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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*

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Summary

Alginate constitutes a significant part of seaweed biomass and thus a crucial nutrient for numerous marine heterotrophic bacteria. However, the mechanisms for alginate assimilation remain largely unknown in marine microorganisms. We show here that the genome of the marine flavobacterium *Zobellia galactanivorans* contains seven putative alginate lyase genes, five of them localized within two clusters comprising additional carbohydrate-related genes. The transcription of these genes and the alginolytic activity were strongly induced when *Z. galactanivorans* used alginate as sole carbon source. These clusters were shown to be transcribed as polycistronic mRNAs and thus to constitute operons. Several candidate enzymes were successfully overexpressed in *Escherichia coli*, purified and their activity tested. Particularly, AlyA1, AlyA4, AlyA5 and AlyA7 are confirmed as active alginate lyases. Zg2622 and Zg2614 are a dehydrogenase and a kinase, respectively, further converting the terminal unsaturated monosaccharides released by alginate lyases into 2-keto-3-deoxy-6-phosphogluconate. In-depth phylogenomic analyses reveal that such alginolytic operons originated from an ancestral marine flavobacterium and were independently transferred to marine proteobacteria and Japanese gut *Bacteroides*. These bacteria thus gained the capacity to assimilate the main polysaccharide of brown algae, an adaptive advantage in coastal environments but also in the gut microbiota of specific human population.

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Introduction

Brown algae are complex multicellular photosynthetic organisms, which are evolutionary distinct from land plants, but also from green and red algae. These seaweeds are the dominant macroalgae in temperate and polar regions and thus constitute a large primary biomass in coastal ecosystems. Polysaccharides account in average for half of the algal tissues, where they are found either as carbon storage compounds (Michel *et al.*, 2010a) or cell wall (Michel *et al.*, 2010b; Popper *et al.*, 2011). Alginate is the main component of the cell wall in brown algae, where it can account for 10% to 45% of the dry weight (Kloareg and Mabeau, 1987). This linear polysaccharide consists of two uronic monosaccharides, the β-D-mannuronic acid and its C5 epimer the α-L-guluronic acid, arranged in blocks along the polysaccharide chain. Alginates form gels in the presence of divalent cations and their self-assembling properties depend on the relative proportion of guluronate homopolymeric blocks (Haug *et al.*, 1974). Some terrestrial or pathogenic bacteria of the family *Pseudomonadaceae* also produce an acetylated type of alginate as an exopolysaccharide (Ramsey and Wozniak, 2005). The genomic analysis of the brown alga *Ectocarpus siliculosus* (Cock *et al.*, 2010) has recently revealed that brown algae acquired the capacity to produce alginate by lateral gene transfer from an ancestral *Actinobacteria* (Michel *et al.*, 2010b). Alginate, as a polysaccharide of bacterial origin, has then further evolved in brown algae in order to interact with the other cell wall components, such as cellulose, sulfated fucans and polyphenols (Popper *et al.*, 2011).

Because of its abundance, alginate also constitutes a crucial nutrient for numerous marine heterotrophic bacteria. Bacterial degradative processes increase the nutritional value of algal tissues and allow the dissolution of organic matter into the water column, where it can be ingested by filter feeders and other animals (Norderhaug

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et al., 2003; Azam and Malfatti, 2007). Compared with the microbial degradation of land plant biomass, the molecular mechanisms in marine bacteria for the detection, the depolymerization and the uptake of algal polysaccharides and oligosaccharides are largely unknown. Enzymatic pathway for the complete degradation of bacterial alginate and for its assimilation into the central metabolism was studied in the early 1960s in *Pseudomonas* spp. (Preiss and Ashwell, 1962a,b). It has since been found in other terrestrial species such as *Sphingomonas* spp. and *Agrobacterium* spp. (Ochiai *et al.*, 2006; Hashimoto *et al.*, 2010). The first step in alginate catabolism is the depolymerization of the polysaccharide by alginate lyases, releasing unsaturated oligosaccharides that are further degraded down to the monosaccharide 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH). Dehydrogenases then convert DEH into 2-keto-3-deoxy-D-gluconate, which is further phosphorylated by a carbohydrate kinase. The final product is 2-keto-3-deoxy-6-phosphogluconate (KDPG) that can be directly assimilated through the Entner-Doudoroff pathway (Preiss and Ashwell, 1962a,b). To date, this complete pathway for alginate degradation has only been studied in a few terrestrial or pathogenic species, which degrade bacterial alginates in their natural environment. Several alginate lyases were also characterized in various marine bacteria (Lombard *et al.*, 2010). However, systems integrating perception and complete degradation of algal alginate by marine bacteria have never been reported in the literature.

Among marine heterotrophic bacteria, members of the *Bacteroidetes* phylum are increasingly recognized as specialists for the degradation of polysaccharides (Kirchman, 2002; Thomas *et al.*, 2011a). Within this phylum, the flavobacterium *Zobellia galactanivorans* is being developed in our group as a model organism for the degradation of algal biomass (Thomas *et al.*, 2011b). *Zobellia galactanivorans* was isolated in Roscoff (Brittany, France) from the red alga *Delesseria sanguinea* (Barbeyron *et al.*, 2001) and utilizes a wide range of algal polysaccharides as a sole carbon source (Michel *et al.*, 2006; Thomas *et al.*, 2011a). This suggests the existence of complete utilization pathways for these complex polymers. The recent sequencing of *Z. galactanivorans* genome confirmed this huge potential for polysaccharide bioconversion, with the presence of more than 130 Carbohydrate Active Enzymes (CAZymes) and 72 sulfatases (Barbeyron *et al.*, unpublished). In the present study we report the identification and transcriptomic characterization of the complex alginolytic system in *Z. galactanivorans*, demonstrating its organization into two genuine alginolytic operons. Moreover, activity assays performed on several recombinant enzymes confirmed that this Alginate Utilization System (AUS) can account for the complete breakdown of alginate into KDPG. In addition, we provide phylogenomic evidence that the AUS emerged in an ancestral marine flavobacterium and was transferred through independent lateral gene transfers (LGT) to marine *Proteobacteria*, but also to several Japanese gut *Bacteroides* due to the traditional consumption of brown algae in Japan.

Results

Census of genes potentially involved in alginate degradation

Seven genes encoding alginate lyases were predicted in the genome of *Z. galactanivorans* (*alyA1–alyA7*) (Fig. 1). These putative alginate lyases belong to four different CAZy families (Lombard *et al.*, 2010). AlyA1 (Zg1182), AlyA2 (Zg2618) and AlyA5 (Zg4131) are classified in family 7 of the polysaccharide lyases (PL7), where known activities are alginate lyase (EC 4.2.2.3) and α-L-guluronate lyase (EC 4.2.2.11). Their sequences contain a cleaved lipoprotein signal, predicting localization in the outer membrane. AlyA2 and AlyA5 have similar molecular masses (35 and 39 kDa, respectively), whereas AlyA1 is larger due to the presence of a carbohydrate binding module (CBM) belonging to family 32. Because CBM usually evolved similar substrate specificity than their appended catalytic module (Michel *et al.*, 2009), this CBM32 likely binds alginate and potentiates the enzymatic activity of the PL7 module. AlyA3 (Zg2624) belongs to the PL17 family, in which the only known activity is alginate lyase. This enzyme possesses a cleavable signal peptide, and is thus predicted as a periplasmic or extracellular protein (Fig. 1). AlyA4 (Zg4130) and AlyA6 (Zg4132) both belong to the PL6 family in which known activities are alginate lyase and chondroitinase B (EC 4.2.2.4) (Michel *et al.*, 2004). To date, the only characterized alginate lyase in this family is Q06365 from *Pseudomonas* sp. OS-ALG-9, which has a mass of 44.5 kDa (Maki *et al.*, 1993). AlyA4 and AlyA6 are twice as large as the *Pseudomonas* enzyme, both featuring an additional C-terminal module of unknown function. AlyA4 contains an uncleavable signal peptide, and is thus likely localized in the periplasm anchored to the cytoplasmic membrane. AlyA6 displays a cleavable signal peptide, suggesting that this protein is exported in the periplasm or in the extracellular medium. AlyA7 (Zg4327) is classified in the PL14 family, which comprises alginate lyases, exo-oligoalginate lyases (EC 4.2.2.-) and β-1,4-glucuronan lyases (EC 4.2.2.14). This protein features a cleavable signal peptide, and is predicted to be either periplasmic or extracellular. Moreover, another protein, Zg3452, displays 24% sequence identity on 492 amino acids (total length 768 amino acids) with the PL15 exotype alginate lyase Atu3025 from *Agrobacterium tumefaciens* C58 (Ochiai

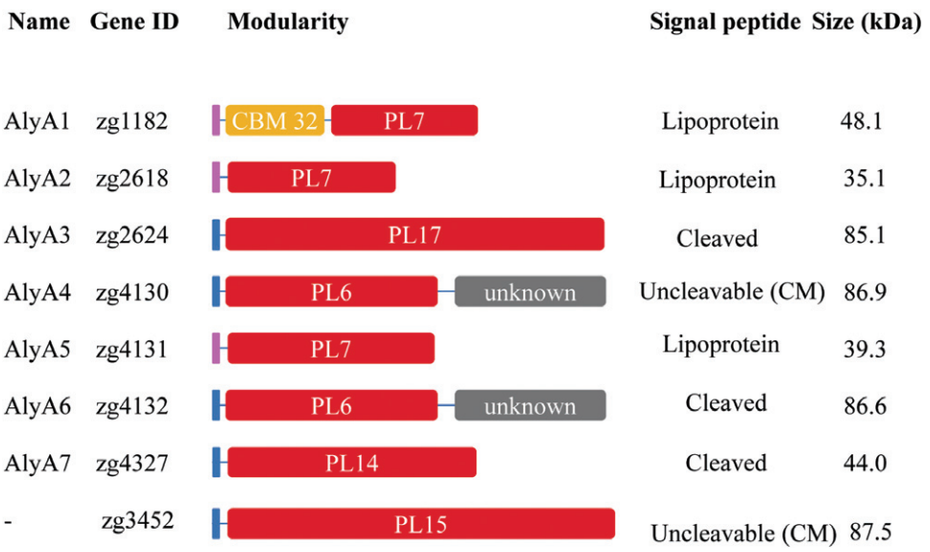


Fig. 1. Modular analysis of the putative alginate lyases predicted in *Z. galactanivorans* genome. PL, polysaccharide lyase (CAZy family); CBM, Carbohydrate Binding Module; CM, cytoplasmic membrane.

et al., 2010). Currently, the only known activities in the PL15 family are oligo-alginate lyase and alginate lyase, suggesting that Zg3452 may be an alginate lyase despite its distant relationship with Atu3025. Finally, *Z. galactanivorans* possesses two enzymes, Zg3445 and Zg3804 (named UglA1 and UglA2, respectively), which belong to the GH88 family that encompasses glycoside hydrolases specific for unsaturated oligosaccharides released by polysaccharide lyases (Itoh *et al.*, 2006). Thus, UglA1 and UglA2 may be good candidates to further process the degradation products of alginate lyases and were included in the present analysis.

The genes *alyA1*, *alyA7*, *uglA1*, *uglA2* and *zg3452* are isolated in the genome of *Z. galactanivorans*, without any neighbouring gene obviously related to alginate utilization. In contrast, *alyA4*, *alyA5* and *alyA6* constitute a small gene cluster (6 kbp), while *alyA2* and *alyA3* are localized in a large cluster of 13 genes (15.9 kbp, Fig. 2A). Most of these genes have a predicted function related to carbohydrate metabolism: a sugar permease (*zg2616*), two sugar oxidoreductases (*zg2615* and *zg2622*), two carbohydrate kinases (*zg2613* and *zg2614*) and a lipoprotein comprised of a PKD module and a putative CBM (*zg2619*). The large cluster also contains four genes homologous to the genes *gntR* (*zg2617*), *susC* (*zg2621*), *susD* (*zg2620*) and *kdgF* (*zg2623*). GntR is a transcriptional factor negatively regulating the *gntRKU* operon involved in the uptake and catabolism of gluconate in *Escherichia coli* (Izu *et al.*, 1997). SusC is a TonB-dependent receptor (TBDR) of *Bacteroides thetaiotaomicron* specific for the uptake of starch degradation products, while SusD is an associated outer membrane lipoprotein shown to bind malto-oligosaccharides (Anderson and Salyers, 1989; Koropatkin *et al.*, 2008). KdgF is involved in the pectinolytic system of *Dickeya dadantii* (formerly known as *Erwinia chrysanthemi*), but its exact function is unknown (Condemine and Robert-Baudouy, 1991). The last gene of the cluster, *zg2612*, encodes a putative divalent cation transporter of the NRAMP family (Natural Resistance-Associated Macrophage Protein).

Transcriptomic analyses of the alginate-related gene clusters

The genetic organization of the two identified clusters (Fig. 2A) suggested they could be transcribed as polycistronic mRNAs. To test this hypothesis, total RNAs were extracted from bacteria grown with either glucose or alginate as sole carbon source, and used in RT-PCR reactions to amplify regions covering several genes. Reactions using three different primer pairs gave positive results with samples from the culture condition with alginate (Fig. 2B, lanes 1A, 2A and 3A). This is consistent with the presence of messenger RNAs corresponding to genes *zg2624* to *zg2618*, *zg2618* to *zg2613* and *zg4130* to *zg4132* respectively. Because the primer sets 1 (fw1 + rv1) and 2 (fw2 + rv2) were designed to give two overlapping amplification products (Fig. 2A), results of experiments with these oligonucleotides showed that the 12 genes from *zg2624* to *zg2613* can be transcribed on a 15.9 kb polycistronic mRNA. No PCR products were detected using samples from the culture with glucose as sole carbon source, indicating the absence of the polycistronic transcripts in this condition (Fig. 2B, lanes 1G, 2G and 3G).

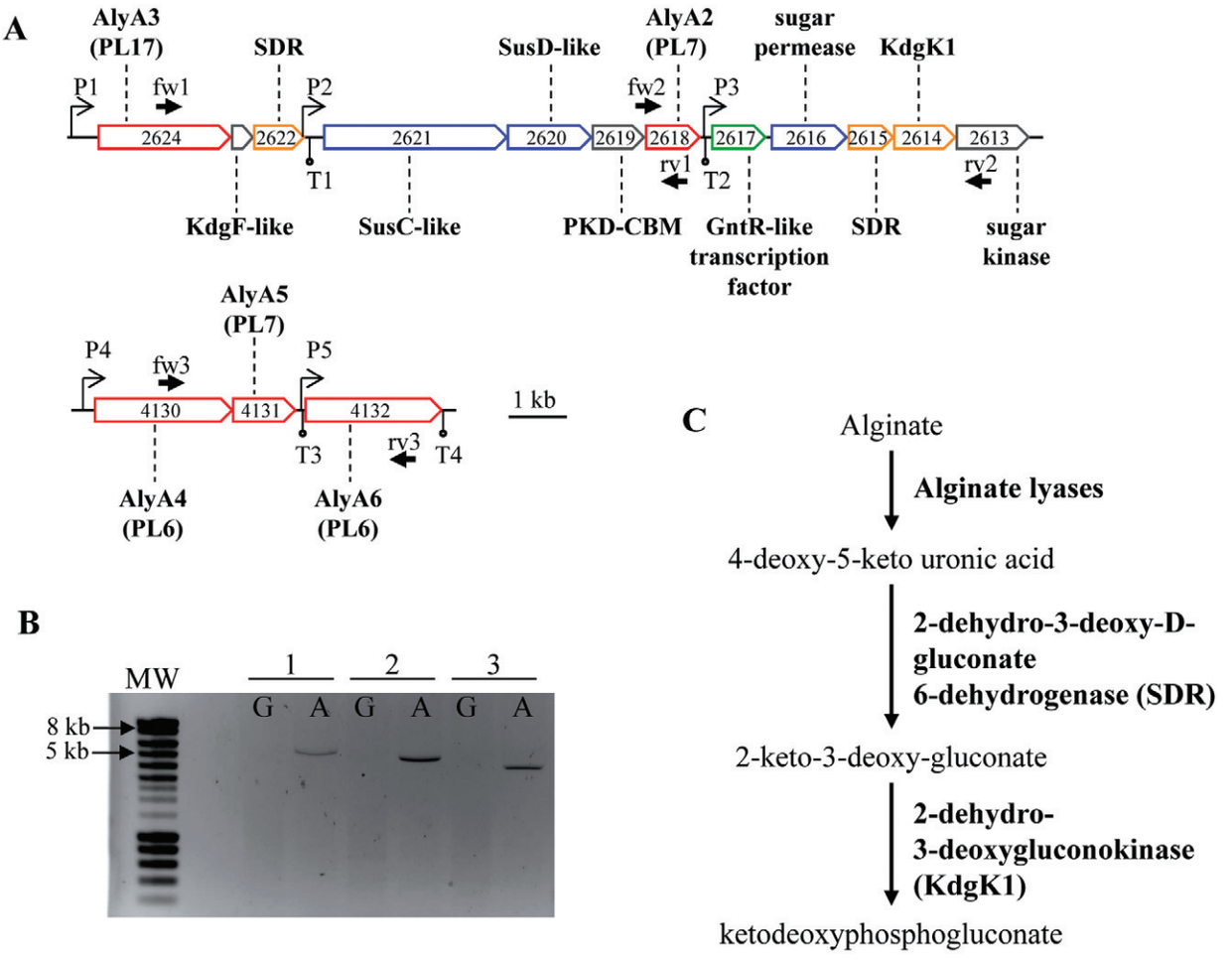


Fig. 2. Organization of the two alginolytic operons of *Z. galactanivorans*.
A. Genetic organization of the two alginolytic clusters found in *Z. galactanivorans* genome. The functions of the proteins are colour-coded: red, alginate lyases; orange, other degradation enzymes; green, putative regulation factor; blue, membrane proteins involved in binding/transport; grey, unknown function. Predicted promoters (P1 to P5) and Rho-independent terminators (T1 to T4) are depicted, as well as primers used for the RT-PCR analysis (black arrows; fw: forward; rv: reverse).
B. RT-PCR analysis of the transcription pattern for the two clusters. Agarose gel of RT-PCR products resulting from primer pairs shown in (A). RT-PCR were performed on RNA extracted from culture with glucose (G) or alginate (A). Lanes: MW, molecular mass standard; 1, primer set 1; 2, primer set 2; 3, primer set 3.
C. Biochemically validated utilization pathway for alginate in *Z. galactanivorans*.

DNA sequence analyses of the two clusters were performed to identify patterns that mediate transcription, such as promoters and Rho-independent terminators. Two and three sequences were found matching the consensus promoter identified in other *Bacteroidetes* species (Chen *et al.*, 2007a,b), for the 6 kb and 15.9 kb clusters respectively (Fig. 2A and Table S1A). These five putative promoters fulfil the TTG/TAnnTTTG consensus, except for P2, which contains one mismatch. The length of the spacer separating the –33 and –7 motifs was always 19 bp. Moreover, the identified sequences were relatively poor in GC (between 12% and 34% of GC) compared with the mean value for the whole genome (43% of GC). These parameters are consistent with studies in *Flavobacterium* species, showing that the promoter strength is influenced by the spacer length (19 bp being the optimal value), and is higher with AT-rich sequences (Chen *et al.*, 2007a). Sequence analyses also revealed two putative terminators (T1 and T2, Fig. 2A and Table S1B) in the intergenic regions of the 15.9 kb operon: the first one between *zg2622* and *zg2621* and the second one between *zg2618* and *zg2617*. Two other terminators (T3 and T4) were predicted in the 6 kb operon, between *zg4131* and *zg4132* and after *zg4132*.

Regulation at the transcriptomic level during alginate degradation was further studied in *Z. galactanivorans*

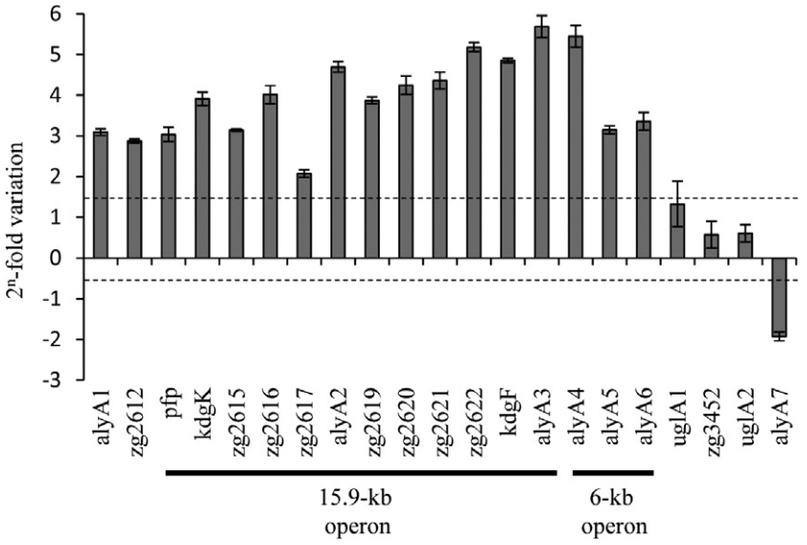


Fig. 3. Differential gene expression in the presence of alginate compared with glucose. Transcript levels were quantified by RT-qPCR. Values are mean ± SEM ($n = 3$). Dotted lines denote the significance threshold (2^n-fold-variation > 1, upregulated; 2^n-fold-variation < 1, downregulated).

using RT-qPCR. The relative expression of 20 genes predicted to be involved in alginate degradation was measured in triplicate (Table S2). Namely, the 12 genes from the 15.9 kb cluster, the three alginate lyase genes from the 6 kb cluster and five other alginate-related (AR) genes isolated in the genome, were included in this study. Furthermore, the gene *zg2612*, which encodes a divalent cation transporter, was added to the set of genes tested. Although its predicted function was not related to carbohydrate metabolism, its position directly upstream of the 15.9 kb operon suggested it could be co-regulated with adjacent genes. Expression of most of the target genes was indeed higher in the presence of alginate compared with glucose, with 2^n-fold variations between 2.1 for *zg2617* and 5.7 for *alyA3* (Fig. 3 and Table S2). The transcription levels were relatively similar between glucose- and alginate-containing media (2^n-fold variation between 0 and 1.3) for *uglA1*, *uglA2* and the gene *zg3452* encoding the PL15 enzyme. Interestingly, the expression level of genes encoding alginate lyases was relatively low in absence of alginate, except for *alyA7*, which was significantly higher than the six others (Tukey-Kramer test, P-value < 0.001). To assess the possibility that the observed effects were due to catabolic repression by glucose, the expression of the target genes was also measured when *Z. galactanivorans* was grown in the complex Zobell medium. Expression values were similar to results obtained with glucose as sole carbon source, except for the genes *alyA1* and *alyA7* (Table S2).

Biochemical characterization of enzymes involved in alginate catabolism

To confirm the influence of alginate on the expression of alginate-related proteins, alginate lyase activities were measured in the extracellular, intracellular and membrane-bound fractions of *Z. galactanivorans*. Very low enzymatic activities were recovered in the extracellular and intracellular soluble fractions produced from cultures containing glucose as sole carbon source, and no activity was detected in the membrane-bound fraction (Table 1). In contrast, the activities were higher by two orders of magnitude in the extracellular and soluble intracellular fractions obtained from cultures grown with alginate, and activity was also detected in the membrane-bound fraction. The alginate lyase activity detected in the culture supernatant (0.32 U $ml^{-1)}$ was purified by anion exchange chromatography. SDS-PAGE analysis revealed that this enzymatic activity corresponded to a single protein with an estimated molecular mass of 40 kDa (Fig. 4A). The protein was digested with trypsin and 35 peptides were identified by mass spectrometry. These peptides perfectly matched AlyA1, covering 60% of the whole sequence (Fig. 4B). In addition, the calculated molecular mass of AlyA1 after cleavage of its signal peptide was in agreement with the experimental mass determined on SDS-PAGE. Consequently, the protein purified from the culture supernatant was identified to be the alginate lyase AlyA1.

Table 1. Total alginate lyase activity in the extracellular, intracellular soluble and membrane-bound fractions.

Carbon source	Total activity in fraction (mU)[a]		
	Extracellular	Intracellular soluble	Membrane-bound
Glucose	4.9	5.7	not detected
Alginate	266.1	535.9	27.3

a. One unit of activity was defined as an increase of 1.0 in absorbance at 235 nm per min.

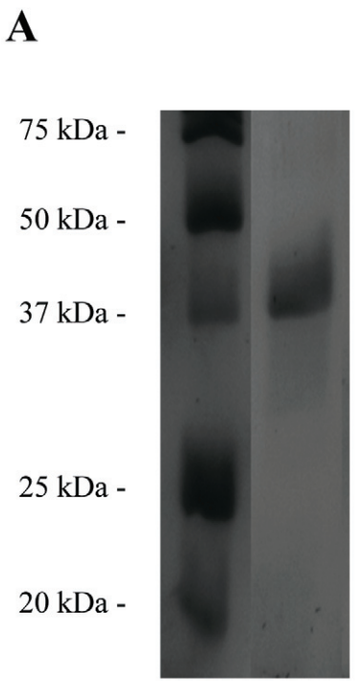


Fig. 4. Purification and identification of AlyA1 in the extracellular fraction.
A. SDS-PAGE analysis of the active, purified protein.
B. Superposition of AlyA1 protein sequence and peptides identified in mass spectrometry (red).

A biochemical validation strategy was applied for 10 genes of *Z. galactanivorans* encoding enzymes potentially involved in the various steps of alginate degradation (Table S3). The coding regions of the seven predicted alginate lyases (AlyA1-AlyA7), one GH88 enzyme (UglA1, Zg3445), one sugar oxidoreductase (Zg2622) and one carbohydrate kinase (KdgK1, Zg2614) were cloned into the pFO4 expression vector (N-terminal His-Tag fusion). Seven recombinant enzymes were successfully expressed in a soluble form in the *E. coli* BL21(*DE3*) strain, namely AlyA5, AlyA7, UglA1, KdgK1, Zg2622 and the catalytic modules of AlyA1 and AlyA4 (Table S3). AlyA3 could not be amplified using the designed primers, whereas AlyA2 and AlyA6 were not expressed in *E. coli* BL21(*DE3*) strains (Table S3). The seven soluble recombinant enzymes were purified by nickel ion affinity chromatography (Fig. S1). The alginate lyase activity was monitored by measuring the increase in absorbance at 235 nm, corresponding to the release of unsaturated oligosaccharides. Using this assay, AlyA1, AlyA4, AlyA5 and AlyA7 were found to be able to degrade alginate, with specific activities of 189.7, 1.2, 17.4 and 49.7 U mg^{-1}, respectively, confirming that these proteins are indeed active alginate lyases. In-depth biochemical and structural characterization of AlyA1 and AlyA5 (family PL7) is underway. Notably, AlyA5 has been shown to be an exo-type alginate lyase, mainly releasing unsaturated monosaccharides, which are spontaneously converted into 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH) (Thomas *et al.*, unpublished). In absence of commercially available DEH, the activity of putative oxidoreductase Zg2622 was tested by monitoring the oxidation of NADH in the presence of DEH released by AlyA5. In contrast to the control condition, a fast and large decrease of absorbance at 340 nm (A_{340}) was observed in the presence of Zg2622 (Fig. S2), indicating that the cofactor was oxidized. Therefore, DEH was reduced into 2-dehydro-3-deoxy-D-gluconate (KDG), confirming that Zg2622 is a 2-dehydro-3-deoxy-D-gluconate 6-dehydrogenase (EC 1.1.1.126). Similarly, KDG produced by Zg2622 was used as substrate to test the putative kinase activity of Zg2614, with ATP as a cofactor. Activity was determined by enzyme-coupled assay indirectly linking the phosphorylation of KDG to the oxidation of NADH into NAD^+ (Kim and Lee, 2006). In four independent trials, a significant decrease of A_{340} was observed in the presence of Zg2614 in comparison with the boiled enzyme ($\Delta A_{340} = -0.213 \pm 0.017$). Therefore, Zg2614 is able to phosphorylate KDG into 2-keto-3-deoxy-6-phospho-D-gluconate (KDPG) and is thus an active 2-dehydro-3-deoxygluconokinase (EC 2.7.1.45). Finally the putative glycoside hydrolase UglA1 was tested on unsaturated oligoalginates released by AlyA1, but no activity could be detected at any pH tested.

Table 2. Distribution of the Alginate Utilization System in the different bacterial classes.

Taxon	Number of genomes	Species with alginolytic operon	%
Bacteroidetes	135	20	14.8
Flavobacteriia	36	13	36.1
Cytophagia	8	1	12.5
Sphingobacteriia	9	1	11.1
Bacteroidia	79	5	6.3
Proteobacteria	668	19	2.8
Gammaproteobacteria	279	16	5.7
Alphaproteobacteria	193	3	1.6

Phylogenetic distribution of the alginate-related clusters

Homologues of the alginate lyases from *Z. galactanivorans* were searched for in all the genomes of Archae (93 genomes) and bacteria (1743 genomes) available on the NCBI genomic blast server (10 June, 2011). The genomic vicinity of each identified alginate lyase gene was then screened for homologues of the other alginate-related (AR)-genes from *Z. galactanivorans*, in order to determine the presence of AR-clusters in these microbes. Such AR-clusters are absent from Archae and are only present in two bacterial phyla, the *Bacteroidetes* (20/136 genomes) and the *Proteobacteria* (19/668 genomes). However, the AR-cluster distribution is not even in the different bacterial classes (Table 2): they are very frequent in the *Flavobacteriia* (36.1% of the sequenced genomes), while they are uncommon in the *Alphaproteobacteria* and *Gammaproteobacteria* (1.6% and 5.7% respectively). The marine habitat largely dominates the environmental distribution of the bacteria containing AR-clusters (31/39 genomes), including North Atlantic Ocean, North Pacific Ocean, Southern Ocean, North Sea and English Channel, Mediterranean Sea and Sea of Japan (Fig. S3). The exceptions are limited to three terrestrial microorganisms (the sphingobacterium *Pedobacter saltans* DSM 12145 and two strains of *Xanthomonadales, Stenotrophomonas maltophilia* K279a and R551-3) and five species of human gut *Bacteroides*. Among these gut bacteria two species were isolated from individuals of Japanese origin (*Bacteroides clarus*, from Japanese volunteers, and *Bacteroides eggerthii* from Japanese Hawaiian volunteers) (Holdeman and Moore, 1974; Watanabe *et al.*, 2010). The three other *Bacteroides* strains were isolated in USA from female patients during medical analyses, but there is no information concerning the ethnicity of these individuals (http://www.broadinstitute.org/annotation/genome/bacteroides_group/GenomeDescriptions.html).

With the exception of the putative phosphotransferase *zg2613*, which is only conserved in *Cellulophaga algicola*, the large AR-cluster of *Z. galactanivorans* is almost entirely conserved throughout 12 flavobacterial genera (Fig. 5 and Table S4). The synteny of this gene cluster is also well preserved in these marine flavobacteria. In most cases, there is only one large AR-cluster, which also encompasses homologues of the alginate lyase genes localized in the small AR-cluster of *Z. galactanivorans* (*alyA4*, *alyA5* and *alyA6*). In contrast, the AR-clusters are much shorter in the other bacterial lineages and their gene composition is more variable. For instance, the *susD*/*susC*-like genes (*zg2620* and *zg2621*) are only conserved in other *Bacteroidetes* classes (*Bacteroidia, Cytophagia* and *Sphingobacteriia*). Although there is no *susD*-like gene in *Proteobacteria*, a TBDR gene is nonetheless present in the AR-cluster of the *Alphaproteobacteria* and most of the *Gammaproteobacteria*. However, these TBDRs are too distantly related to *zg2621* to be its orthologous genes. The closest gene from *Z. galactanivorans* is *zg1355* (~ 25% sequence identity with its proteobacterial homologues), an isolated TBDR gene without an associated *susD*-like gene. The *Vibrio* species constitute an exception among the *Proteobacteria*, as they do not possess significant homologue of alginate-related TBDR (*Bacteroidetes*- or *Proteobacteria*-type) and of the sugar permease Zg2616. Furthermore, the PKD-CBM protein Zg2619 is unique to *Flavobacteriia*, while the alginate lyase AlyA2 (PL7) is only conserved in two *Vibrio* species (Fig. 5 and Table S4). The synteny is also less preserved among *Proteobacteria*, even between different species of a same genus (e.g. *Pseudoalteromonas haloplanktis* and *Pseudoalteromonas atlantica*), with frequent interruption of the AR-cluster by unrelated genes.

Because some AR-genes were not found in several gene clusters, a systematic search for isolated genes, homologous to the AR-genes from *Z. galactanivorans* (> 40% sequence identity), was performed in each genome. Few isolated AR-genes were indeed found in some species, but even with these additional genes the AR-pool of genes from *Sphingobacteriia*, *Bacteroidia, Cytophagia* and *Proteobacteria* remain incomplete in comparison with the flavobacterial operons. Finally, phylogenetic trees of each protein family of the alginolytic system (with the exception of Zg2613 and Zg2619 that are unique to *Flavobacteriia*) were reconstructed using a maximum likelihood method (Fig. S4). Flavobacterial proteins cluster as a single group for three protein families (NRAMP, GntR-like and AlyA3), but they emerge in two distinct clades in the other cases. These intriguing results are explained by gene duplication events followed by a divergent evolution of the duplicated genes. Indeed, several protein families comprise at least two divergent paralogous genes in the genomes of extant *Flavobacteriia*. The presence of duplicated *kdgK-, susD-* and *susC*-like genes is limited to two species, *Flavobacteriales* bacterium ALC-1 and *Polaribacter dokdonensis* MED152.

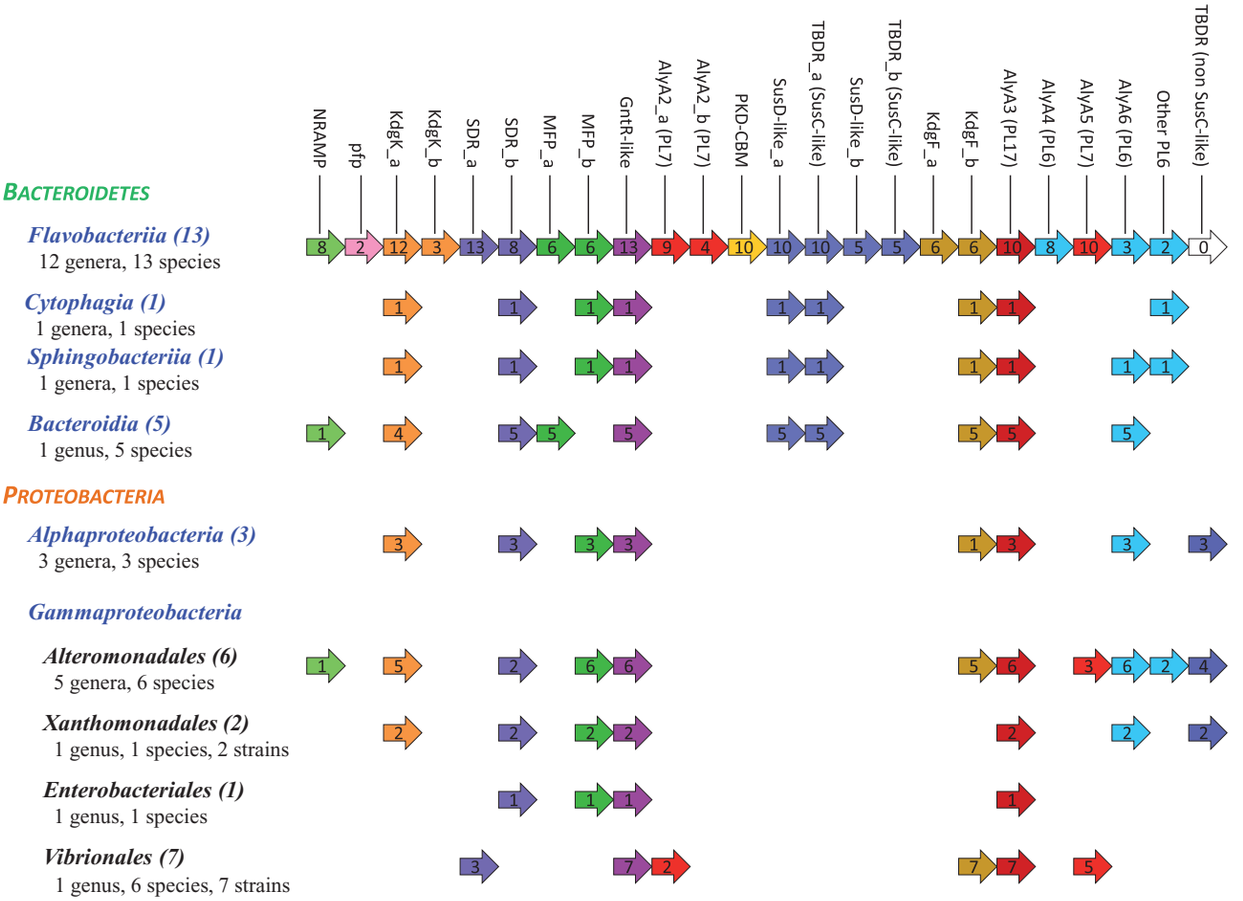


Fig. 5. Phylogenetic distribution of the Alginate Utilization System. The number of strains for which each gene was found is indicated in the arrow. The number of strains considered in each bacterial group is given between brackets. The gene organization was arbitrarily represented as found in *Z. galactanivorans*. Duplicated genes share the same name, followed by '_a' or '_b'. By definition, the a-type genes are found in *Z. galactanivorans*. This figure was built from the information presented in Table S4.

In contrast, the sugar oxidoreductases *zg2615* and *zg2622*, and the alginate lyases *alyA4* and *alyA6* (PL6 family) are conserved as paralogous genes in most flavobacterial species. The phylogenetic analysis also revealed several cryptic gene duplications, defined here as the occurrence of a gene duplication in the most recent common ancestor of a group of organisms, followed by the loss of one copy (referred to as *gene a*) in one subgroup of descendants and of the second copy (*gene b*) in the other subgroup. This is clearly the case for the sugar permease (*zg2616*) and the *kdgF*-like gene (*zg2623*). A cryptic duplication of *susD*- and *susC*-like genes also occurred in most flavobacterial species, but only *Flavobacteriales* bacterium ALC-1 and *P. dokdonensis* MED152 conserved both copies. In the subsequent paragraphs, the duplicated genes will be distinguished by the letters a and *b*, the *a*-group including by definition the gene(s) from *Z. galactanivorans*.

Interestingly, the AR-proteins from the gut *Bacteroides* constitute single clades, which are sister groups of the proteins from marine *Flavobacteriia* or are even rooted by them. The gut *Bacteroides* and the marine *Flavobacteriia* have in common only one copy of each duplicated gene, namely KdgK_a, SDR_b, MFP_a, *susD*-like_a *susC*-like_a, KdgF_b and *alyA6* (Fig. 5). In most phylogenetic trees the AR-proteins from *Vibrio* species emerge into monophyletic clades distinct from the other *Gammaproteobacteria*, which rather cluster with the *Alphaproteobacteria* (e.g. SDR, MFP, GntR-like, AlyA3, AlyA6). In several cases, the proteobacterial proteins constitute sister groups of the AR-proteins from marine *Flavobacteriia* or even emerge within a flavobacterial clade (e.g. SDR, MFP, AlyA3). As observed for the *Bacteroidia*, *Cytophagia* and *Sphingobacteriia*, the *Proteobacteria* have conserved only one copy of the flavobacterial duplicated genes. It is noteworthy that the *Vibrio* species have not conserved the

same gene copies than the other *Gammaproteobacteria* (Fig. 5).

Discussion

Z. galactanivorans *possesses a complex Alginate Utilization System organized into two operons*

The genome of *Z. galactanivorans* contains numerous genes predicted to act in alginate catabolism, including seven putative alginate lyases belonging to four different PL families and with various modular architectures (Fig. 1). In this work, we have experimentally confirmed that at least four of these genes (*alyA1*, *alyA4*, *alyA5* and *alyA7*) indeed encode active alginate lyases. This diversity of polysaccharide lyases is reminiscent of the complex enzymatic systems involved in the deconstruction of plant cell walls (Gilbert, 2010). It likely reflects the difficulty to degrade alginates in the cell walls of brown algae. Indeed, alginates form fibres in tight interaction with the other components of brown algal cell walls (Popper *et al.*, 2011), whereas they constitute a simple exopolysaccharide in bacteria. We have also demonstrated the biochemical function of two additional proteins involved in alginate catabolism. Zg2622 is a 2-dehydro-3-deoxy-D-gluconate 6-dehydrogenase, which reduces the DEH released by the exo-type alginate lyase AlyA5 to produce KDG. This compound is further phosphorylated into KDPG by the 2-dehydro-3-deoxygluconokinase Zg2614 (KdgK1). KDPG is finally assimilated through the Entner-Doudoroff pathway (Preiss and Ashwell, 1962a,b). To date, the only other characterized 2-dehydro-3-deoxy-D-gluconate 6-dehydrogenase originates from the soil bacteria *Sphingomonas* sp. A1 (Takase *et al.*, 2010) and is a distant homologue of Zg2622 (36% sequence identity). Few 2-dehydro-3-deoxygluconokinases were characterized in the context of the microbial degradation of gluconate and pectins (Hugouvieux-Cotte-Pattat *et al.*, 1994; Ohshima *et al.*, 2004; Potter *et al.*, 2008). KdgK1 from *Z. galactanivorans* is distantly related to these kinases (~ 30% sequence identity) and is the first to be linked to the catabolism of alginate. Both *zg2622* and *zg2614* possess a paralogous gene in *Z. galactanivorans* genome, *zg2615* and *zg4703* respectively. *zg2615* is localized in the same gene cluster as *zg2622* and the two corresponding proteins display 41% sequence identity. The functional redundancy or differences between these paralogous dehydrogenases remain to be established. In contrast, Zg4703 (KdgK2) is highly similar to Zg2614 (94% sequence identity). Interestingly, KdgK2 is located next to a putative KDPG aldolase (Zg4702), which may catalyse the step just after the KDG phosphorylation within the Entner-Doudoroff pathway (conversion of KDPG into pyruvate and glyceraldehyde 3-phosphate). Finally, we have invalidated our initial hypothesis that the putative glycoside hydrolase Zg3445 (UglA1, family GH88) could cleave unsaturated oligoalginates. Therefore, the exo-type activity of AlyA5 seems sufficient to release unsaturated monosaccharides, which spontaneously convert into DEH. The exact specificity of UglA1 remains unclear.

Most of the alginate-related genes from *Z. galactanivorans* are colocalized within a large and a small cluster. By using RT-PCR, we have demonstrated that these gene clusters are transcribed as polycistronic mRNAs in the presence of alginate and thus constitute genuine operons (Fig. 2). Furthermore, the expression of all the genes within the two operons and of *alyA1* and *zg2612* was strongly upregulated (2^n-fold variation > 2) when alginate was used as sole carbon source instead of glucose (Fig. 3). This transcriptomic shift coincided with a drastic increase in the alginolytic activities detected in the extracellular, intracellular and membrane-bound fractions (Table 1). Moreover, the extracellular enzymatic activity was shown to be due essentially to AlyA1 (Fig. 4). We have already experimentally confirmed the role of six of these genes in the catabolism of alginate (see above). It is highly probable that the remaining genes also participate in alginate assimilation, due to their position within these operons and/or to the fact that alginate induces their transcription. Although it remains to be experimentally proven, we can confidently predict the function of the proteins encoded by some of these genes (Table S5). Clusters containing *susC*/*susD* homologues and other carbohydrate-related genes are referred to as Polysaccharide Utilization Loci (PUL) and are abundant in environmental and gut *Bacteroidetes* (Martens *et al.*, 2009; Thomas *et al.*, 2011a). However, very few PULs have been experimentally shown to be genuine operons. The transcription of the xylan utilization clusters from *Prevotella bryantii* B14 was recently analysed, but these gene clusters were not experimentally proven to be transcribed as polycistronic mRNAs (Dodd *et al.*, 2010; 2011). To our knowledge, the *zg2613-zg2624* cluster is thus the first polysaccharide utilization operon characterized in a marine bacterium and the second characterized in the *Bacteroidetes* phylum, after the starch utilization system of *B. thetaiotaomicron* (Anderson and Salyers, 1989).

Finally, the transcription level of *uglA1* was only slightly induced by alginate, whereas *zg3452* (PL15) and *uglA2* were not affected. This suggests that these three genes are not involved in alginate catabolism. Consistently, no activity could be detected for the recombinant UglA1 on unsaturated oligo-alginates. More surprisingly, the transcription of *alyA7* was repressed in the presence of alginate, whereas this gene was significantly expressed in cells growing in the minimal medium containing glucose (Fig. 3). However, we have experimentally demonstrated

that AlyA7 is an active alginate lyase. This unusual expression pattern suggests a seductive hypothesis: AlyA7 might be a 'sentry' enzyme, waiting to recognize alginate in the extracellular medium to release oligoalginates acting as initial signals to trigger the alginate degradation pathway of *Z. galactanivorans*.

Origin and evolution of the Alginate Utilization System

Comparative genomics revealed that the Alginate Utilization System (AUS) identified in *Z. galactanivorans* is conserved at various degrees in 39 bacteria and that its taxonomic distribution is strictly restricted to *Bacteroidetes* and *Proteobacteria.* A hypothesis suggesting that the AUS existed in the common ancestor of these two distant bacterial phyla would require multiple gene losses and is therefore highly unlikely. The most parsimonious scenario is the occurrence of one or several independent lateral gene transfers (LGT) between these diverse bacterial species. The completeness of the alginolytic operon in marine *Flavobacteriia*, its high frequency in this taxonomic group, and its high degree of conservation (both in term of gene composition and of synteny) strongly support that this AUS arose from an ancestral marine flavobacterium (Fig. 5). Furthermore, our phylogenetic analyses have unveiled gene duplications in the common ancestor of the AUS-containing flavobacteria. Several gene duplications are still visible in the genome of extant flavobacterial species (Fig. 5, Table S4), but some are now cryptic (i.e. MFP, *kdgF*-like, *susD*-like and *susC*-like). The conservation of specific gene copies in the various bacterial groups supports the occurrence of independent LGT events from distinct ancestral marine flavobacteria differing by the exact composition of their AUS. For instance, extant *Bacteroidia* display a sugar permease belonging to the MFP_a subgroup, whereas all the other bacteria possess the MFP_b subgroup. Similarly, the *Vibrio* species contain the a-type SDR (Zg2615), which is strictly conserved in the marine flavobacteria, whereas the other microorganisms have the b-type SDR (Zg2622), which has been lost by several extant marine flavobacteria.

Most of the AUS-containing microorganisms were isolated in coastal regions all around the world (Fig. S3). All these marine ecosystems host various species of brown algae (see algaeBASE, http://www.algaebase.org/), and thus comprise significant alginate resources. In this context, the acquisition of an efficient alginolytic pathway allows bacteria to take advantage of this prominent source of carbon. Marine *Flavobacteriia*, *Alphaproteobacteria* and *Gammaprotobacteria* usually dominate the microbial communities colonizing the surface of macroalgae (Staufenberger *et al.*, 2008; Salaun *et al.*, 2010; Tujula *et al.*, 2010). Therefore, this ecological promiscuity has certainly favoured the AUS transfers from marine *Flavobacteriia* to marine *Proteobacteria*. It is noteworthy that the alginate-producing bacteria (e.g. the *Gammaproteobacteria Pseudomonas aeruginosa* and *Azotobacter vinelandii*) and the terrestrial microorganisms degrading bacterial alginate (e.g. the *Alphaproteobacteria Sphingomonas* spp.) do not feature alginate-related operons similar to the large alginolytic operon identified in *Z. galactanivorans*. In *P. aeruginosa*, the alginate lyase activities are involved in the alginate production process and the gene *algL* encoding a family PL5 alginate lyase is localized in the alginate biosynthesis operon (Ramsey and Wozniak, 2005). The gene *aly* of *Sphingomonas* sp A.1, which encodes three different alginate lyases from a single precursor, is localized 1 kb upstream of the operon encoding the components of the pit-dependent ABC transporter. This complex directly imports alginate into the cells and is unique to this species (Mishima *et al.*, 2001).

The acquisition of an AUS of marine origin by several human gut *Bacteroides* is more surprising at first sight. Our group has recently demonstrated transfers of genes involved in the degradation of red algal polysaccharides, from marine *Flavobacteriia* to the Japanese gut bacterium *Bacteroides plebeius*. The traditional consumption of edible red algae (e.g. *Porphyra*) likely favoured these LGT events (Hehemann *et al.*, 2010; Rebuffet *et al.*, 2011). Interestingly, the present study indicates that AUS are present in two species isolated from Japanese and Japanese-Hawaiian individuals (*B. clarus* and *B. eggerthii*, respectively). Because several brown algae are part of the daily diet of Japanese, the most plausible scenario explaining this LGT is the uptake of AUS-containing marine *Flavobacteriia* at the surface of edible brown algae. Such transfers of individual CAZYme genes (Hehemann *et al.*, 2010; Rebuffet *et al.*, 2011) or even of complete polysaccharide utilization operons (this study) suggest that these LGTs are more frequent than initially expected (Hehemann *et al.*, 2010) and may be associated to the consumption of seafood in general.

Finally, the phylogenetic proximity between the *Flavobacteriia* and the *Bacteroidia* has likely facilitated the conservation of the core alginolytic operon observed in the *Bacteroidetes* (*kdgK*-like, SDR, sugar permease, *gntR*-like, *susD*-/*susC*-like, *kdgF*-like, *alyA3*, *alyA6*). In contrast, the core operon conserved in the evolutionary distant *Proteobacteria* (except the *Vibrio* species) is more limited (SDR, sugar permease, *gntR*-like, *alyA3*). In particular, the TonB-dependent receptor of the *Bacteroidetes* was replaced by another TBDR, specific for the *Proteobacteria*. This surprising event could be explained by the fact that SusD-like proteins are unique to *Bacteroidetes* (Thomas *et al.*, 2011a). Moreover, they interact physically and functionally with their associated TBDR (Martens *et al.*, 2009). For yet unknown reasons, SusD-like proteins seem incom-

patible with the physiology of the *Proteobacteria* and these transferred genes were lost by the proteobacterial species together with their associated *susC*-like genes. The AUS-containing *Proteobacteria* appear to have subsequently recruited another TBDR of proteobacterial origin, which evolved to fulfil the role of the lost alginate SusC/SusD homologues. The *Vibrio* species constitute an even more radical case, as these microorganisms lack obvious alginate-related TBDR and Zg2616-like sugar permease. This suggests that alginolytic *Vibrio* species have developed a different system for the uptake of alginate oligosaccharides. At the very moment of submitting our work, this hypothesis has been confirmed in the context of a bioengineering project aiming at building an *E. coli* strain able to produce bioethanol from brown seaweed (Wargacki *et al*., 2012). By an indirect genetic approach, these authors have identified that the outer membrane porin KdgMN (V12B01_24269 and V12B01_309) and the sodium/solute symporter V12B01_24194 (plasma membrane) are responsible for the uptake of oligoalginates in *Vibrio splendidus* 12B01 (Wargacki *et al*., 2012). While the porin KdgMN has no homologue in *Z. galactanivorans,* BLAST searches with V12B01_24194 as a query identifies various distantly related sodium/solute symporters, but none of them are obviously related to alginate metabolism. These results support our hypothesis that the alginate uptake systems in *Flavobacteriaceae* and *Vibrionaceae* are unrelated.

Conclusion

We have identified the first Alginate Utilization System in a marine bacterium, which is mainly organized in two operons inducible by the presence of alginate. This AUS comprises all the enzymes needed for the complete breakdown of the cell wall polysaccharide into the metabolic intermediate KDPG. The activities of these recombinant enzymes were assessed biochemically, confirming the bioinformatic predictions. Furthermore, the AUS encodes other proteins, which represent good candidates for detection and uptake of alginate degradation products. Such AUS emerged in an ancestral marine flavobacterium and was acquired by diverse bacteria, which thus gained the capacity to assimilate the main polysaccharide of brown algae, an adaptive advantage in the coastal environment but also in the gut microbiota of specific human populations.

Experimental procedures

Reagents

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich.

Bacterial strain and culture conditions

The type strain Dsij^T of *Z. galactanivorans* was routinely grown at 20°C in Zobell medium 2216E (5 g l^{-1} tryptone, 1 g l^{-1} yeast extract, sea water; ZoBell, 1941) until mid-exponential phase (A_{600} around 0.3). Cells were transferred either in fresh Zobell medium or in the Marine Mineral Medium supplemented with 2 g l^{-1} glucose or alginate as sole carbon sources. Briefly, one litre of Marine Mineral Medium is composed of 24.7 g NaCl, 6.3 g $MgSO_4.7H_2O$, 4.6 g $MgCl_2.H_2O$, 2 g NH_4Cl, 0.7 g KCl, 0.6 g $CaCl_2$, 200 mg $NaHCO_3$, 100 mg K_2HPO_4, 50 mg yeast extract, 20 mg $FeSO_4.7H_2O$, in Tris-HCl 50 mM pH 8.0 (Thomas *et al*., 2011b). Cultures were grown at 20°C and 150 r.p.m. For RNA isolation, 5 ml cultures were grown in 50 ml Falcon tubes. For protein extraction and purification, cultures were grown in flasks with baffles, allowing a 1:5 liquid to air ratio.

RT-PCR and quantitative PCR

Total RNA isolation and cDNA synthesis were performed as described previously (Thomas *et al*., 2011b). Briefly, RNA was isolated from 2 ml of cultures in mid-log phase (A_{600} 0.3–0.5) using RNA Mini Kit (Qiagen) and treated with Turbo DNase (Ambion). Total elimination of genomic DNA was checked by PCR on RNA samples. Eight hundreds ng of RNA were retro-transcribed using the Phusion RT-PCR Kit (Finnzymes) with random hexamer primers. cDNA samples were stored at −20°C and used in PCR reactions within a month. PCR reactions were performed using the Advantage 2 PCR Kit (Clontech) with 0.2 μM of each primer (Table S6) for 30 cycles (95°C for 30 s, 68°C for 6 min). Samples were analysed by electrophoresis on 0.8% agarose gel in TAE buffer and visualized by ethidium bromide. Quantitative PCR reactions were performed on cDNA samples using gene-specific primers (Table S6). Primers were validated (Table S7) as previously described (Thomas *et al*., 2011b). The geometric average of the transcript abundance of *glyA*, *icdA* and *gmkA* was used to normalize the expression of the genes of interest, as described previously (Thomas *et al*., 2011b); 2^n-fold variations of expression in alginate relative to growth in glucose were calculated.

Protein extraction

After microscopic observation to ensure that no significant cellular lysis had occurred, 8 ml of late-log phase (A_{600} 0.9–1.0) cultures in Marine Mineral Medium supplemented with either glucose or alginate were centrifuged 10 min at 3000 *g*. Supernatants (hereafter referred to as 'extracellular fraction') were conserved at 4°C. Cells were resuspended in 5 ml of ice-cold Tris-HCl buffer (0.1 M, pH 7.5) and disrupted in a French pressure cell under 1.6 kbar. Suspensions were centrifuged for 30 min at 20 000 *g* and at 4°C to remove cell debris. Supernatants were conserved at 4°C (hereafter referred to as 'intracellular soluble fraction'). Cell debris were washed in 3 ml of Tris-HCl buffer (0.1 M, pH 7.5) and centrifuged for 30 min at 20 000 *g* and 4°C. Pellets were resuspended in 1 ml of Tris-HCl buffer containing 0.1%

Tween 20 and incubated overnight to solubilize membranes. After centrifugation (22 000 *g*, 25 min, 4°C), supernatants were conserved at 4°C (hereafter referred to as 'membrane-bound fraction'). Protein concentrations in each fraction were determined by the Bradford method (Bradford, 1976) using BSA as a standard.

Purification and identification of a secreted enzyme

Bacteria were grown until early stationary phase (A_{600} 1.2) in mineral medium supplemented with alginate. After microscopic observation to ensure that no significant lysis had occurred, cells were removed by centrifugation for 20 min at 12 000 *g* and 4°C. Unless otherwise stated, the following treatments were conducted at 4°C. The alginate present in the supernatant was precipitated by slow addition of 0.25 volume of 100% ice-cold ethanol under agitation, and recovered by centrifugation for 30 min at 30 000 *g*. Alginate lyase activity was measured on the supernatant and the pellet. The pellet, which contained most of the alginolytic activity was resuspended in 10 ml of Buffer A (Tris-HCl 0.1 M, pH 7.5), incubated for 4 h at 30°C and passed through 0.2 μm filter. The sample was brought to 80% ammonium sulfate saturation and centrifuged for 20 min at 15 000 *g*. The supernatant (9.7 ml) was dialysed against Buffer A in membrane with 6–8 kDa MWCO (Spectrum Laboratories, USA) and injected on an anion exchange column (MonoQ, 1 ml, GE Healthcare) equilibrated with the same buffer. Proteins were eluted at 1 ml min^{-1} with a 20 ml linear gradient of 0 to 1 M NaCl in Buffer A and 0.5 ml fractions were collected. Elution was followed by the absorbance at 280 nm. Ten microlitres of each fraction were assessed for alginate lyase activity. An active fraction was obtained at around 300 mM NaCl and precipitated with three volumes of acetone before analysis by SDS-PAGE on 15% polyacrylamide gel. Coomassie blue-stained protein slices were cut into small pieces, washed with distilled water and destained using acetonitrile (ACN). The cysteine residues were reduced by 100 μl of 10 mM DTT at 56°C and alkylated by 150 μl of 55 mM iodoacetamide at room temperature. Iodoacetamide solution was replaced by 100 μl of 100 mM NH_4HCO_3 and gel dehydration was achieved with ACN. After evaporation in a Speed Vac (Thermo), proteins were digested overnight at 37°C by a solution containing 0.9 μg of modified bovine trypsin (Promega, Madison, WI, USA) prepared in 25 mM NH_4HCO_3. Finally, a double extraction was performed, first with 1% (v/v) trifluoroacetic acid (TFA) solution, and subsequently with 100% (v/v) ACN. The resulting peptide mixture was extracted and dried under vacuum and resuspended in 50% ACN, 0.1% TFA solution. Peptide Mass Fingerprinting by MALDI-TOF MS was performed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems). Each pooled tryptic digest (1 μl) was mixed with an equal volume of α-Cyano-4-hydroxycinnamic acid matrix (10 mg ml^{-1} in 50% ACN, 0.1% TFA) and spotted onto the MALDI target. Spectra were acquired in positive ion reflector mode under 20 kV accelerating voltage and a mass range of 450–5000 Da. Internal calibration was performed using trypsin autolysis fragments at m/z 1433.70, 2163.05 and 2289.1.

Cloning, expression and purification of recombinant enzymes

The genes encoding the seven putative alginate lyases (*alyA1-alyA7*), the sugar dehydrogenase/reductase (*zg2622*), the sugar kinase (*kdgK1*) and the GH88 enzyme (*uglA1*) were selected for a medium-throughput cloning strategy performed as previously described (Groisillier *et al.*, 2010). Briefly, primers were designed to amplify the coding region corresponding to the mature proteins, except for AlyA1 and AlyA4 for which only the catalytic module was cloned. The genes were amplified by PCR from *Z. galactanivorans* genomic DNA using the oligonucleotide primers reported in Table S3. Using the restriction sites indicated in Table S3, the PCR products were then ligated into the expression vector pFO4, resulting in a gene encoding the protein flanked by an N-terminal hexa-histidine tag. The recombinant plasmids were transformed into *E. coli* BL21(DE3) expression strains. Recombinant strains were cultured at 20°C during 3 days in 200 ml of ZYP medium (Studier, 2005) supplemented with 100 mg ml^{-1} ampicillin. The cells were resuspended in 25 mM Tris-HCl pH 7.5, 200 mM NaCl and 5 mM imidazole buffer, supplemented with one dose of an anti-protease cocktail (Complete EDTA free, Roche) and 0.1 mg ml^{-1} of DNase. Cells were disrupted in a French pressure cell under 1.6 kbar. The samples were centrifuged for 2 h at 20 000 *g* and at 4°C. The supernatant was loaded onto a HyperCell PAL column charged with $NiSO_4$ (0.1 M) and pre-equilibrated with resuspension buffer. The His-tagged proteins were eluted with a linear imidazole gradient (5–500 mM imidazole) at a flow rate of 1 ml min^{-1}.

Enzymatic activity assays

Alginate lyase activity was assayed by measuring the increase in absorbance at 235 nm (A_{235}), due to the release of unsaturated uronic acids, for 5 min at 25°C in a 1 cm quartz cuvette (Fineran *et al.*, 2005). Reaction mixtures (1 ml) were composed of 0.1% (w/v) alginate in Tris-HCl buffer (0.1 M, pH 7.5). One unit of enzyme activity was defined as an increase of 1 in A_{235}, at 25°C, and per min.

The 2-dehydro-3-deoxy-D-gluconate 6-dehydrogenase activity was assayed as described by Takase and coworkers (Takase *et al.*, 2010). In absence of commercially available 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEH), this compound was produced by the exo-alginate lyase AlyA5. This enzyme releases unsaturated monosaccharides, which spontaneously are converted into DEH. Two hundreds microlitres of purified AlyA5 (199 μg ml^{-1}) are added to a reaction medium (total volume: 5 ml) containing 0.25% (w/v) alginate sodium salt (Mannuronate/Guluronate ratio: 0.5, Danisco), 100 mM MOPS pH 7.0 and 200 mM $CaCl_2$. After incubation at 30°C for 19 h, the mixture is ultrafiltrated on an YM3 filter (3 kDa cut-off, Amicon) and the filtrate (5 ml) is conserved as the DEH solution. Reaction mixture for the dehydrogenase assay (total volume: 500 μl) consisted in 100 μl of DEH solution, 50 μl of NADH 2 mM, 250 μl potassium phosphate buffer 100 mM, 50 μl H_2O Milli-Q and 50 μl of purified Zg2622 (240 μg ml^{-1}). The kinetic was performed at 30°C and activity was monitored by measuring the decrease of the absorbance at 340 nm (A_{340}).

The 2-dehydro-3-deoxygluconokinase activity was tested by a coupled assay, as previously described (Kim and Lee, 2006). KDG was produced from DEH using Zg2622 (reaction mixture: 50 µl of purified Zg2622 (240 µg ml^{-1}), 100 µl of DEH solution, 50 µl of NADH 2 mM, 250 µl potassium phosphate buffer 100 mM and 50 µl H_2O Milli-Q). After 30 min at 25°C the reaction was stopped by heating the mixture at 100°C during 5 min. After cooling the medium at 25°C, 100 µl of KdgK1 (970 µg ml^{-1}) and 6 µl ATP 100 mM were added. After 30 min, 8 µl of PEP 100 mM, 40 µl of NADH 10 mM, 1.2 Units of lactate dehydrogenase from rabbit, 1 unit of pyruvate kinase from rabbit muscle and 1.5 µl of $MgSO_4$ 500 mM were added to the medium. The mixture was incubated for 40 min. at 37°C. The reaction mixture was then diluted with 1 ml H_2O and the formation of NAD was measured by the decrease of A_{340} as compared with a control made in the same way with the exception that the lactate dehydrogenase and the pyruvate kinase were previously boiled.

The UglA1 activity was tested on purified unsaturated alginate disaccharides (ΔG) released by AlyA1 by monitoring the decrease of A_{235}, as previously described (Hashimoto *et al.*, 1999). The reaction mixture (500 µl) contained 470 µl of reaction buffer [200 mM NaCl, 25 mM buffer (see below)], 5 µl of UglA1 (191 µg ml^{-1}) and 25 µl of ΔG (3 mg ml^{-1}). Different buffers were tested: Citrate pH 6.0, MOPS pH 7.0, Tris-HCl pH 7.5 or glycine NaOH pH 8.5. The reaction was monitored for 20 min at 30°C.

Sequence analyses

The alginate-related (AR) genes of *Z. galactanivorans* were identified in its complete genome (sequenced by Genoscope: http://www.genoscope.cns.fr/spip/-Marine-cytophagales-.html; Barbeyron *et al.*, unpublished) using the program GenDB (Meyer *et al.*, 2003). For each corresponding protein, evidence of conserved protein modules was queried on the Pfam database (Bateman *et al.*, 1998) and by BlastP searches against the GenBank nr database. Signal peptide and transmembrane helices were predicted using SignalP v2.0 (Bendtsen *et al.*, 2004) and TMHMM (Krogh *et al.*, 2001) respectively. The presence of homologous AR genes in all available prokaryotic genomes was screened using the genomic BlastP (Altschul *et al.*, 1997) at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The corresponding proteins were subjected to multiple sequence alignment using MAFFT (Katoh *et al.*, 2002), with the iterative refinement method and the scoring matrix Blosum62. These alignments were manually edited using Bioedit (Tom Hall). The different phylogenetic trees were derived from these refined alignments using the Maximum Likelihood method with the program PhyML (Guindon and Gascuel, 2003). The reliability of the trees was tested by bootstrap analysis using 100 resamplings of the dataset. The trees were displayed with MEGA 5 (Tamura *et al.*, 2011). To identify putative promoters, intergenic regions were searched using DNAMAN 4.15 for the motif –33 (TTG)/-7 (TAnnTTTG) with a spacer length ranging between 18 and 21 bp. This motif has been identified as the consensus promoter in *Flavobacterium* species, and is conserved in *Bacteroides fragilis* (Chen *et al.*, 2007a,b). The TransTermHP software was used to predict putative Rho-independent transcriptional terminators (Chen *et al.*, 2007a).

Accession numbers

The nucleotide sequence for the genome of *Z. galactanivorans* has been deposited at EMBL with the Accession No. FP476056.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. SDS-PAGE analyses of recombinant alginate-related proteins purified by nickel affinity chromatography. (1) PL7 module of AlyA1 (Zg1182); (2) AlyA5 (Zg4131); (3) Zg2622; (4) KdgK1 (Zg2614); (5) PL6 module of AlyA4 (Zg4130); (6) UglA1 (Zg3445); (7) AlyA7 (Zg3445).

Fig. S2. Activity assay of the putative dehydrogenase Zg2622. The 2-dehydro-3-deoxy-D-gluconate 6-dehydrogenase activity was assayed as described by Takase and coworkers (Takase *et al.*, 2010). Reaction mixture (450 μl) consisted in 100 μl of DEH solution (prepared with AlyA5), 50 μl of NADH 2 mM, 250 μl potassium phosphate buffer 100 mM and 50 μl of purified Zg2622 (240 μg ml^{-1}). The kinetic was performed at 30°C and activity was monitored by measuring the decrease of the absorbance at 340 nm (A_{340}). The blue curve corresponds to the active Zg2622, while the pink curve corresponds to the boiled enzyme.

Fig. S3. Geographical distribution of the species containing an Alginate Utilization System Each colour-coded spot locates the site of isolation of the sequenced strain. Zg,

Zobellia galactanivorans; Cal, *Cellulophaga algicola*; Cly, *Cellulophaga lytica*; Gf, '*Gramella forsetii*'; Ka, *Kordia algicida*; Ca, *Croceibacter atlanticus*; Dd, *Dokdonia donghaensis*; ALC-1, Flavobacteriales bacterium ALC-1; 2A, Flavobacteria MS024-2A; 3C, Flavobacteria MS024-3C; SCB49, Bacteroidetes SCB49; Pd, *Polaribacter dokdonensis* MED152; BBFL7, Flavobacteria bacterium BBFL7; Ps, *Pedobacter saltans*; Rm, *Rhodothermus marinus*; Bc, *Bacteroides clarus*; Be, *Bacteroides eggerthii*; Mm, *Maricaulis maris*; Hb, *Hirschia baltica*; Bs, *Brevundimonas subvibrioides*; Ph, *Pseudoalteromonas haloplanktis*; Pa, *Pseudoalteromonas atlantica*; Am, *Alteromonas macleodii*; Sd, *Saccharophagus degradans* 2–40; TW7, Alteromonadales bacterium TW-7; Sf, *Shewanella frigidimarina*; K279a, *Stenotrophomonas maltophilia* K279a; Vs, *Vibrio sinaloensis*; SWAT3, Vibrionales bacterium SWAT-3; Va, *Vibrio alginolyticus*; Vh, *Vibrio harveyi* 1DA3; MED222, *Vibrio* sp. MED222; 12B01, *Vibrio splendidus* 12B01; LGP32, *Vibrio splendidus* LGP32.

Fig. S4. Phylogenetic trees of the protein families related to the alginolytic system.
Table S1. Predicted promoters and terminators in the alginolytic clusters.
Table S2. Quantification of the transcript levels for the 21 genes analysed.
Table S3. Cloning strategy and expression results for recombinant alginate lyases.
Table S4. Census of genes of the Alginate Utilization System in different species.
Table S5. Putative functions of genes of the large alginolyic operon of *Z. galactanivorans.*
Table S6. Primers used in this study.
Table S7. Validation data for qPCR primer pairs.

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