on one hand, and adhesion and motility proteins on the other (Fig. 3).

Toxin-like Proteins—Proteins annotated with a putative toxin-like function were found in all exoproteomes except *R. lacuscaerulensis* ITI1157 and *Roseobacter* sp. MED193 but their global abundances were highly diverging. As mentioned above, the diversity of sequences can be indicative of an elevated diversity of functions or cell targets. These proteins generally represented a large ratio of the MS/MS detected peptides with a range from 6% to 45% for *S. stellata* E-37 and *R. denitrficans* OCh114, respectively (supplemental Table S3). Remarkably, two strains, namely *R. lacuscaerulensis* ITI1157 and *Roseobacter* sp MED193, presented for such polypeptides a drastically lower number of spectral counts, 0.2 and 1.3% respectively. Curiously, among the three toxin-like proteins validated in these two strains, two presented an invasion associated locus B (IalB) domain, namely ZP_05786225 and ZP_01058515, showed similarities to hemolysin-like annotated proteins. Despite this, we verified that no RTX motif or serralysin peptidase domain was present in any of the three sequences. In the other nine *Roseobacter* clade strains, we only found nine IalB-similar proteins (non-RTX), whereas all other 45 toxin-like proteins detected presented RTX characteristics. Some of these RTX proteins were detected with a high number of spectral counts, e.g. ZP_01441112 of *P. bermudensis* HTCC2601, ZP_02155013 of *O. indolifex* HEL45, or ZP_00999898 from *O. batsensis* HTCC2597, was well covered (12%) along the whole sequence from residue 178 to residue 11715 with 77 distinct peptides (supplemental Table S3).

The abundance of the different protein categories revealed the plausible trophic strategy used by each strain in order to uptake nutrients or to compete with the community in its environment. Four strategies were established according to the proteomic data: production of transporters for the uptake of nutrients, synthesis of flagellum structures to become mobile in the milieu, secretion of adhesion proteins to form plausible symbiosis or convert to a sessile life mode, and export of toxins in order to avoid community competition (Fig. 3). Threshold for classification was defined when one category represented over 40% of spectral counts. Lower percentages were accepted as main categories (i.e. 35% for *R. nubinhibens* ISM and *D. shibae* DFL12) only if other categories were much lower represented. In other cases a combination of the two highest protein groups detected were considered.

### Table II

| Culture conditions | Cytosolic proteins | Transport$^a$ | Motility/Adhesion$^a$ | Toxic-like$^{a,b}$ | Others$^a$ |
|--------------------|--------------------|---------------|----------------------|-------------------|-----------|
| MB exponential phase | 19%                | 13.6%         | 0.2%                 | 82.1% (67.2)      | 4.1%      |
| MB stationary phase | 36%                | 24.8%         | 4.6%                 | 53.3% (45.4)      | 17.3%     |
| MB:LB exponential phase | 33%            | 13.4%         | 1.1%                 | 79.2% (64.9)      | 6.3%      |
| MMM succinate | 2%                 | 8.2%          | 0.5%                 | 90.7% (82.6)      | 0.6%      |
| Autoclaved SW succinate | 2%             | 10.1%         | 0%                   | 88.8% (80.7)      | 1.1%      |

$^a$ Ratios based on MS/MS spectral counts.

$^b$ In brackets is the percentage of the RTX-like protein PaxA.

The result of a large intein removal. Another large protein (1307 kDa), namely ZP_00999898 from *O. batsensis* HTCC2597, was well covered (12%) along the whole sequence from residue 178 to residue 11715 with 77 distinct peptides (supplemental Table S3).

Comparative Proteomics of *R. pomeroyi* DSS-3 Exoproteome Under Different Culture Conditions—Five different culture conditions were assayed (Fig. 2B) in order to determine how the cellular physiology could influence the exoproteome of a *Roseobacter* clade representative, e.g. *R. pomeroyi* DSS-3, and if this had an effect of its trophic distinctness, according to the categories made above. These variations were clearly established by nanoLC-MS/MS analysis through our label-free semiquantitative proteomic approach (data compiled in supplemental Table S4). We assessed whether if the stressing nature of the different culture media could be directly linked to the ratio of predicted cytosolic proteins in terms of spectral counts. As shown in Table II, this ratio increased from 2% (poor-nutrient MMM medium or autoclaved sea water supplemented with succinate) and 19% (MB exponential phase), until 33% (nonoptimal broth made with LB and MB mixed 1:1), and 36% (MB stationary phase condition). Interestingly, *R. pomeroyi* DSS-3 gave a much higher cytosolic spill into the milieu when it was grown in rich broth (doubling time ~2 h when calculated from growth curves in MB) than when it was grown in a poor media with much slower doubling times (~4 h when calculated from growth curves in MMM supplemented with succinate). Therefore,
cells growing faster appeared as less resilient than cells cultivated in conditions mimicking their natural environment where cell leakage was plausibly reduced because of starvation.

We found a high ratio of toxin-like proteins in all conditions (Table II). The main contributor to this ratio was PaxA as clearly evidenced in Fig. 2B. The exoproteomes of exponentially grown cells prepared in rich broths (MB and MB:LB) showed similar category ratios in terms of abundance. For cells entering in stationary phase, we observed a significant decrease in the ratio of toxin-like proteins. This was directly correlated with the higher presence of transporters, motility proteins, and other proteins with putative functions related to stationary phase adaptation. We found that the highest relative toxin production (~90% of the spectral counts) was detected when _R. pomeroyi_ DSS-3 was grown in a poor culture medium, _i.e._ MMM or autoclaved seawater supplemented with succinate. We also noted that only RTX-like toxins contribute to the toxin fraction of the exoproteomes of _R. pomeroyi_ DSS-3. Fig. 4 shows the genomic context and the degree of conservation among other Roseobacters of the 14 RTX-like proteins found in _R. pomeroyi_ DSS-3 genome. Only six of these proteins were detected by MS/MS in our experiment. We found that all six were detected with relatively small variations between the different culture conditions, resulting in a similar ranking. Beside the preponderant PaxA protein (YP_165496), YP_165625 and YP_168868 were in significant amounts whereas the three others were accounting only for low ratios. In Fig. 4, the similarity that each RTX toxin of _R. pomeroyi_ DSS-3 presented with its closest Roseobacter clade counterpart is shown in terms of blast E-values. In most cases, similarities were low as homologous regions were restricted to the characteristic GGxGxD repetitive motives in the serralysin peptidase domain, _e.g._ the PaxA protein. As seen in Fig. 1, neighboring domains have few or no similarity leading to a large heterogeneity of toxins coded in the Roseobacter clade. Despite, half of the RTX toxins encoded in _R. pomeroyi_ DSS-3 genome were somehow conserved in all the Roseobacter clade isolates considered here (e.g._ YP_168868), or at least in a restricted number of strains (e.g. YP_167926). Experimentally, we only detected the YP_168868 homologs in two Roseobacter clade exoproteomes: _O. batsensis_.
HTCC2597 and P. bermudensis HTCC2601. The diversity of genes flanking the RTX coding regions in the genome of R. pomeroyi DSS-3 revealed a nonconserved structural organization (Fig. 4). This differs to what has been stated for this kind of protein (28), where a conserved cluster containing genes coding for auxiliary proteins for toxin export or post-translational modifications is found. In this case, only three of the RTX genes had a plausible transporter gene coded in the close neighborhood (Fig. 4). Plausible transcriptional regulators or genes coding for enzymes involved in protein modification were also observed although not generalized. Interestingly, genes coding for transposition functions were coded near three RTX genes with being two of them (YP_168868 and YP_167926) detected in the proteomic survey. This could indicate a horizontal transfer acquisition for these proteins. Such acquisition is of importance as the products are abundant.

R. pomeroyi DSS-3 Exoproteome in Conditions Mimicking Natural Seawater—The exoproteome produced by R. pomeroyi DSS-3 under mimicked natural conditions was analyzed in order to determine the influence caused by either the natural community (presence and absence of the natural indigenous microbial community) or the water sampling area (port and open seawater) on the secreted proteins of this bacterium (MS/MS data compiled in supplemental Table S5). Blank samples (seawater without R. pomeroyi DSS-3 inoculum) gave no false positive identifications when the R. pomeroyi DSS-3 sequence database was used. Apparently, the incubation in seawater supplied a stress for R. pomeroyi DSS-3 as almost 50% of the detected peptides from the exoproteome belong to cytosolic proteins. This ratio was seen in all natural conditions independently of the seawater origin or the presence of the natural community. In these conditions we also found an elevated abundance of transporters (~37% in cape and ~50% in marina seawater) and motility related proteins (~14% in cape and ~6% in marina seawater) in the exoproteomes. These ratios were higher than those observed in the laboratory stationary phase condition (Table II). Although nutrient levels were not measured, this would agree with a poor-nutrient condition because of the typical oligotrophy seen in seawater samples. Again, RTX toxins were found among the most abundant proteins detected by MS/MS although with a much lower global abundance ratio (10–17% in all conditions except for the marina samples in the presence of the natural community). PaxA (YP_165496), abundantly detected in laboratory conditions, was found in much lower abundance in these samples (~3%). Such difference could be directly related to the physiological state of the cells and the available energy source. The YP_165625 polypeptide was also less detected than previous conditions tested (~3%). On the other hand, the 830-kDa YP_167926 polypeptide increased its relative abundance up to over 11% of total spectral counts in marina seawater in absence of the microbial community. Surprisingly, these three RTX-proteins were abundantly detected in all conditions except when the marina community was present. Furthermore, no MS/MS spectra could be assigned to any of the 14 RTX-like proteins from R. pomeroyi in this condition (marina seawater with the natural community, supplemental Table S5). Supplemental Table S6 shows the statistical analysis carried out to determine protein variations because of the natural microbial community on the first hand, and to the seawater sampling area on the other. The spectral count-based folds and statistical significance for RTX toxins between the different conditions are presented in Table III. The microbial community present in the marina seemed to have a strong effect on the depletion of these proteins. The direct interaction of these proteins with members of the microbial community and further sample filtration to remove the cellular content of the water could explain such depletion. Alternatively, the presence of an abundant microbial community in these samples could inhibit the synthesis of such proteins in R. pomeroyi DSS-3 or accelerate their degradation. On the other hand, the cape community from open seawater, probably in much less number, showed no influence on the abundance of these toxins. Furthermore, when incubated in marina seawater in absence of the natural community, R. pomeroyi DSS-3 slightly increased the secretion of these proteins when compared with the open cape seawater conditions (2–3×, Table III). Whether this slight increase is due to a higher nutrient concentration found in ports or any released material from the community is an open question. Interestingly, the secreted microcystin-dependent protein (YP_165745) of R. pomeroyi DSS-3 with a putative function in pathogenicity or plant-microbe interaction also exhibited a different pattern of detection. As shown in supplemental Table S6, its detection in marine systems sig-

| Community influence | Sampling area influence |
|----------------------|------------------------|
| **Cape Non vs Com.** | **Marina Non vs Com.** |
| YP_165496            | ~                      |
| YP_165625            | 11× (~0.01)            |
| YP_167926            | 13× (~0.01)            |
| YP_165496            | ~                      |
| YP_165625            | 13× (~0.01)            |
| YP_167926            | 46× (~0.01)            |
| YP_165496            | ~                      |
| YP_165625            | 11× (~0.01)            |
| YP_167926            | 2× (~0.01)             |
| YP_165496            | ~                      |
| YP_165625            | 9× (~0.01)             |
| YP_167926            | 7× (~0.02)             |

*~* indicates folds comprised between −2 and 2 and with low statistical significance (p value > 0.05)
compared with the open-cape community seawater samples.

DISCUSSION

Trophic strategies for marine bacteria have been discussed at the molecular level with the analysis of numerous genomes of copiotrophic or oligotrophic organisms (49). Ecological lifestyles have also been discussed for micro-organisms within the generalist Roseobacter clade (50). Nevertheless these strategies have not been discussed in the light of experimental proteomic data until now. Analysis of bacterial exoproteomes is able to reveal the proteins secreted by cells in order to interact with their environment and biotic community. In the present study, we have shown how the exoproteins secreted by an organism can give a clear picture of the physiological state of cells and, furthermore, the trophic strategy they used to face changes of their environmental conditions. Using comparative genomics, we listed the theoretical arsenal the marine Roseobacter clade strains may use to live in their environment. Our proteomic experimental results gave a much more precise view on the ecology of the organisms as we pointed out which proteins from this arsenal were likely to be more important. It was also possible to catalog and quantify the proteome dynamics cells have developed to adapt to variations in their environment as we have shown by incubating R. pomeroyi DSS-3 in different natural seawater conditions. Based on our comparative proteomic analysis (Fig. 3), we could define up to four tropic strategies followed by members of the Roseobacter clade: (1) synthesis of a large number of transporters in order to uptake nutrients present in the milieu, e.g. S. stellata E-37; (2) adoption of a mobile mode to search for nutrients or more adequate environmental niches, e.g. O. batsensis HTCC2597; (3) export adhesion-like proteins as a mechanism to favor symbioses or surface colonization, e.g. D. shibae DFL12; and/or (4) secrete toxin-like compounds to take advantage of other members of the community, e.g. R. pomeroyi DSS-3. We noted that genomic data and proteomic evidences were not systematically correlated. As an example, R. lacuscaerulensis ITI1157 showed a small variety of genes coding for transporters in its genome leading to the conclusion that these transporters may not be of importance for its adaptation to the environment. However, this bacterium largely takes profit from its small transporter repertory as transporter-like proteins were abundantly detected in its exoproteome in our assays, representing an almost exclusive item in its exoproteome. The plausible lifestyle of a given microorganism deduced from inspection of its exoproteome matched relatively well with the bacterial sampling origin. For example, R. littoralis OCh149 and D. shibae DFL12 were isolated from seaweed and dinoflagellate, respectively. Both microorganisms showed an abundant secretion of adhesion exoproteins that could be involved in favoring possible symbioses.

Although the marine Roseobacter lineage has proven a robust clade, phylogeny within the group was not yet clearly resolved (15). Newton and coworkers conclude from their genomic analysis of the clade that ecological lifestyle, and not phylogeny, could be an appropriate method to collapse the Roseobacter members into a smaller number of groups and, by this way, predict their genome content (50). Based on our proteomic data we confirmed that phylogeny does not necessarily determine the ecological strategy used by a group of closely related strains. R. pomeroyi DSS-3 and R. lacuscaerulensis ITI1157, both belonging to the genus Ruegeria, showed a totally different exoprotein secretion strategy. Although R. pomeroyi DSS-3 secreted large amounts of toxin-related proteins, R. lacuscaerulensis ITI1157 limited its secretion to transport-related polypeptides. Speciation of Roseobacters is probably enhanced by the ecological niche they are based in and the distinctness this confers to the bacterial lifestyle.

Apart from the tropic strategy, exoproteomes could also give hints on the physiological state of the cells. Adverse culture conditions derived in increased levels of cellular lysis. As a consequence, cytosolic spill into the milieu could be detected. This was the case for R. pomeroyi DSS-3 cells in the stationary phase or grown in nonoptimal broth conditions. In this case, over 30% of cytosolic polypeptides were identified in the exoproteomes (Table II). This cytosolic spill was also noted in simulated natural conditions in which R. pomeroyi DSS-3 cells grown in rich media were incubated in seawater with a poor content of nutrients. In this case, the modification of conditions that trigger drastic adaptive cellular changes led to an exoproteome with ~50% of cytosolic polypeptides. We have also shown that cells grown in rich media and thus with fast growth are subjected to a much higher cytosolic spill than cells grown in poor media (19% versus 2%). This is also observed for R. lacuscaerulensis ITI1157, which showed an elevated cytosolic content in its exoproteomes, whereas this strain exhibit one of the fastest doubling times in MB medium. The exoproteome of R. pomeroyi DSS-3 showed an adaptation to the stationary phase, being an increase of the relative abundance of transport and motility polypeptides clearly detected. These variations indicated that in these conditions cells adapted by searching remaining nutrients in the milieu.

Several Roseobacter clade strains are able to secrete antimicrobial compounds, i.e. TDA (17) or tryptanthrin (18). Their genome analysis also revealed the presence of type IV virulence factors widely spread among the clade (26). These factors are thought to confer Roseobacter clade strains inhibitory capabilities directed against other prokaryotes, preventing algae blooms or converting them into aggressive colonizers (20, 21, 23–25). We were the first in describing R. pomeroyi DSS-3 as an important RTX-toxin secretor (13). We assume these proteins are rapidly secreted by their extremely low detection within the bacterial cytosolic proteome (13, 32). As seen in the data presented here, secretion of such RTX-toxins seems a common feature in the whole clade. These polypeptides, secreted by type I secretion systems, comprise
repeated conserved motives for binding Ca\(^{2+}\) (28). The encoded RTX genes in the Roseobacter clade strains comprise a variable number of such repeated motives in serralysin peptidase-like domains. Additional accompanying conserved domains in these polypeptides are highly diverse in terms of nature, size, and structure. However, Zn-metalloproteases or a large diversity of adhesion-like domains seemed to be most predominant (Fig. 1). This could define the specificity of these toxins and thus the ecological traits of the different members of the community. The mechanisms of action of these RTX toxins are still largely unknown and definitively deserve further investigation. In conditions mimicking natural seawater conditions, we observed an almost complete depletion of all RTX toxins commonly secreted by \textit{R. pomeroyi} only when the marina community was present (Table III). Further experiments are now required to establish whether this depletion is because of secretion inhibition or toxin interaction with targeted cells and, hence, removed from the exoproteome fraction. RTX degradation is probably not responsible for this depletion as we found a normal ratio of all other exoproteome components of \textit{R. pomeroyi} DSS-3.

As presented in the introduction, environmental exoproteomes are largely unknown although they represent a key parameter to get insights into how marine microorganisms interact in association or competition in their natural environment. The present study is the largest reported analysis of marine exoproteomes with data obtained from 12 Roseobacter clade representatives. Our shotgun proteomic approach allowed an extensive description of the toolbox actually used by these Roseobacter clade isolates. Such approaches will probably foster novel investigations to understand marine systems and community interactions in their whole complexity. Different ecological trophic strategies have been defined from these proteomic data highlighting a common secretion of RTX polypeptides among the different clade members. How these toxins function, whether they represent an advantage for Roseobacter adaptation to their marine environment and in which case, are now important questions to be answered.

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This article contains supplemental Tables S1 to S6.

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