Genetic analyses unravel the crucial role of a horizontally acquired alginate lyase for brown algal biomass degradation by Z obellia galactanivorans

Yongtao Zhu, François Thomas, Robert Larocque, Nan Li, Delphine Duffieux, Lionel Cladière, Florent Souchaud, Gurvan Michel, Mark J. Mcbride

Genetic analyses unravel the crucial role of a horizontally acquired alginate lyase for brown algal biomass degradation by *Zobellia galactanivorans*

Yongtao Zhu[1#], François Thomas[2#], Robert Larocque[2], Nan Li[3], Delphine Duffieux[2], Lionel Cladière[2], Florent Souchaud[2], Gurvan Michel[2]*, and Mark J. McBride[1]*.

Author affiliations:

[1]Department of Biological Sciences, University of Wisconsin-Milwaukee, P. O. Box 413, Milwaukee, WI 53201

[2] Sorbonne Université, UPMC, Centre National de la Recherche Scientifique, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France

[3] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province 430072, China

*Corresponding authors

#These authors contributed equally to this work

Corresponding Author

Mark J. McBride

Department of Biological Sciences

University of Wisconsin-Milwaukee

3209 N. Maryland Ave

181 Lapham Hall

Milwaukee, WI USA

53211

email:mcbride@uwm.edu

Co-corresponding Author:

Gurvan MICHEL

Station Biologique de Roscoff

Place Georges Teissier

29682 Roscoff

Brittany, FRANCE

e-mail: gurvan.michel@sb-roscoff.fr

Running title: *Zobellia galactanivorans* alginate degradation

Originality-Significance Statement

Zobellia galactanivorans belongs to the phylum *Bacteroidetes,* whose members are important in polymer digestion in many environments. *Z. galactanivorans* efficiently digests marine algal polysaccharides. Gene deletion and complementation techniques were developed and used to demonstrate the role of alginate lyase AlyA1 in digestion of algal cell walls and alginate gels. The genetic techniques described here function in diverse members of the phylum and allow genetic dissection of the complex polymer digestion strategies employed by these bacteria.

Summary

Comprehension of the degradation of macroalgal polysaccharides suffers from the lack of genetic tools for model marine bacteria, despite their importance for coastal ecosystem functions. We developed such tools for *Zobellia galactanivorans*, an algae-associated flavobacterium that digests many polysaccharides, including alginate. These tools were used to investigate the biological role of AlyA1, the only *Z. galactanivorans* alginate lyase known to be secreted in soluble form and to have a recognizable carbohydrate-binding domain. A deletion mutant, Δ*alyA1*, grew as well as the wild type on soluble alginate but was deficient in soluble secreted alginate lyase activity and in digestion of and growth on alginate gels and algal tissues. Thus, AlyA1 appears to be essential for optimal attack of alginate in intact cell walls. *alyA1* appears to have been recently acquired via horizontal transfer from marine *Actinobacteria,* conferring an adaptive advantage that might benefit other algae-associated bacteria by exposing new substrate niches. The genetic tools described here function in diverse members of the phylum *Bacteroidetes* and should facilitate analyses of polysaccharide degradation systems and many other processes in these common but understudied bacteria.

Introduction

Marine macroalgae are abundant in many coastal environments. The worldwide standing stock of macroalgae was estimated around 280 million tons (Carpenter and Liss, 2000). A large fraction of this biomass is composed of cell wall polysaccharides, which constitute an abundant nutrient source for heterotrophic bacteria. Marine algal polysaccharides contain unique sugars and are often heavily sulfated, differing dramatically from terrestrial plant polysaccharides. Examples of marine algal polysaccharides include the agars, porphyrans, and carrageenans of red algae, the alginates and fucose-containing sulfated polysaccharides of brown algae, and the ulvans, galactans, and arabinogalactans of green algae (Popper et al., 2011; Ficko-Blean et al., 2015). Although bacterial turnover of terrestrial plant cell wall polysaccharides has been extensively studied (Cragg et al., 2015) digestion of marine algal polysaccharides is less well understood (Hehemann et al., 2014; Martin et al., 2014).

Alginates are major cell wall polysaccharides of brown algae (Mabeau and Kloareg, 1987) and can account for up to 45% of the dry weight. Alginates are anionic polymers of β-D-mannuronate (M) and α-L-guluronate (G), arranged in homopolymeric (polyM, polyG) or heteropolymeric (polyMG) blocks. Chelation of divalent metal ions by polyG blocks (but not polyM and polyMG) allows the formation of strong gels of interconnected alginate molecules. In brown algae, G and M content is controlled by mannuronan C-5 epimerases (Fischl et al., 2016; Inoue et al., 2016) and varies depending on species, life cycle stage, tissue, and season, resulting in alginates with a variety of rheological properties (Haug et al., 1974). Alginates can also be cross-linked by phenols in cell walls and are part of a complex network including fucose-containing sulfated polysaccharides, cellulose and proteins (Deniaud-Bouet et al., 2014). Because of this, marine bacteria may need complex systems to

degrade alginate in brown algal biomass. Recent studies on natural marine communities have shown that alginolytic potential is widespread among the *Bacteroidetes*, *Proteobacteria*, and other poorly characterized taxa (Wietz et al., 2015; Matos et al., 2016; Mitulla et al., 2016). Polysaccharide-utilizing bacteria from the phylum *Bacteroidetes* are often associated with marine algae and are thought to be important for the turnover of algal polysaccharides in nature (Kirchman, 2002; Williams et al., 2013).

Zobellia strains are marine *Bacteroidetes* commonly associated with red, brown, and green algae and are considered a normal component of the microbiota of healthy macroalgae (Hollants et al., 2013; Martin et al., 2015; Marzinelli et al., 2015). *Zobellia galactanivorans* was originally isolated from the red alga *Delesseria sanguinea* (Barbeyron et al., 2001) and exhibits impressive abilities to digest diverse algal polysaccharides (Barbeyron et al., 2016). Analysis of the *Z. galactanivorans* genome revealed 141 glycoside hydrolases (GHs), 15 polysaccharide lyases (PLs), 18 carbohydrate esterases and 72 sulfatases. Because of its degradative abilities, *Z. galactanivorans* has emerged as a model system for studies of the digestion of marine algal polysaccharides. Biochemical analyses have begun to reveal its novel enzymes and pathways involved in degradation of agars, carrageenans, porphyrans, and alginates (Thomas et al., 2012; Thomas et al., 2013; Hehemann et al., 2014).

Z. galactanivorans possesses a complex alginolytic system comprising seven alginate lyases (AlyA1-AlyA7) and at least five additional enzymes that are thought to act on the products released by the alginate lyases (Thomas et al., 2012). Gene expression of the alginolytic system is not constitutive but is induced by the presence of alginate. The only exception is AlyA7, which appears to be expressed constitutively and therefore speculatively acts as a sentinel enzyme to produce the first oligosaccharides from alginate (Thomas et al., 2012). The large number of alginate lyases from four PL families raises questions regarding potential redundancy or synergy in digestion of different forms of algal cell wall alginate. To

date, only AlyA1 and AlyA5 have been extensively characterized. AlyA1 is an endolytic guluronate lyase, whereas AlyA5 cleaves monomers from the non-reducing end of oligo-alginates in an exolytic fashion (Thomas et al., 2013). AlyA1 is the only alginate lyase of *Z. galactanivorans* that is known to be secreted in soluble form, and the only one with a recognizable carbohydrate-binding module (CBM). These distinctive features of AlyA1 suggest that it may be uniquely important for *Z. galactanivorans* alginate utilization. Surprisingly, the encoding gene, *alyA1,* is not located near other obvious alginate utilization genes in the *Z. galactanivorans* genome. This contrasts with *alyA2*, *alyA3*, *alyA4*, *alyA5*, and *alyA6*, which reside in two polysaccharide utilization loci (PULs) that are predicted to be dedicated to alginate degradation. Since most well-studied polysaccharide utilization genes of members of the *Bacteroidetes* reside in PULs (Martens et al., 2009; Terrapon et al., 2015) the exact biological role of *alyA1* in alginate utilization required investigation.

The development of tools to genetically manipulate *Z. galactanivorans* would allow determination of the roles of individual enzymes in alginate utilization. It would also facilitate the discovery and analysis of novel proteins involved in utilization of other algal polysaccharides. Plasmids, transposons, antibiotic resistance markers, and other genetic elements that function in Gram-negative proteobacteria or in Gram-positive bacteria generally fail to function in members of the *Bacteroidetes* (McBride and Kempf, 1996). However, genetic tools for some members of this phylum have been developed, primarily based on transposons, plasmids, and selectable markers that are native to this group of bacteria (Salyers et al., 1987; McBride and Kempf, 1996; Alvarez et al., 2004; Braun et al., 2005; Belanger et al., 2007; Mally and Cornelis, 2008; Xu et al., 2012; Wang et al., 2014; Li et al., 2015; Zhu and McBride, 2016). Shuttle plasmids developed from these have antibiotic resistance and plasmid replication genes that function in *E. coli* but not in members of the *Bacteroidetes*, and they have another set of genes that function only in the *Bacteroidetes*. The

plasmids are usually transferred from *E. coli* to the target bacterium by conjugation. The ability of these tools to function in diverse members of the phylum has not been fully explored. Here we adapted and developed tools and techniques to allow genetic manipulation of *Z. galactanivorans* and other members of the phylum *Bacteroidetes.* We used these tools to demonstrate that *Z. galactanivorans* AlyA1 is important for digestion of alginate gels and brown algal tissues, a process that has potential biotechnological applications. *alyA1* appears to have been recently acquired by horizontal gene transfer from marine *Actinobacteria*, an event that has implications for the recycling of brown algal biomass in coastal ecosystems.

Results

Development of genetic techniques for Z. galactanivorans*, and deletion of the alginate lyase gene* alyA1

The transposons Tn*4351* and *HimarEm1* each function in several members of the *Bacteroidetes* (Shoemaker et al., 1986; Dyer et al., 1992; McBride and Baker, 1996; McBride and Kempf, 1996; Braun et al., 2005; Mally and Cornelis, 2008; Hu et al., 2012; Zhu and McBride, 2014, 2016). We examined the ability of these transposons to function in *Z. galactanivorans*. pEP4351 (Cooper et al., 1997) and pHimarEm1 (Braun et al., 2005) were introduced into *Z. galactanivorans* by conjugation and nine Tn*4351*-induced and eight *HimarEm1*-induced erythromycin resistant colonies were obtained. Integration of the transposons into different sites in the chromosome was verified by Southern blot analysis (Fig. S1). Each of the mutants appeared to have a single transposon insertion on the chromosome. The frequencies of transposition were low but the results indicate that DNA can be transferred from *E. coli* into *Z. galactanivorans* by conjugation and that Tn*4351* and

HimarEm1 transpose in and confer erythromycin resistance on this bacterium. We used this information to develop methods for site directed mutagenesis and integrative complementation as described below.

Techniques to construct gene deletions and other unmarked site-directed mutations in the chromosomes of several members of the *Bacteroidetes* have been developed (Pumbwe et al., 2006; Koropatkin et al., 2008; Rhodes et al., 2011a; Wang et al., 2014; Zhu and McBride, 2014; Li et al., 2015; Zhu and McBride, 2016). Most of these cannot be used on wild type cells but instead require strains that already have specific chromosomal mutations to allow counterselection. A method for site-directed mutagenesis of wild type cells was recently developed for the bacteroidete, *Flavobacterium columnare* (Li et al., 2015). This involves the *sacB* gene (Link et al., 1997) expressed from the *F. johnsoniae ompA* promoter (Chen et al., 2007), conferring sucrose sensitivity for counter-selection. We adapted this plasmid for use in *Z. galactanivorans* by introducing the *sacB* gene with *ompA* promoter into the *ermF*-containing suicide vector, pLYL03 (Li et al., 1995), generating pYT313 and pYT354 (Fig. 1, and Fig. S2). To test this gene deletion approach, we introduced 2.3 kbp regions upstream and downstream of the alginate lyase gene *alyA1* into pYT313 to generate pYT332. Transfer of pYT332 into *Z. galactanivorans* followed by selection for erythromycin resistance resulted in several strains carrying the plasmid integrated into the chromosome by homologous recombination either upstream or downstream of *alyA1* (Fig. 1). These cells were grown without antibiotic selection to allow plasmid loss by a second recombination event. They were then exposed to sucrose to select colonies that had lost the plasmid (Fig. 1). Plasmid loss can occur by recombination between the duplicated upstream or downstream regions, and this results in either gene deletion or wild type genotype depending on the site of the initial insertional recombination event. It is expected that approximately half of the sucrose resistant colonies will be deletion mutants. Of 24 sucrose-resistant colonies analyzed in our

first use of pYT313, nine were *alyA1* deletion mutants and the remaining fifteen were wild type for the *alyA1* locus.

Development of the integrative plasmid pYT356 for complementation analyses in Z. galactanivorans

Complementation of mutants such as Δ*alyA1* is important to determine if observed phenotypes are caused by the mutated gene or are the result of polar effects or unanticipated mutations elsewhere in the genome. Replicative plasmids are useful for complementation. Several cryptic *Bacteroidetes* plasmids have been used to construct replicative shuttle plasmids for complementation (Salyers et al., 1987; McBride and Kempf, 1996; Mally and Cornelis, 2008). We attempted to transfer pCP11, pNJR5, pFD340, pYT162, pYT172, and pMM105.A (Table S1) into *Z. galactanivorans* by conjugation. Each of these plasmids had origins of transfer for conjugation that were similar or identical to those in pHimarEm1 and pYT313, and each carried the *ermF* gene which as shown above confers erythromycin resistance in *Z. galactanivorans*, but no erythromycin resistant colonies were obtained (Table S1). The results suggest that the replicative functions of these plasmids are not suitable for *Z. galactanivorans*. As an alternative to replicative plasmid-based complementation we developed a non-replicating integrative plasmid for *Z. galactanivorans*. A region spanning ZGAL_4583 to ZGAL_4586 was chosen as a neutral site for gene integrations into the chromosome by homologous recombination. These loci lie within a larger region spanning ZGAL_4579 to ZGAL_4598 that was apparently inserted into the *comM* gene, ZGAL_4578/ZGAL_4599 (Fig. S3). This insertion appears to be recent since it was not found in the related bacteria *Zobellia uliginosa* MAR_2009_138 and *Cellulophaga baltica* NN016038, which instead have intact *comM* genes (Fig. S3). The region between

ZGAL_4579 to ZGAL_4598 includes genes encoding predicted site-specific DNA recombinases, a potential nucleic acid-binding protein, several predicted viral proteins, and many small hypothetical proteins of unknown function. The 2.1 kbp region spanning ZGAL_4583 to ZGAL_4586, and a 186 bp fragment spanning the *F. johnsoniae ompA* promoter and putative ribosome binding site, were cloned into pLYL03 to generate pYT356 (Fig. 2). Regions of DNA for complementation are inserted into the multiple cloning site of pYT356 and expressed using the *ompA* promoter. The plasmid is transferred into *Z. galactanivorans* by conjugation. pYT356 does not replicate in *Z. galactanivorans* but strains with the plasmid integrated into the chromosome at the neutral target site are selected by erythromycin resistance. As a test case the gene encoding superfolder GFP (sfGFP) (Uehara et al., 2009; Dinh and Bernhardt, 2011) was inserted into pYT356 and transferred into wild type cells. The plasmid recombined into the chromosome at the target site as verified by PCR and the resulting cells produced sfGFP and were fluorescent (Fig. S4). Wild type *alyA1* was also cloned into pYT356 and integrated into the chromosome to complement the Δ*alyA1* mutant (Fig. 2).

Deletion of alyA1 *results in partial defects in alginate utilization*

The genetic tools described above were used to determine if *alyA1* is critical for digestion of soluble or jellified alginate. Two types of alginate were used that had 33% and 66% G respectively. The growth rate of *Z. galactanivorans* wild type and Δ*alyA1* strains were compared in liquid cultures either containing tryptone and yeast extract or containing soluble sodium alginate as carbon sources (Table 1). Deletion of *alyA1* had no effect on the bacterial growth rate on media containing either of the types of soluble alginate or on media containing tryptone and yeast extract. Growth was fastest on media containing tryptone and

yeast extract, whereas it was reduced by 13% and 45% when bacteria used alginate containing 33% or 66% G, respectively. We quantified the alginate lyase activity secreted in soluble form by cultures grown with soluble alginate as sole carbon source (Fig. 3A). Activity released by wild type *Z. galactanivorans* was 6-fold higher when grown with 66% G alginate compared to 33% G alginate. With both types of alginate, deletion of *alyA1* reduced the secreted soluble alginate lyase activity approximately 10-fold. Consistent with this, the concentration of alginate degradation products was higher in spent culture fluid of wild type cells compared to Δ*alyA1* mutant cells (Fig. 3B, C). Degradation products accumulated in the culture fluid during the late log-phase of wild type cells (40-55 hours of culture), but did not accumulate in culture fluids from the Δ*alyA1* mutant. The accumulation of degradation products was more pronounced when cells used alginate containing 66% G (up to 1095 µM products accumulated) than when cells used alginate containing 33% G (maximum 766 µM products).

The addition of calcium to soluble alginate results in gel formation (Hecht and Srebnik, 2016), which partially mimics alginate in algal cell walls. Gel formation could impact the ability of some alginate lyases to access and digest the polysaccharide. Growth of *Z. galactanivorans* was tested on a mineral medium Ca-alginate gel with alginate as the sole carbon source. The wild type strain liquefied the alginate more rapidly than did the Δ*alyA1* mutant (Movie S1). Both the wild type and mutant strains exhibited spreading growth characteristic of gliding bacteria (Fig. 4A, inset). Colony expansion was about 50% slower for Δ*alyA1* compared to wild type on alginate gels containing either 33% or 66% G (Fig. 4). This was apparently not a result of a major defect in gliding motility for the Δ*alyA1* mutant, since both strains spread similarly on Cytophaga agar medium (Fig. S5). Ectopic integration of plasmid pYT359 (carrying wild-type *alyA1*) into the chromosome of the Δ*alyA1* mutant restored colony growth and alginate degradation to wild type levels (Fig. 4B). The

complemented strain exhibited a slight apparent growth delay compared to the wild type. This might be due to differences in *alyA1* expression levels since ectopic expression of *alyA1* was controlled by the *F. johnsoniae ompA* promoter and therefore was disconnected from the normal induction by alginate.

AlyA1 is important for growth on fresh algal biomass

To investigate the biological function of *alyA1* in the interaction between *Z. galactanivorans* and a brown alga we tested the effect of *alyA1* deletion on the degradation of fresh tissues of the kelp *Laminaria digitata* (Fig. 5). Pieces of algal blade or stipe in filter-sterilized seawater were inoculated with either *Z. galactanivorans* wild type or Δ*alyA1* mutant strains. The seawater was supplemented with kanamycin and streptomycin (to which *Z. galactanivorans* is resistant) to limit growth of other bacteria present on the algal tissues. The samples were examined after 8 d of incubation. Wild type *Z. galactanivorans* grew in seawater containing *L. digitata* tissues (Fig. 5A) whereas no growth was observed in controls lacking *L. digitata* tissues (data not shown). Growth of the Δ*alyA1* mutant on *L. digitata* was significantly less than growth of the wild type (ANOVA, Treatment effect: $F=103.3$, $P<0.001$; pairwise post-hoc Tukey test: all $P<0.01$). Larger amounts of unsaturated uronic acid degradation products accumulated in cultures containing blade or stipe tissues that were inoculated with the wild type strain compared to cultures inoculated with the Δ*alyA1* mutant (Fig. 5B). Macroscopic observations revealed that blade pieces that had been in contact with wild type *Z. galactanivorans* for 8 d were strongly damaged (Fig. 5C). Epidermal and meristodermal layers were detached and the tissues were soft and translucent. In contrast, tissues from the non-inoculated controls showed an intact external aspect, no detachment of cell layers, and normal pigmentation and mechanical resistance. Blade tissues from cultures inoculated with cells of the Δ*alyA1* mutant were slightly translucent but their external aspect

was otherwise similar to non-inoculated controls with preserved epidermal attachment and mechanical resistance. To further investigate the ability of wild type and Δ*alyA1* mutant cells to degrade algal tissues, stipes were recovered after 8 d of incubation and stained with Toluidine Blue O (TBO) which binds to negatively charged polysaccharides including alginate and sulfated fucans. Stipes from the non-inoculated control condition and from the Δ*alyA1* treatment showed typical algal tissue anatomy with dense cell wall material at cortical cell junctions (Fig. 5D). In contrast, the diffuse TBO staining in stipes inoculated with wild type *Z. galactanivorans* indicated loss of tissue integrity due to alginate degradation. To determine if these dramatic differences could partly be due to other alga-associated bacteria that resisted the antibiotic treatment, we analyzed the composition of bacterial communities after 8 days of growth with stipes (Fig. 6). The background community found in non-inoculated controls exhibited limited diversity (3 detected taxa, Shannon index 2.79) and the 16S rRNA amplicon library was largely dominated by sequences related to *Winogradskyella* (Fig. 6). Communities growing in cultures inoculated with *Z. galactanivorans* were more diverse (inoculated with wild type: 13 detected taxa, Shannon index 5.17; inoculated with Δ*alyA1*: 10 detected taxa, Shannon index 5.84). Sequences related to *Zobellia* were only detected from samples that had been inoculated with *Z. galactanivorans* strains. Blastn search against the NCBI 16S Microbial database confirmed that all sequences assigned to *Zobellia* had their best Blast hit with *Z. galactanivorans*. These sequences accounted respectively for 36% and 22% of the libraries from cultures inoculated with wild type or Δ*alyA1*, confirming the reduced ability of the mutant strain to utilize stipe biomass as a carbon source. Interestingly, sequences affiliated to 4 flavobacterial taxa were recovered from samples of algal biomass inoculated with the wild type strain but not from those inoculated with Δ*alyA1* or from non-inoculated controls. This suggests that the growth of these bacteria was favored by the degradation of algal biomass by *Z. galactanivorans* only

when a functional *alyA1* gene was present. Among these opportunistic taxa *Tenacibaculum* was particularly successful and contributed 23% of the 16S rRNA amplicon library from samples inoculated with wild type *Z. galactanivorans*.

alyA1 acquisition by horizontal gene transfer

The unique features of AlyA1 questioned its evolutionary history in the *Flavobacteriaceae* and suggested it might have emerged independently from the alginolytic PULs. To test this hypothesis, we searched for full-length homologs of AlyA1 (displaying a predicted PL7 catalytic module fused with a putative CBM32 module) in members of the *Flavobacteriaceae* that possessed a predicted alginolytic PUL (Table S2). Among 67 alginate-PUL-containing *Flavobacteriaceae*, only 7 strains contained a full-length homolog of AlyA1. In all but one case (*Aquimarina* sp. RZW4-3-2), the *alyA1* homolog was in a genomic region distant from the predicted alginate PUL(s). In contrast, we found homologs of the PL7 catalytic module of AlyA1 alone (without CBM32) in 34 alginate-PUL-containing *Flavobacteriaceae*. In general, these homologs were more closely related to *Z. galactanivorans* AlyA2, which only features a PL7 module and resides within an alginolytic PUL (Table S2). *Z. galactanivorans alyA1* is located in a region of the chromosome that is not dedicated to alginate degradation, but rather contains genes predicted to encode rod-shape determining proteins (*mreC*, *mreD*, *mrdA*, *mrdB*), an endonuclease, peptide methionine sulfoxide reductases (*msrA1*, *msrA2*, *msrB2*) and peptidases (Fig. S6). This region was highly conserved in other marine *Flavobacteriaceae*, except for the absence of *alyA1*. Altogether, this suggests that *alyA1* was recently acquired in *Z. galactanivorans* and in a few other alginate-PUL-containing members of the *Flavobacteriaceae*. We extended the search for full-length homologs of AlyA1 to the entire nonredundant NCBI database. This strategy retrieved

29 sequences (query cover >80%, e-value<10^{-6}), including the 7 *Bacteroidetes* proteins mentioned above, 2 additional proteins from *Bacteroidetes*, 18 proteins from *Actinobacteria* and 2 proteins from *Proteobacteria* (Table S3). Twenty-two of the thirty AlyA1 homologs (including *Z. galactanivorans* AlyA1) were from marine bacteria with the remainder contributed by terrestrial bacteria. Separate phylogenies of the PL7 and CBM32 modules showed globally congruent topologies (Fig. 7). In both cases, proteins from *Bacteroidetes* and *Proteobacteria* species emerged as sister groups to proteins from *Micromonospora* species, rooted by other actinobacterial proteins mostly from marine *Streptomyces* strains. This suggests that the recent acquisition of *alyA1* in a few marine members of the *Flavobacteriaceae* resulted from horizontal gene transfer (HGT) from marine *Actinobacteria*.

A genetic toolset for the Bacteroidetes

The genetic tools developed here for *Z. galactanivorans,* and those previously available for related bacteria, were analyzed for their ability to function in diverse members of the phylum *Bacteroidetes* (Table S1, Table S4). The transposons Tn*4351* (Shoemaker et al., 1985) and *HimarEm1* (Braun et al., 2005) were broadly useful across the entire phylum, including in members of the Classes *Flavobacteriia*, *Cytophagia*, *Sphingobacteriia*, and *Bacteroidia*. Tn*4351* functioned in each of eighteen species examined, and *HimarEm1* functioned in each of eight species examined. This included members of four genera (*Gramella, Robiginitalea, Chitinophaga,* and *Zobellia*) for which gene transfer of any type had not previously been demonstrated. In contrast, the replicative plasmids each exhibited more narrow host ranges. The lack of replicative plasmids complicates complementation experiments for some of these bacteria, including *Z. galactanivorans*. However, the chromosomal-integration approach described above allows complementation experiments to

be performed on these diverse bacteria. The *sacB* and *ermF*-containing suicide vector pYT313 is also likely to be generally useful throughout the phylum. To test this we determined the ability of pYT313 to facilitate targeted gene deletion in wild type cells of three distantly related members of the phylum *Bacteroidetes*. Markerless in-frame deletions of *Cytophaga hutchinsonii* endoglucanase-encoding gene *cel5A* (Zhu et al., 2016), *F. johnsoniae* motility gene *sprF* (Rhodes et al., 2011b), and *Cellulophaga algicola* motility gene *gldB* (Zhu and McBride, 2016) were obtained (Fig. S7 and Fig. S8). Since *ermF* from Tn*4351* functions in many members of the phylum (Table S1), pYT313 is likely to be suitable for construction of gene deletions in many other members of the phylum *Bacteroidetes*. Slight modifications to the approach described above would result in other types of site-directed mutations such as point mutations or insertion of foreign DNA into the chromosome. Other antibiotic resistance genes that function in members of the *Bacteroidetes*, including *tetQ* (Fletcher and Macrina, 1991) and *cfxA* (Smith and Parker, 1993), are available and allow similar genetic manipulations for bacteria such as *F. columnare* for which *ermF* fails to confer sufficient resistance to erythromycin (Staroscik et al., 2008; McBride, 2014; Li et al., 2015).

Discussion

New genetic tools for Z. galactanivorans *and other* Bacteroidetes

In this study genetic techniques were developed for *Z. galactanivorans* to allow analyses of its polysaccharide utilization systems. Plasmids that replicate in some other members of the phylum *Bacteroidetes* failed to function in *Z. galactanivorans*. However, *HimarEm1* and Tn*4351* transposed in and conferred erythromycin resistance on *Z.*

galactanivorans, demonstrating that DNA can be transferred into this bacterium by conjugation. This knowledge allowed the development of techniques for site-directed mutagenesis and integrative complementation. The *sacB*-containing plasmid pYT313 was used to construct an in-frame markerless chromosomal gene deletion. An advantage of the *sacB*-based system (Li et al., 2015) over the gene deletion systems developed for some related bacteria (Koropatkin et al., 2008; Rhodes et al., 2011a; Zhu and McBride, 2014) is that deletions can be constructed in wild type cells. The *sacB*-based system used here also has advantages over the FLP-FRT system developed for *C. hutchinsonii* (Wang et al., 2014) since it does not leave a scar at the site of the mutation. The absence of antibiotic resistance markers in the resulting mutants allows this approach to be used iteratively to generate strains with multiple mutations. This facilitates analysis of complex processes that involve genes with redundant or semi-redundant functions. Since replicative plasmids are not available for *Z. galactanivorans* an integrative plasmid was developed to allow complementation of mutants. This approach is only slightly more complicated than complementation using a replicative plasmid, and it has several advantages. Complementation using multicopy replicative plasmids can result in pleiotropic effects resulting from the increased gene dosage (Braun et al., 2005). In contrast, genes expressed from the integrative plasmid pYT356 are present in single copies on the chromosome. Replicative plasmids may also be lost in the absence of antibiotic selection, whereas plasmids integrated into the chromosome are less likely to be lost. For even greater stability pYT313 could be used to insert the gene of interest into the chromosome by a double recombination event, analogous to the gene deletion approach described above.

The genetic tools described above, including the transposons and the site-directed gene deletion plasmid pYT313, functioned in diverse members of the phylum *Bacteroidetes*. pYT313 was used to delete genes not only in *Z. galactanivorans*, but also in the marine algal

polysaccharide digesting psychrophile *C. algicola*, the soil and freshwater chitin-digesting bacterium *F. johnsoniae*, and the cellulolytic soil bacterium *C. hutchinsonii*. Until now genetic manipulation of members of the *Bacteroidetes* had been primarily confined to bacteria associated with animals or humans (Salyers et al., 1987; Alvarez et al., 2004; Mally and Cornelis, 2008; Hu et al., 2013; Li et al., 2015) and to a few model systems for studies of gliding motility or cellulose utilization (McBride and Baker, 1996; McBride and Kempf, 1996; Zhu and McBride, 2014). The phylum *Bacteroidetes* is large and diverse (Krieg et al., 2011; Thomas et al., 2011b). Members of this phylum are often among the most abundant in many marine and freshwater environments and they are also common in many other habitats (Kirchman, 2002; Williams et al., 2013). The tools described here allow genetic analyses of novel processes in *Z. galactanivorans* and they should facilitate similar studies of many other environmental members of the phylum *Bacteroidetes*.

alyA1, a horizontally acquired gene essential for efficient degradation of brown algal cell walls

Genetic analysis of *Z. galactanivorans* revealed the importance of AlyA1 in alginate utilization. AlyA1 is one of seven *Z. galactanivorans* alginate lyases, and is the only one known to be secreted in soluble form (Thomas et al., 2012). Accordingly, when cells were grown on soluble alginate the deletion of *alyA1* resulted in decreased levels of soluble secreted alginate lyase and lower accumulation of degradation products. However, this did not affect the growth rate. Presumably, other alginate lyases that may reside on the bacterial cell surface can access and readily digest soluble alginate. The oligosaccharides produced locally at the cell surface could directly be imported into the periplasm through the TonB-dependant receptor / SusD-like complex encoded by the alginolytic PUL (Thomas et al.,

2012), therefore preventing accumulation of degradation products in the medium. Insoluble alginate in brown algal cell walls is likely to be less accessible. Deletion of *alyA1* resulted in defects in digestion of and growth on insoluble alginate gels and algal tissues. Several biochemical properties can explain the importance of AlyA1 for optimal use of algal cell wall alginate. First, the soluble secreted AlyA1 may be more effective than cell surface enzymes at penetrating deep into the algal cell wall. Second, AlyA1 is an endo-acting guluronate lyase that attacks insoluble alginate between consecutive G residues (Thomas et al., 2013). Its action will rapidly decrease the degree of polymerization and generate free ends and soluble oligomers as substrates for the other alginate lyases. Its preference for G-G motifs directly targets the polyG blocks that are responsible for the gelling properties of alginate, and it is thus expected to rapidly solubilize alginate gels. Third, AlyA1 is the only *Z. galactanivorans* alginate lyase containing a recognizable non-catalytic CBM. CBMs are known to promote the association of an enzyme with its substrate (Boraston et al., 2004). Recent work on the *Flammeovirga* sp. strain MY04 AlyA1 ortholog showed that truncation of the non-catalytic region resulted in the accumulation of larger oligosaccharides from soluble alginate, and that the CBM was essential for enzyme binding and degradation of small substrates (Han et al., 2016). The CBM might have an even greater role during attack of alginate gels within brown algal biomass, where alginate interacts with other cell wall components to maintain the physical structure of the cell wall.

Examination of the genomic context of *alyA1*, its absence from the vast majority of *Flavobacteriaceae* possessing alginolytic PULs, and the phylogenies of homologs from distantly related bacteria suggested that *alyA1* was recently acquired via HGT from marine *Actinobacteria*. Although *Actinobacteria* are not generally considered as major marine macroalgal polysaccharide degraders, a recent census of GHs and PLs in 126 genomes of marine heterotrophic bacteria (Barbeyron et al., 2016) showed that on average, marine

actinobacterial genomes encoded 47 such enzymes (4 genomes available), which is comparable to the average 51 found in marine *Bacteroidetes* (21 genomes available). Therefore, the role of marine *Actinobacteria* in polysaccharide turnover may previously have been underestimated. The congruence of the separate PL7 and CBM32 domain phylogenies indicates that both were likely acquired simultaneously by *Z. galactanivorans* rather than being fused after transfer. The presence of *alyA1* homologs in only a few other *Bacteroidetes* and *Proteobacteria* suggests that independent HGT events of *alyA1* homologs occurred in distantly related bacteria. This finding extends a growing body of evidence that HGT plays a major role in the evolution of marine polysaccharide catabolism by bacteria, either through the transfer of complete catabolic pathways or of single genes. Notably, the alginolytic PUL (which encodes a set of proteins enabling alginate detection, degradation and import of products) emerged in *Flavobacteriaceae* and was subsequently transferred to marine *Proteobacteria* and human gut *Bacteroides* (Thomas et al., 2012). Similarly, several genes involved in the degradation of red algal porphyran and agar were transferred from a marine bacterium to gut bacteria of surgeonfish (Rebuffet et al., 2011) and humans (Hehemann 2010). Recently, analysis of a clade of *Vibrionaceae* revealed a complex series of independent acquisitions and transfers of alginate lyases and oligo-alginate lyases between closely related populations, leading to fine-scale differentiation of the alginolytic potential (Hehemann et al., 2016). Here, we show that addition of a single exogenous gene to a pre-existing alginolytic system could confer an adaptive advantage on *Z. galactanivorans* by improving access to alginate gels and increasing its ability to degrade fresh algal biomass.

In the environment, polysaccharide degradation is thought to involve at least three ecophysiological types of bacteria: (i) *pioneers*, which have full degradation pathways and secrete soluble enzymes that break down polymers and produce diffusible products (ii) *harvesters*, which possess a complete set of degradation enzymes but do not secrete soluble

enzymes and may benefit from the soluble enzymes secreted by pioneers, and (iii) *scavengers*, which lack enzymes that cleave polysaccharides but can use small oligomers released by pioneers and harvesters (Hehemann et al., 2016). The presence of AlyA1-containing bacteria acting as pioneers for the initial attack on algal biomass could influence community assembly by exposing new substrate niches for harvesters and scavengers. Although we only examined the antibiotic-resistant subset of the algae-associated microbiome in pooled composite samples, our results showing that growth of *Z. galactanivorans* favored genera that were not detected in the presence of the Δ*alyA1* mutant or in non-inoculated controls provide preliminary evidence to support this hypothesis. Dissemination of *alyA1*-like genes within algae-associated bacterial communities could boost the recycling of algal biomass and impact the carbon cycle in coastal ecosystems. Furthermore, the search for sustainable bioenergy alternatives has recently fostered efforts towards using kelp biomass as feedstock for the production of biofuels and renewable chemicals. One of the main challenges to exploit the full potential of brown macroalgae is to bioengineer microbial systems that efficiently process alginate from fresh biomass (Wargacki et al., 2012). The demonstrated role of *Z. galactanivorans* AlyA1 in algal tissue degradation makes it a promising candidate to improve the efficiency of conversion of fresh kelp tissue into useful products by such bioengineered microorganisms.

Conclusion

Genetic techniques were developed for *Z. galactanivorans* and were shown to function in diverse members of the phylum *Bacteroidetes*. These techniques were used to determine the role of *Z. galactanivorans* AlyA1 in alginate utilization. An *alyA1* deletion mutant was deficient in soluble secreted alginate lyase activity and in digestion of alginate

gels and brown algal tissues. Secreted AlyA1 may perform the initial attack on the cell wall polymer, and other cell-associated alginate lyases may assist in further digestion of the released products. *alyA1* appears to have been recently acquired via HGT from marine *Actinobacteria*. Further study is needed to determine if AlyA1 acquisition has influenced the functional evolution and regulation of the enzymes of the alginolytic PULs to optimize degradation efficiency. Ecologically, dissemination of AlyA1-like enzymes in macroalgae-associated bacteria might influence carbon cycling in coastal environments by favoring degradation of kelp tissues. Future biotechnological applications may take advantage of AlyA1-like enzymes for the biorefinery of macroalgal biomass.

Experimental Procedures

Bacterial strains, plasmids, and primers

Z. galactanivorans strain Dsij^T (DSM 12802) (Barbeyron et al., 2001), was the wild type strain used in this study. The donor strain of *E. coli* used for conjugative transfer of plasmids was S17-1 λ *pir* (de Lorenzo and Timmis, 1994). Strains and plasmids used in this study are listed in Table S5, and primers are listed in Table S6 in the supplemental material.

Chemicals and substrate materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. Sodium alginate samples with M/G ratios of 0.5 and 2.0 were provided by Danisco (Landerneau, France). The G content was confirmed by ^{1}H-NMR spectroscopy to be 66% and 33%, respectively (data not shown).

L. digitata (L) Lamouroux sporophytes (30-50 cm in length) were harvested at low tide close to Ile Verte (+48°43', -3°59') in Roscoff (France) on March 8, 2016. Pieces of blade (2.5 cm^2) and stipes (1 cm in length) were cut with an ethanol-sterilized blade and rinsed three times for 2 h in a large volume of filter sterilized seawater at 13°C before use.

Growth conditions for genetic manipulations

Z. galactanivorans strains were grown at 30°C in Cytophaga medium (DSMZ medium 172) which consisted of 1.0 g/L yeast extract, 1.0 g/L tryptone, 24.7 g/L NaCl, 0.7 g/L KCl, 6.3 g/L $MgSO_4 \cdot 7H_2O$, 4.6 g/L $MgCl_2 \cdot 6H_2O$, 1.2 g/L $CaCl_2 \cdot 7H_2O$, 0.2 g/L $NaHCO_3$, pH 7.2. Marine conjugation medium (Zhu and McBride, 2016) consisted of 1.0 g/L yeast extract, 1.0 g/L tryptone, 5 g/L NaCl, 0.35 g/L KCl, 3.15 g/L $MgSO_4 \cdot 7H_2O$, 2.3 g/L $MgCl_2 \cdot 6H_2O$, 0.6 g/L $CaCl_2 \cdot 7H_2O$, 0.1 g/L $NaHCO_3$ (pH7.2). Marine conjugation agar was identical except for the addition of 15 g agar/L. Marine conjugation medium has salt concentrations that both *E. coli* and *Z. galactanivorans* tolerate and was developed to allow conjugative transfer of DNA from *E. coli* into marine bacteria. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C (Sambrook et al., 1989). Antibiotics were used at the indicated concentrations when needed: ampicillin, chloramphenicol, 20 µg/ml; 100 µg/ml; erythromycin, 50 µg/ml; kanamycin, 35 µg/ml; tetracycline, 20 µg/ml.

Conjugative gene transfer into Z. galactanivorans

E. coli S17-1 λ *pir* strains containing mobilizable plasmids were grown overnight in LB at 37°C and *Z. galactanivorans* was grown overnight in Cytophaga medium at 30°C. *Z. galactanivorans* cells (10-20 ml) were harvested (3,700 × g, 10 min) and washed once with

Cytophaga medium. *E. coli* cells (5-10 ml) were harvested (3,700 × g, 10 min) and washed once with LB medium. *Z. galactanivorans* and *E. coli* cells were suspended in marine conjugation medium and mixed together (approximately 1:1 ratio; approximately 100 µl final volume), and spotted on 0.45 µm nitrocellulose filter membrane (Merck Millipore, MA, USA) that was overlaid on marine conjugation agar and allowed to dry. The membranes prevented *Z. galactanivorans* from penetrating into the agar and thus allowed maximum recovery of cells. Following overnight incubation at 30°C, cells were scraped off of the filter membranes, diluted in Cytophaga medium and plated on Cytophaga agar containing erythromycin. Plates were incubated for 3 to 4 days at 30°C.

Construction of sacB-*containing suicide vectors for gene deletion in* Z. galactanivorans

A 1.6-kbp fragment spanning the *F. johnsoniae ompA* promoter and *sacB* gene from pMS75 (Li et al., 2015) was amplified by PCR using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) and primers 1769 and 1770. The fragment was digested with EcoRI and ligated into pLYL03 that had been digested with the same enzyme, generating the suicide vector pYT313 (Fig. 1). The orientation of the inserted fragment was confirmed by DNA sequencing. pYT313 was modified to introduce convenient restriction sites. A 174-bp fragment spanning the multiple cloning site of pBC SK+ (Agilent Technologies, Santa Clara, CA) was amplified by primers 1678 and 1679. The fragment was ligated into pYT313, which had been digested with BamHI and SalI and treated with end-conversion mix from the Perfectly blunt cloning kit (Novagen, Madison, WI), to generate pYT354 and pYT355 (Fig. S2). The orientation of the inserted fragment was confirmed by DNA sequencing.

Deletion of Z. galactanivorans alyA1

A 2.3-kbp fragment spanning ZGAL_1179, ZGAL_1180, ZGAL_1181, and the first 54 bp of *alyA1* was amplified using primers 1833 (introducing a BamHI site) and 1834 (introducing a XbaI site). The fragment was digested with BamHI and XbaI and ligated into pYT313, which had been digested with the same enzymes, to generate pYT330. A 2.3-kbp fragment spanning a part of ZGAL_1183 and the last 36 bp of *alyA1* was amplified using primers 1835 (introducing a XbaI site) and 1836 (introducing a SphI site). The fragment was cloned into XbaI and SphI sites of pYT330 to generate the deletion construct pYT332 (Fig. 1). Plasmid pYT332 was introduced into *Z. galactanivorans* by conjugation. Selection for erythromycin resistance resulted in colonies that had pYT332 inserted in the genome by homologous recombination. These were streaked for isolation, and a single colony was grown overnight in Cytophaga medium in the absence of antibiotics to allow a second recombination event resulting in loss of the plasmid. These cells were plated on Cytophaga agar containing 5% sucrose. Isolated sucrose-resistant colonies were picked and streaked for isolation on Cytophaga agar containing sucrose to eliminate background cells that had not lost the plasmid. Colonies were screened by PCR using primers 1856 and 1857 which flank *alyA1*, to identify the Δ*alyA1* mutant. Streaking colonies for isolation on selective media at both the plasmid integration and plasmid loss steps was critical to eliminate nonselected cells.

Construction of the suicide vector pYT356 for integrative complementation in Z. galactanivorans

A 2.1-kbp fragment spanning ZGAL_4583, ZGAL_4584, ZGAL_4585, and ZGAL_4586, was amplified from *Z. galactanivorans* using primers 1963 and 1964. A 186-bp fragment spanning the *ompA* promoter region was amplified from *F. johnsoniae* using

primers 1965 and 1966. The two fragments were fused by crossover PCR, digested with BamHI and XbaI, and cloned into pLYL03 that had been digested with the same enzymes, generating pYT356 (Fig. 2). To test the ability of pYT356 to deliver constructs to the *Z. galactanivorans* chromosome, the promoterless super folder *gfp* gene (sf*gfp*) (Dinh and Bernhardt, 2011) was amplified with primers 1390 and 1984 from pTB263 (Uehara et al., 2009) and cloned into XbaI and SphI sites of pYT356, generating pYT362. pYT362 was introduced into wild type *Z. galactanivorans* by conjugation and observed for fluorescence. To complement Δ*alyA1*, promoterless *alyA1* was amplified with primers 1976 and 1977 and cloned into the XbaI and SphI sites of pYT356, generating pYT359, which was introduced into the Δ*alyA1* mutant by conjugation. Control strains with the empty vector, pYT356, integrated into the chromosome were generated in a similar way. In each case insertion of the suicide vector at the neutral target site was confirmed by PCR.

Growth conditions for physiological experiments

Z. galactanivorans strains were routinely grown from glycerol stocks in Zobell medium 2216E (5 g/L tryptone, 1 g/L yeast extract, filtered seawater (Zobell, 1941)). Before use, all precultures were collected by centrifugation, washed in 2 volumes of sterile saline solution, and suspended to the same OD_{600}. For determination of growth rates, strains were grown in 30 ml liquid cultures in 250 ml flasks at 20°C with shaking at 180 rpm, in Zobell medium or in a marine mineral medium (MMM) supplemented with either 33% G or 66% G alginate (4 g/L). Briefly, MMM was composed of (per liter) 24.7 g NaCl, 6.3 g $MgSO_4 \cdot 7H_2O$, 4.6 g $MgCl_2 \cdot H_2O$, 2 g NH_4Cl, 0.7 g KCl, 0.6 g $CaCl_2$, 200 mg $NaHCO_3$, 100 mg K_2HPO_4, 20 mg $FeSO_4 \cdot 7H_2O$, 1 ml 1000X vitamin mix, in Tris-HCl 50 mM pH 8.0 (Thomas et al., 2011a). To test the growth on jellified alginate, plates were prepared by

adding 15 mM $CaCO_3$ and 20 mM glucono-D-lactone to a 2% alginate solution in MMM (Draget et al., 1989). Erythromycin (10 µg/ml) was added to plates when testing the *ermF*-carrying strains mZG0031 (WT+pYT356), mZG0033 (Δ*alyA1*+pYT356) and mZG0035 (Δ*alyA1*+pYT359). Plates were inoculated with two-microliters of cell suspensions (OD_{600} normalized to 1.2) deposited on the center and incubated at 20°C. The diameter of the area covered by bacteria was measured as a proxy for bacterial growth. To test the degradation of algal biomass, one piece of *L. digitata* blade or stipe was added to 40 ml flasks containing 5 ml MMM supplemented with kanamycin and streptomycin (100 µg/ml). These two antibiotics to which *Z. galactanivorans* is resistant (Barbeyron et al., 2001) were used to prevent uncontrolled growth of other alga-associated bacteria. Growth experiments confirmed that deletion of *alyA1* did not alter the resistance to these antibiotics (data not shown). Flasks were inoculated with *Z. galactanivorans* wild type or Δ*alyA1* strains to a final OD_{600} of 0.05 and incubated at 20°C with shaking at 180 rpm. Non-inoculated control cultures were handled in the same fashion.

Biochemical measurements

Alginate lyase activity was assessed using a spectrophotometer by measuring the increase in absorbance at 235 nm (A_{235}) for 5 min at 30°C, due to the release of unsaturated uronic acid degradation products (Thomas et al., 2012). Reaction mixtures (600 µl) prepared in quartz cuvettes contained 100 µl of alginate solution (0.08% (w/v) final concentration) and 500 µl of culture supernatant recovered by centrifugation (5 min, 14500 g). One unit of enzyme activity was defined as an increase of 1 in A_{235} per min. Soluble unsaturated uronic acids were quantified in a microplate reader (TECAN Spark 10M) by measuring A_{235} on

culture supernatant diluted 1:10 with distilled water. The molar extinction coefficient ε=8500 l mol^{-1} cm^{-1} was used to infer product concentration.

Microscopic observations

Cross-sections of *L. digitata* stipes were prepared on a cryostat microtome (Leitz 1320, Ernst Leitz Wetzlar GmbH, Weztlar, Germany), deposited on poly-L-lysine pretreated glass slides, directly incubated in the fixative solution (paraformaldehyde 4%, sterile seawater 50% in distilled water) overnight at 4°C and stored in fixative solution diluted 1:4 in sterile seawater until analysis. Before staining, slides were rinsed with phosphate buffer saline (PBS). Sections were stained for 10 min in 0.1% Toluidine Blue O (TBO) in 0.1 M phosphate buffer pH 6.8 and rinsed thoroughly with 15 ml 0.1 M phosphate buffer pH 6.8. Slides were mounted with coverslips and examined using an Olympus BX60 microscope (Paris, France) equipped with an EXI Aqua digital camera. A minimum of 21 pictures were analyzed for each treatment.

16S rRNA amplicon sequencing

Samples (2 ml) were collected from cultures with fresh algal biomass after 8 days of incubation, centrifuged for 5 min at 14,500 × g and frozen at -20°C until analysis. Samples from biological triplicates were pooled to obtain one composite sample for each of the three conditions (inoculated with wild-type cells, inoculated with Δ*alyA1*, and non-inoculated control). Genomic DNA was extracted from cell pellets suspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) supplemented with 1 mg/ml lysozyme and incubated for 30 min at 37°C. SDS (0.1%) and proteinase K (0.2 mg/ml) were added, followed by further

incubation for 30 min at 37°C. After phenol/chloroform extraction, DNA was recovered by isopropanol precipitation, rinsed with 70% ethanol, dried for 5 min in a vacuum concentrator and suspended in 30 µl 10 mM Tris-HCl pH 8.0. DNA was quantified with the Qubit DNA Assay kit on a Qubit fluorometer (Thermo Fisher Scientific). The V3-V4 hypervariable regions of the 16S rRNA gene were amplified using primers 347F and 800R and sequenced using the Illumina MiSeq PE300 platform at Eurofins Genomics (Ebersberg, Germany). Reads were deposited in the SRA database under BioProjectID PRJNA356506. Paired-end reads were trimmed to a minimum QScore of 25 using trimmomatic v0.36 with options LEADING:25 TRAILING:25 SLIDINGWINDOW:4:25 (Bolger et al., 2014), and joined using PANDAseq (Masella et al., 2012) with the following criteria: sequence length between 400 and 500 bp, quality threshold 0.8, no ambiguous bases. De novo chimera detection was performed in QIIME v1.9 (Caporaso et al., 2010) using UCHIME (Edgar et al., 2011). Sequences were dereplicated using UCLUST (Edgar, 2010) with similarity set to 100%. Taxonomic assignment of each unique sequence was performed using RDP (Wang et al., 2007) against the Silva 111 database. Singletons and sequences affiliated to chloroplasts were removed from the dataset. The final dataset comprised a total of 1,737,069 reads. Relative proportions and Shannon index were calculated on a dataset rarefied at 407,186 sequences per sample (lowest number of reads in one sample).

alyA1 *genomic context and phylogeny*

To identify alginolytic PULs in *Flavobacteriaceae*, homologs of *Z. galactanivorans* AlyA3 (MicroScope accession ZGAL_2624, NCBI accession ZOBGAL_RS12440) and ZGAL_2617 (NCBI accession ZOBGAL_RS12405) were searched in all publicly available genomes (1001 genomes) using microbial tblastn at NCBI (Acland et al., 2013). These two

proteins are encoded by genes from the *Z. galactanivorans* alginolytic PUL and are conserved in alginate digesting members of the *Flavobacteriaceae* (Thomas et al., 2012). For each blast hit, the genomic region was manually checked to confirm it belonged to a predicted alginolytic PUL. Homologs of *Z. galactanivorans* AlyA1 were retrieved by tblastn searches against the genome of each identified alginolytic PUL-containing member of the *Flavobacteriaceae* and by BlastP against the non-redundant NCBI protein database. Modules were delimited by scanning sequences on the dbCAN web server (Yin et al., 2012). For each module, alignments obtained using M-COFFEE (Moretti et al., 2007) were manually edited in Jalview v2.9 (Waterhouse et al., 2009). Final alignments contained 112 and 207 residues for CBM32 and PL7 modules, respectively. After testing for the best substitution model in MEGA7 (Tamura et al., 2013), phylogenetic trees were constructed using RaxML with 1,000 resamplings (Stamatakis, 2014). Trees with the highest log-likelihood (-3373.4 for CBM32, -5793.6 for PL7) are shown in Fig. 7.

Statistical analyses

Statistical analyses were performed using R 3.2.2. Welch test, ANOVA and post-hoc Tukey tests were performed on log-transformed values. For all tests, the significance threshold was set to α=5%.

Genetic nomenclature

Locus tags for *Z. galactanivorans* genes correspond to those assigned by MicroScope (Vallenet et al., 2013). Corresponding National Center for Biotechnology Information locus tags are indicated in Fig. S3 and Fig. S6.

Acknowledgements

The authors thank Dr. Tristan Barbeyron for help with bacterial cultures, Dr. Frédérique Leroux for advice on mutagenesis and fruitful discussions, Dr. Cécile Hervé for advice on microscopic observation of algal tissues, and Dr. Thomas G. Bernhardt for providing pTB263. This research was funded by National Science Foundation grant MCB-1516990 to MJM, and by the French National Research Agency grant ANR-14-CE19-0020-01 to GM. The authors have no conflicts of interest regarding the research described in this paper.

References

Acland, A., Agarwala, R., Barrett, T., Beck, J., Benson, D.A., Bollin, C. et al. (2013) Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research* **41**: D8-D20.

Alvarez, B., Secades, P., McBride, M.J., and Guijarro, J.A. (2004) Development of genetic techniques for the psychrotrophic fish pathogen *Flavobacterium psychrophilum*. *Appl Environ Microbiol* **70**: 581-587.

Barbeyron, T., L'Haridon, S., Corre, E., Kloareg, B., and Potin, P. (2001) *Zobellia galactanovorans* gen. nov., sp. nov., a marine species of *Flavobacteriaceae* isolated from a red alga, and classification of [*Cytophaga*] *uliginosa* (ZoBell and Upham 1944) Reichenbach 1989 as *Zobellia uliginosa* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **51**: 985-997.

Barbeyron, T., Thomas, F., Barbe, V., Teeling, H., Schenowitz, C., Dossat, C. et al. (2016) Habitat and taxon as driving forces of carbohydrate catabolism in marine heterotrophic bacteria: example of the model algae-associated bacterium *Zobellia galactanivorans* DsijT. *Environ Microbiol* **18**: 4610-4627.

Belanger, M., Rodrigues, P., and Progulske-Fox, A. (2007) Genetic manipulation of *Porphyromonas gingivalis*. *Current Protocols in Microbiology* **13C**: 2.1-2.24.

Bolger, A.M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114-2120.

Boraston, A.B., Bolam, D.N., Gilbert, H.J., and Davies, G.J. (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochem J* **382**: 769-781.

Braun, T.F., Khubbar, M.K., Saffarini, D.A., and McBride, M.J. (2005) *Flavobacterium johnsoniae* gliding motility genes identified by *mariner* mutagenesis. *J Bacteriol* **187**: 6943-6952.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K. et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335-336.

Carpenter, L.J., and Liss, P.S. (2000) On temperate sources of bromoform and other reactive organic bromine gases. *Journal of Geophysical Research-Atmospheres* **105**: 20539-20547.

Chen, S., Bagdasarian, M., Kaufman, M.G., Bates, A.K., and Walker, E.D. (2007) Mutational analysis of the *ompA* promoter from *Flavobacterium johnsoniae*. *J Bacteriol* **189**: 5108-5118.

Cooper, A.J., Kalinowski, A.P., Shoemaker, N.B., and Salyers, A.A. (1997) Construction and characterization of a *Bacteroides thetaiotaomicron recA* mutant: Transfer of *Bacteroides* integrated conjugative elements is RecA independent. *J Bacteriol* **179**: 6221-6227.

Cragg, S.M., Beckham, G.T., Bruce, N.C., Bugg, T.D., Distel, D.L., Dupree, P. et al. (2015) Lignocellulose degradation mechanisms across the Tree of Life. *Curr Opin Chem Biol* **29**: 108-119.

de Lorenzo, V., and Timmis, K.N. (1994) Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods in enzymology* **235**: 386-405.

Deniaud-Bouet, E., Kervarec, N., Michel, G., Tonon, T., Kloareg, B., and Herve, C. (2014) Chemical and enzymatic fractionation of cell walls from Fucales: insights into the structure of the extracellular matrix of brown algae. *Ann Bot* **114**: 1203-1216.
Dinh, T., and Bernhardt, T.G. (2011) Using superfolder green fluorescent protein for periplasmic protein localization studies. *J Bacteriol* **193**: 4984-4987.
Draget, K.I., Ostgaard, K., and Smidsrod, O. (1989) Alginate-based solid media for plant-tissue culture. *Applied Microbiology and Biotechnology* **31**: 79-83.
Dyer, D.W., Bilalis, G., Michel, J.H., and Malek, R. (1992) Conjugal transfer of plasmid and transposon DNA from *Escherichia coli* into *Porphyromonas gingivalis*. *Biochemical and Biophysical Research Communications* **186**: 1012-1019.
Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**: 2460-2461.
Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194-2200.
Ficko-Blean, E., Herve, C., and Michel, G. (2015) Sweet and sour sugars from the sea: the biosynthesis and remodeling of sulfated cell wall polysaccharides from marine algae. *Perspectives in Phycology* **2**: 51-64.
Fischl, R., Bertelsen, K., Gaillard, F., Coelho, S., Michel, G., Klinger, M. et al. (2016) The cell-wall active mannuronan C5-epimerases in the model brown alga *Ectocarpus*: From gene context to recombinant protein. *Glycobiology* **26**: 973-983.
Fletcher, H.M., and Macrina, F.L. (1991) Molecular survey of clindamycin and tetracycline resistance determinants in *Bacteroides* species. *Antimicrob Agents Chemother* **35**: 2415-2418.
Han, W.J., Gu, J.Y., Cheng, Y.Y., Liu, H.H., Li, Y.Z., and Li, F.C. (2016) Novel alginate lyase (Aly5) from a polysaccharide-degrading marine bacterium, *Flammeovirga* sp Strain MY04: Effects of Module Truncation on Biochemical Characteristics, Alginate Degradation Patterns, and Oligosaccharide-Yielding Properties. *Applied and Environmental Microbiology* **82**: 364-374.
Haug, A., Larsen, B., and Smidsrod, O. (1974) Uronic acid sequence in alginate from different sources. *Carbohydr Res* **32**: 217-225.
Hecht, H., and Srebnik, S. (2016) Structural characterization of sodium alginate and calcium alginate. *Biomacromolecules* **17**: 2160-2167.
Hehemann, J.H., Boraston, A.B., and Czjzek, M. (2014) A sweet new wave: structures and mechanisms of enzymes that digest polysaccharides from marine algae. *Curr Opin Struct Biol* **28**: 77-86.
Hehemann, J.H., Arevalo, P., Datta, M.S., Yu, X., Corzett, C.H., Henschel, A. et al. (2016) Adaptive radiation by waves of gene transfer leads to fine-scale resource partitioning in marine microbes. *Nat Commun* **7**: 12860.
Hollants, J., Leliaert, F., De Clerck, O., and Willems, A. (2013) What we can learn from sushi: a review on seaweed-bacterial associations. *FEMS Microbiol Ecol* **83**: 1-16.
Hu, Q.H., Miao, S., Ni, X.T., Lu, F.Y., Yu, H., Xing, L.L., and Jiang, P. (2013) Construction of a shuttle vector for use in *Riemerella anatipestifer*. *Journal of Microbiological Methods* **95**: 262-267.
Hu, Q.H., Zhu, Y.Y., Tu, J., Yin, Y.C., Wang, X.L., Han, X.G. et al. (2012) Identification of the genes involved in *Riemerella anatipestifer* biofilm formation by random transposon mutagenesis. *Plos One* **7**.
Inoue, A., Satoh, A., Morishita, M., Tokunaga, Y., Miyakawa, T., Tanokura, M., and Ojima, T. (2016) Functional heterologous expression and characterization of mannuronan C5-

epimerase from the brown alga *Saccharina japonica*. *Algal Research-Biomass Biofuels and Bioproducts* **16**: 282-291.
Kirchman, D.L. (2002) The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**: 91-100.
Koropatkin, N.M., Martens, E.C., Gordon, J.I., and Smith, T.J. (2008) Starch catabolism by a prominent human gut symbiont is directed by the recognition of amylose helices. *Structure* **16**: 1105-1115.
Krieg, N.R., Ludwig, W., Euzeby, J., and Whitman, W.B. (2011) Phylum XIV. *Bacteroidetes* phyl. nov. In *Bergey's Manual of Systematic Bacteriology*. Krieg, N.R., Staley, J.T., Brown, D.R., Hedlund, B.R., Paster, B.J., Ward, N.L. et al. (eds). New York: Springer, p. 25.
Li, L.-Y., Shoemaker, N.B., and Salyers, A.A. (1995) Location and characterization of the transfer region of a *Bacteroides* conjugative transposon and regulation of the transfer genes. *J Bacteriol* **177**: 4992-4999.
Li, N., Qin, T., Zhang, X.L., Huang, B., Liu, Z.X., Xie, H.X. et al. (2015) Gene deletion strategy to examine the involvement of the two chondroitin lyases in *Flavobacterium columnare* virulence. *Applied and environmental microbiology* **81**: 7394-7402.
Link, A.J., Phillips, D., and Church, G.M. (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* **179**: 6228-6237.
Mabeau, S., and Kloareg, B. (1987) Isolation and analysis of the cell walls of brown algae: *Fucus spiralis*, *Fucus ceranoides*, *Fucus vesiculosus*, *Fucus serratus*, *Bifurcaria bifurcata* and *Laminaria digitata*. *Journal of Experimental Botany* **38**: 1573-1580.
Mally, M., and Cornelis, G.R. (2008) Genetic tools for studying *Capnocytophaga canimorsus*. *Applied and environmental microbiology* **74**: 6369-6377.
Martens, E.C., Koropatkin, N.M., Smith, T.J., and Gordon, J.I. (2009) Complex glycan catabolism by the human gut microbiota: the *Bacteroidetes* Sus-like paradigm. *J Biol Chem* **284**: 24673-24677.
Martin, M., Portetelle, D., Michel, G., and Vandenbol, M. (2014) Microorganisms living on macroalgae: diversity, interactions, and biotechnological applications. *Appl Microbiol Biotechnol* **98**: 2917-2935.
Martin, M., Barbeyron, T., Martin, R., Portetelle, D., Michel, G., and Vandenbol, M. (2015) The cultivable surface microbiota of the brown alga *Ascophyllum nodosum* is enriched in macroalgal-polysaccharide-degrading bacteria. *Front Microbiol* **6**: 1487.
Marzinelli, E.M., Campbell, A.H., Zozaya Valdes, E., Verges, A., Nielsen, S., Wernberg, T. et al. (2015) Continental-scale variation in seaweed host-associated bacterial communities is a function of host condition, not geography. *Environ Microbiol* **17**: 4078-4088.
Masella, A.P., Bartram, A.K., Truszkowski, J.M., Brown, D.G., and Neufeld, J.D. (2012) PANDAseq: PAired-eND Assembler for Illumina sequences. *Bmc Bioinformatics* **13**.
Matos, M.N., Lozada, M., Anselmino, L.E., Musumeci, M.A., Henrissat, B., Jansson, J.K. et al. (2016) Metagenomics unveils the attributes of the alginolytic guilds of sediments from four distant cold coastal environments. *Environ Microbiol*: In Press.
McBride, M.J. (2014) The family *Flavobacteriaceae*. In *The Prokaryotes*. Rosenberg, E., Stackebrandt, E., DeLong, E., Lory, S., and Thompson, F.L. (eds). Berlin Heidelberg: Springer, pp. 643-676.
McBride, M.J., and Baker, S.A. (1996) Development of techniques to genetically manipulate members of the genera *Cytophaga*, *Flavobacterium*, *Flexibacter*, and *Sporocytophaga*. *Appl Environ Microbiol* **62**: 3017-3022.
McBride, M.J., and Kempf, M.J. (1996) Development of techniques for the genetic manipulation of the gliding bacterium *Cytophaga johnsonae*. *J Bacteriol* **178**: 583-590.

Mitulla, M., Dinasquet, J., Guillemette, R., Simon, M., Azam, F., and Wietz, M. (2016) Response of bacterial communities from California coastal waters to alginate particles and an alginolytic *Alteromonas macleodii* strain. *Environ Microbiol*: In Press.
Moretti, S., Armougom, F., Wallace, I.M., Higgins, D.G., Jongeneel, C.V., and Notredame, C. (2007) The M-Coffee web server: a meta-method for computing multiple sequence alignments by combining alternative alignment methods. *Nucleic Acids Res* **35**: W645-648.
Popper, Z.A., Michel, G., Herve, C., Domozych, D.S., Willats, W.G., Tuohy, M.G. et al. (2011) Evolution and diversity of plant cell walls: from algae to flowering plants. *Annu Rev Plant Biol* **62**: 567-590.
Pumbwe, L., Ueda, O., Yoshimura, F., Chang, A., Smith, R.L., and Wexler, H.M. (2006) *Bacteroides fragilis* BmeABC efflux systems additively confer intrinsic antimicrobial resistance. *J Antimicrob Chemother* **58**: 37-46.
Rebuffet, E., Groisillier, A., Thompson, A., Jeudy, A., Barbeyron, T., Czjzek, M., and Michel, G. (2011) Discovery and structural characterization of a novel glycosidase family of marine origin. *Environ Microbiol* **13**: 1253-1270.
Rhodes, R.G., Pucker, H.G., and McBride, M.J. (2011a) Development and use of a gene deletion strategy for *Flavobacterium johnsoniae* to identify the redundant motility genes *remF*, *remG*, *remH*, and *remI*. *J Bacteriol* **193**: 2418-2428.
Rhodes, R.G., Nelson, S.S., Pochiraju, S., and McBride, M.J. (2011b) *Flavobacterium johnsoniae sprB* is part of an operon spanning the additional gliding motility genes *sprC*, *sprD*, and *sprF*. *J Bacteriol* **193**: 599-610.
Salyers, A.A., Shoemaker, N.B., and Guthrie, E.P. (1987) Recent advances in *Bacteroides* genetics. *CRC Crit Rev Microbiol* **14**: 49-71.
Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, N. Y.: Cold Spring Harbor Laboratory.
Shoemaker, N.B., Guthrie, E.P., Salyers, A.A., and Gardner, J.F. (1985) Evidence that the clindamycin-erythromycin resistance gene of *Bacteroides* plasmid pBF4 is on a transposable element. *J Bacteriol* **162**: 626-632.
Shoemaker, N.B., Getty, C., Gardner, J.F., and Salyers, A.A. (1986) Tn*4351* transposes in *Bacteroides* spp. and mediates the integration of plasmid R751 into the *Bacteroides* chromosome. *J Bacteriol* **165**: 929-936.
Smith, C.J., and Parker, A.C. (1993) Identification of a circular intermediate in the transfer and transposition of Tn*4555*, a mobilizable transposon from *Bacteroides* spp. *J Bacteriol* **175**: 2682-2691.
Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**: 1312-1313.
Staroscik, A.M., Hunnicutt, D.W., Archibald, K.E., and Nelson, D.R. (2008) Development of methods for the genetic manipulation of *Flavobacterium columnare*. *BMC Microbiol* **8**: 115.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725-2729.
Terrapon, N., Lombard, V., Gilbert, H.J., and Henrissat, B. (2015) Automatic prediction of polysaccharide utilization loci in *Bacteroidetes* species. *Bioinformatics* **31**: 647-655.
Thomas, F., Barbeyron, T., and Michel, G. (2011a) Evaluation of reference genes for real-time quantitative PCR in the marine flavobacterium *Zobellia galactanivorans*. *Journal of Microbiological Methods* **84**: 61-66.

Thomas, F., Hehemann, J.H., Rebuffet, E., Czjzek, M., and Michel, G. (2011b) Environmental and gut *Bacteroidetes*: the food connection. *Front Microbiol* **2**: 93.
Thomas, F., Barbeyron, T., Tonon, T., Genicot, S., Czjzek, M., and Michel, G. (2012) Characterization of the first alginolytic operons in a marine bacterium: from their emergence in marine *Flavobacteriia* to their independent transfers to marine *Proteobacteria* and human gut *Bacteroides*. *Environ Microbiol* **14**: 2379-2394.
Thomas, F., Lundqvist, L.C., Jam, M., Jeudy, A., Barbeyron, T., Sandstrom, C. et al. (2013) Comparative characterization of two marine alginate lyases from *Zobellia galactanivorans* reveals distinct modes of action and exquisite adaptation to their natural substrate. *J Biol Chem* **288**: 23021-23037.
Uehara, T., Dinh, T., and Bernhardt, T.G. (2009) LytM-domain factors are required for daughter cell separation and rapid ampicillin-induced lysis in *Escherichia coli*. *Journal of Bacteriology* **191**: 5094-5107.
Vallenet, D., Belda, E., Calteau, A., Cruveiller, S., Engelen, S., Lajus, A. et al. (2013) MicroScope--an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res* **41**: D636-647.
Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**: 5261-5267.
Wang, Y., Wang, Z., Cao, J., Guan, Z., and Lu, X. (2014) FLP-FRT-based method for unmarked deletions of CHU_3237 (*porU*) and large genomic fragments of *Cytophaga hutchinsonii*. *Appl Environ Microbiol* **80**: 6037-6045.
Wargacki, A.J., Leonard, E., Win, M.N., Regitsky, D.D., Santos, C.N., Kim, P.B. et al. (2012) An engineered microbial platform for direct biofuel production from brown macroalgae. *Science* **335**: 308-313.
Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M., and Barton, G.J. (2009) Jalview Version 2-a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**: 1189-1191.
Wietz, M., Wemheuer, B., Simon, H., Giebel, H.A., Seibt, M.A., Daniel, R. et al. (2015) Bacterial community dynamics during polysaccharide degradation at contrasting sites in the Southern and Atlantic Oceans. *Environmental Microbiology* **17**: 3822-3831.
Williams, T.J., Wilkins, D., Long, E., Evans, F., DeMaere, M.Z., Raftery, M.J., and Cavicchioli, R. (2013) The role of planktonic *Flavobacteria* in processing algal organic matter in coastal East Antarctica revealed using metagenomics and metaproteomics. *Environmental microbiology* **15**: 1302-1317.
Xu, Y., Ji, X., Chen, N., Li, P., Liu, W., and Lu, X. (2012) Development of replicative *oriC* plasmids and their versatile use in genetic manipulation of *Cytophaga hutchinsonii*. *Applied microbiology and biotechnology* **93**: 697-705.
Yin, Y., Mao, X., Yang, J., Chen, X., Mao, F., and Xu, Y. (2012) dbCAN: a web resource for automated carbohydrate-active enzyme annotation. *Nucleic Acids Res* **40**: W445-451.
Zhu, Y., and McBride, M.J. (2014) Deletion of the *Cytophaga hutchinsonii* type IX secretion system gene *sprP* results in defects in gliding motility and cellulose utilization. *Appl Microbiol Biotechnol* **98**: 763-775.
Zhu, Y., and McBride, M.J. (2016) Comparative analysis of *Cellulophaga algicola* and *Flavobacterium johnsoniae* gliding motility. *J Bacteriol* **198**: 1743-1754.
Zhu, Y., Han, L., Hefferon, K.L., Silvaggi, N.R., Wilson, D.B., and McBride, M.J. (2016) Periplasmic *Cytophaga hutchinsonii* endoglucanases are required for use of crystalline cellulose as the sole source of carbon and energy. *Appl Environ Microbiol* **82**: 4835-4845.

Zobell, C. (1941) Studies on marine bacteria I: The cultural requirements of heterotrophic aerobes. *J Mar Res* **4**: 42-75.

Table 1. Growth rates in liquid medium

Carbon source	Growth rate (h^{-1})		P-val
	WT strain	Δ*alyA1*	
Tryptone/Yeast Extr.	0.195 ± 0.008	0.212 ± 0.009	0.22
Alginate, 33% G	0.167 ± 0.016	0.186 ± 0.004	0.33
Alginate, 66% G	0.106 ± 0.003	0.116 ± 0.001	0.08

Values are mean ± s.e.m. (n=3). For each carbon source, differences in growth rate between wild type (WT) and mutant strain were tested by Welch-test on log-transformed values.

Figure legends

Figure 1. Deletion of *Z. galactanivorans alyA1*. (A) Map of the *sacB*-containing suicide vector pYT313 used for constructing chromosomal gene deletions. pYT313 was constructed by introducing *sacB* and the promoter of *F. johnsoniae ompA* (P*ompA*) into the *Bacteroidetes* suicide vector pLYL03. Integration into the chromosome of *Z. galactanivorans* results in erythromycin resistance and sucrose sensitivity, and loss of the plasmid from the cell results in erythromycin sensitivity and sucrose resistance. Numbers immediately inside of the ring refer to kilobase pairs of sequence. *ori* refers to the origin of replication that functions in *E. coli*, but not in *Z. galactanivorans*. *oriT* refers to the conjugative origin of transfer. *bla* confers ampicillin resistance on *E. coli* but not on *Z. galactanivorans*. *ermF* confers erythromycin resistance on *Z. galactanivorans*, but not on *E. coli*. **(B)** Map of the region surrounding *alyA1*. Regions upstream (red) and downstream (blue) of *alyA1* were amplified by PCR and inserted into pYT313 to construct pYT332. Numbers below the map refer to kilobase pairs of sequence. Binding sites for PCR primers are shown above and below the map with the blunt ends indicating the actual binding sites. 'Δ' indicates deleted region. **(C)** Introduction of pYT332 into *Z. galactanivorans* by conjugation and growth in the presence of erythromycin selected for colonies that had the plasmid integrated into the chromosome by recombination in either the upstream (shown) or downstream region. Growth without erythromycin allowed a rare second recombination event to occur leading to loss of the integrated plasmid and resulting in either wild type cells or the deletion mutant as shown. Cells that had undergone this second recombination event were selected by growth in the presence of sucrose and deletion mutants were confirmed by PCR. Regions shown in red and blue in panels B and C are those that lie upstream and downstream of *alyA1*. Yellow, purple, and green denote regions of the plasmid pYT313.

Figure 2. Integrative complementation vector pYT356 used for complementation of the *alyA1* deletion mutant. (A) Map of the neutral *Z. galactanivorans* chromosomal site for plasmid integration by homologous recombination. Numbers below the map refer to kilobase pairs of sequence. Binding sites for primers used in PCR reactions to construct pYT356 and to confirm plasmid integration are shown above the map with the blunt ends indicating the actual binding sites. **(B)** The 2.1 kbp fragment spanning ZGAL_4583 through ZGAL_4586 that was inserted into pLYL03 to generate pYT356 is shown by the dotted lines. pYT356 also carries the *F. johnsoniae ompA* promoter (P*ompA*) and putative ribosome binding site (RBS) to express genes inserted into the multiple cloning site. pYT356 does not replicate in *Z. galactanivorans*. **(C)** Promoterless *alyA1* was amplified and inserted into pYT356 to generate pYT359 as shown. Introduction of pYT359 into *Z. galactanivorans* by conjugation and selection for erythromycin resistance resulted in insertion at the neutral integration site on the chromosome. *ori, oriT*, *bla*, and *ermF* are as described in Fig. 1. Numbers immediately inside the rings in (B) and (C) refer to kilobase pairs of sequence.

Figure 3. Effects of *alyA1* deletion on secreted soluble alginate lyase activity. (A) Soluble activity measured in the supernatant of cultures grown with alginate containing 33% or 66% guluronate motifs, for *Z. galactanivorans* wild type (orange) and Δ*alyA1* (white) strains. Values are mean ± s.e.m (n=3). Asterisks denote significant difference between the two strains (Welch t-test, $p < 0.05$). (**B, C**). Concentration of unsaturated uronic acid products measured over time in supernatants of cultures grown with alginate containing 33% (B) or 66% (C) guluronate motifs. Values for the independent biological replicates were plotted individually. For each time point, asterisks denote significant difference between the two strains (Welch t-test, $p < 0.05$).

Figure 4. Effects of *alyA1* deletion (A) and complementation (B) on growth on jellified alginate. (A) Equal amounts of *Z. galactanivorans* wild type (WT, orange circles) or Δ*alyA1* (white circles) cultures where spotted on mineral medium containing either 66% (plain lines) or 33% G (dotted lines) alginate jellified with calcium. The diameter of the area covered by bacteria was measured over time. Values are mean ± s.e.m (n=3). Inset: photographs of representative plates containing 66% G alginate, 14 days after inoculation with WT (top) or Δ*alyA1* (bottom) strain. **(B)** Equal amounts of wild type *Z. galactanivorans* (orange circles), Δ*alyA1* mutant (white circles) and Δ*alyA1* complemented with pYT359 which carries *alyA1* (blue circles) integrated into the chromosome at the neutral target site were spotted on mineral medium containing 66% G alginate jellified with calcium. Values are mean ± s.e.m (n=3). Wild type and Δ*alyA1* mutant strains used in panel B contained empty control vector pYT356 integrated into the chromosome at the neutral target site to allow direct comparison with the complemented strain.

Figure 5. Effects of *alyA1* deletion on the degradation of fresh brown algal biomass. Liquid mineral media were supplemented with fresh pieces of *L. digitata* blade or stipe and inoculated with *Z. galactanivorans* wild type (WT, orange) or Δ*alyA1* (white) strain. Non-inoculated controls (black) were handled in the same way. **(A)** Bacterial density measured after 8 days. Values are mean ± s.e.m (n=3). **(B)** Concentration of unsaturated uronic acid products measured in supernatants after 8 days. Values were corrected for the background level measured from non-inoculated controls (mean ± s.e.m, n=3). For each tissue type, asterisks denote significant difference between the two strains (Welch t-test, $p < 0.05$). **(C)** Photographs of representative blades recovered after 8 days of culture. Scale bars: 1 cm. **(D)**

Brightfield micrographs of Toluidine Blue O stained cross-sections of stipes recovered after 8 days of culture. Scale bars: 10 µm.

Figure 6. Composition of bacterial communities recovered after 8 days of incubation of *L. digitata* stipes in marine minimal medium. Relative abundance of each taxon in 16S rRNA MiSeq amplicon libraries are depicted by bubble area and normalized according to OD_{600} at sampling time. Only taxa representing more than 0.1% (i.e. 407 sequences) in at least one of the treatments are shown for clarity.

Figure 7. Unrooted maximum-likelihood phylogenetic trees of the CBM32 domain (A) and PL7 catalytic domain (B) of homologs of *Z. galactanivorans* AlyA1. Based on testing different models in MEGA7, the LG + G model (G = 1.39) was used for CBM32 and the WAG + G +I model (G = 2.36, I = 0.12) was used for PL7. Numbers represent bootstrap support derived from 1,000 resamplings in RaxML. Proteins from marine and terrestrial bacteria are depicted in blue and green respectively. Three of the terrestrial bacteria (*Kibdelosporangium aridum*, *Streptomyces leeuwenhoekii*, *Streptomyces cyanogriseus subsp. noncyanogenus*) were from desert samples. Domains from *Z. galactanivorans* AlyA1 are depicted in bold face. Details for each sequence are listed in Table S3.

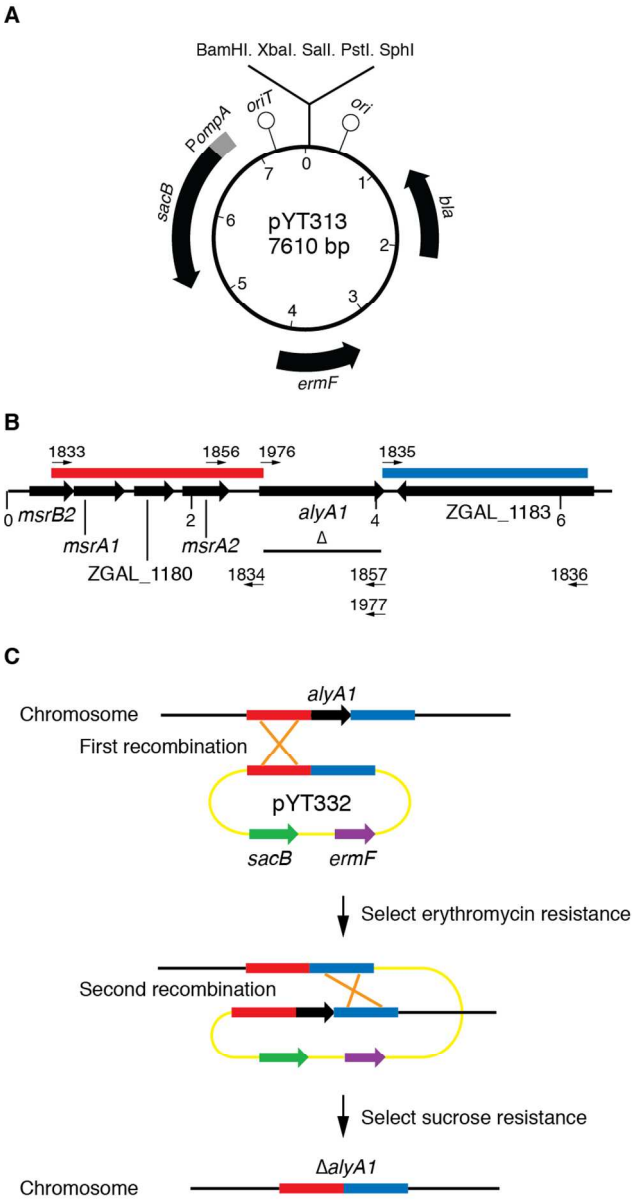

Figure 1. Deletion of *Z. galactanivorans alyA1*

78x149mm (300 x 300 DPI)

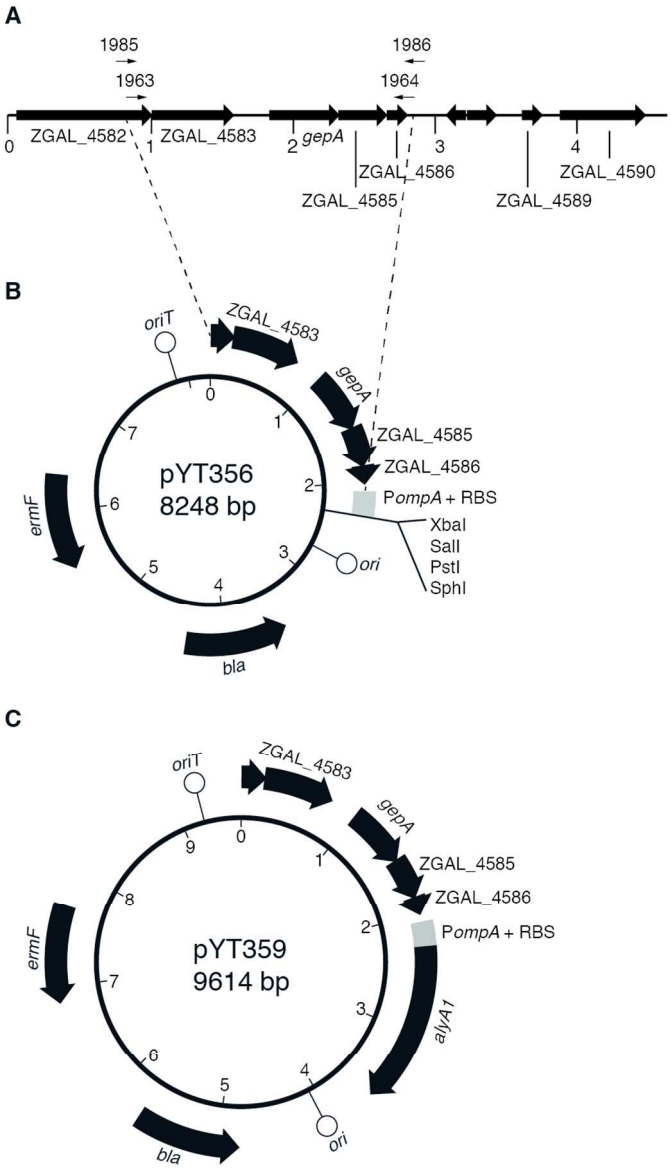

Figure 2. Integrative complementation vector pYT356 used for complementation of the alyA1 deletion mutant

79x142mm (300 x 300 DPI)

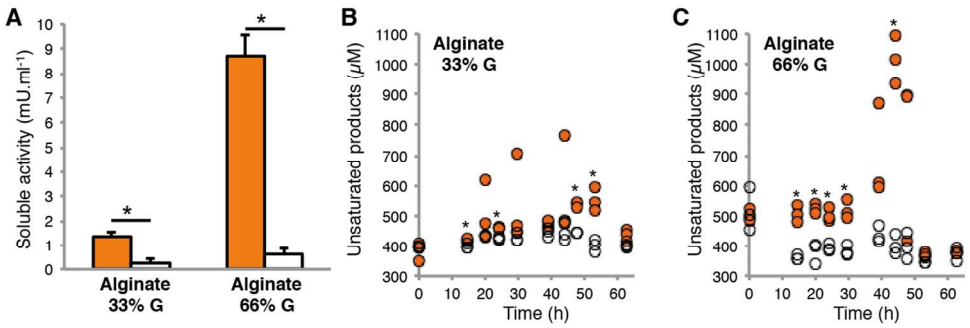

Figure 3. Effects of alyA1 deletion on secreted soluble alginate lyase activity

117x39mm (300 x 300 DPI)

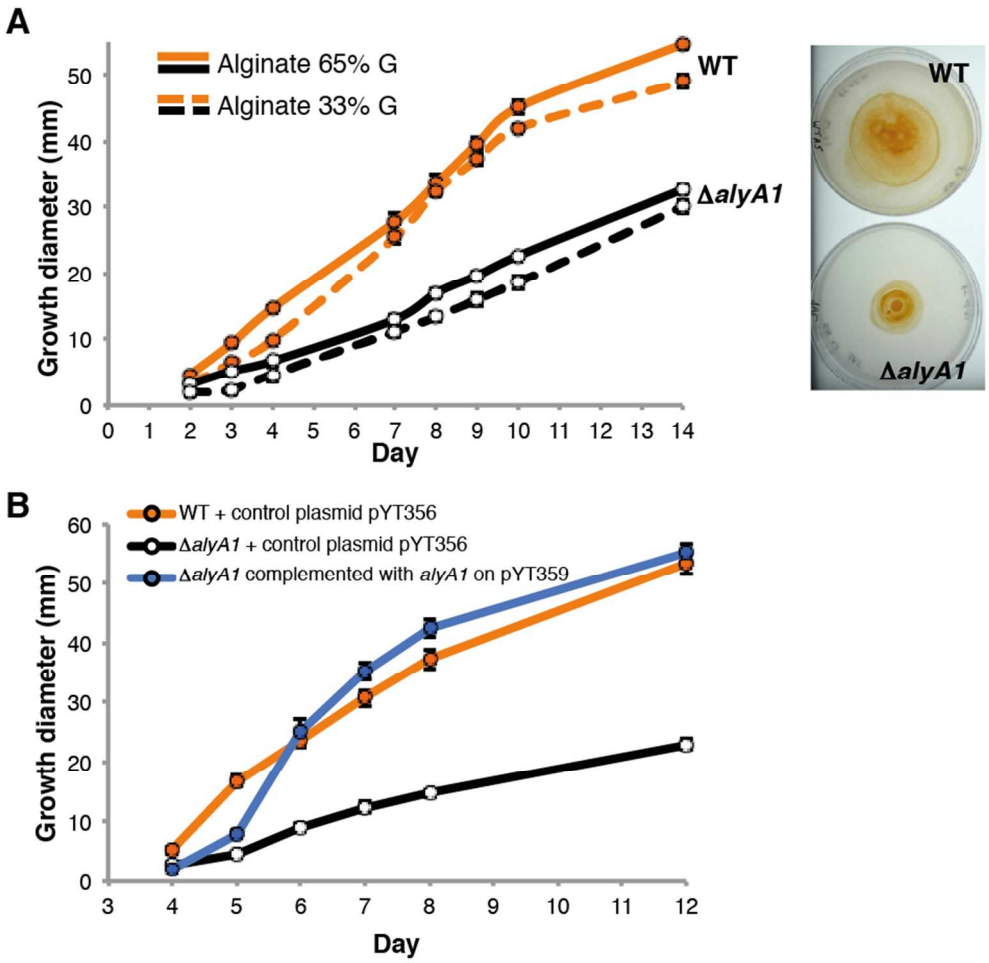

Figure 4. Effects of alyA1 deletion (A) and complementation (B) on growth on jellified alginate

81x79mm (300 x 300 DPI)

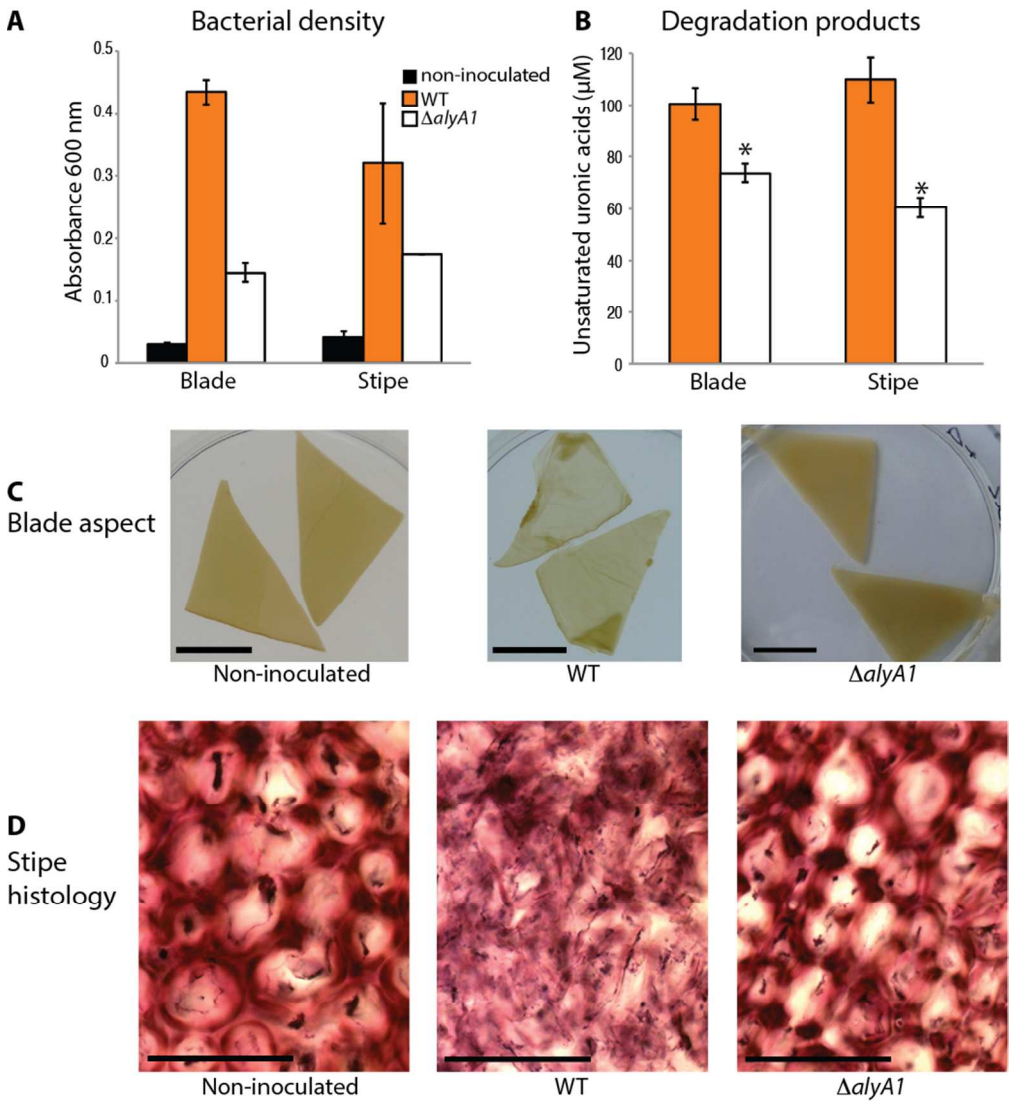

Figure 5. Effects of alyA1 deletion on the degradation of fresh brown algal biomass

175x191mm (150 x 150 DPI)

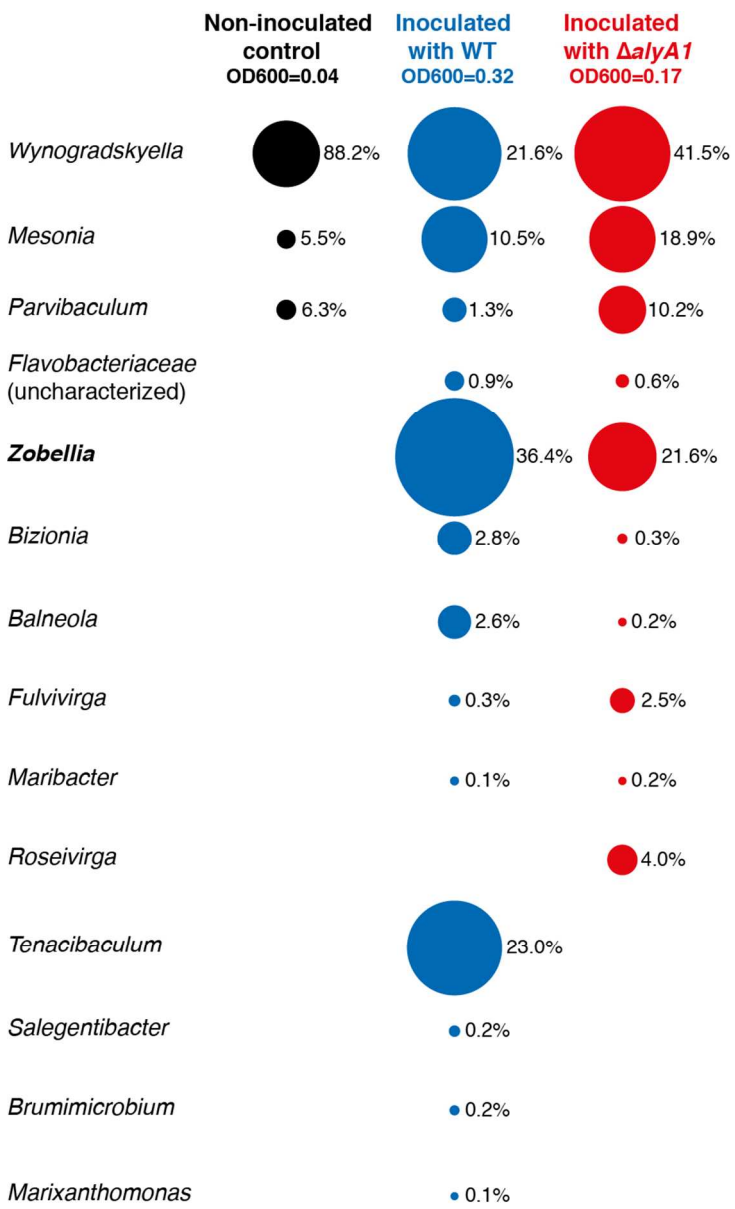

Figure 6. Composition of bacterial communities recovered after 8 days of incubation of L. digitata stipes in marine minimal medium

77x128mm (300 x 300 DPI)

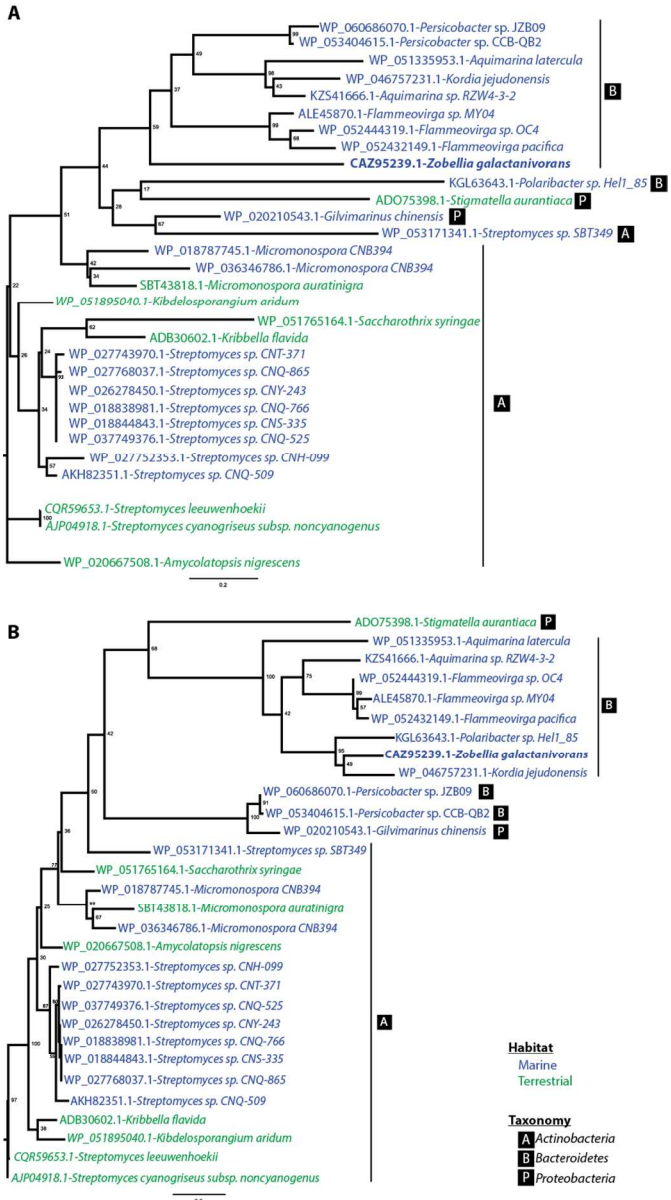

Figure 7. Unrooted maximum-likelihood phylogenetic trees of the CBM32 domain (A) and PL7 catalytic domain (B) of homologs of Z. galactanivorans AlyA1

156x282mm (150 x 150 DPI)