Exploiting fine-scale genetic and physiological variation of closely related microbes to reveal unknown enzyme functions

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Polysaccharide degradation by marine microbes represents one of the largest and most rapid heterotrophic transformations of organic matter in the environment. Microbes employ systems of complementary carbohydrate-specific enzymes to deconstruct algal or plant polysaccharides (glycans) into monosaccharides. Because of the high diversity of glycan substrates, the functions of these enzymes are often difficult to establish. One solution to this problem may lie within naturally occurring microdiversity; varying numbers of enzymes, due to gene loss, duplication, or transfer, among closely related environmental microbes create metabolic differences akin to those generated by knock-out strains engineered in the laboratory used to establish the functions of unknown genes. Inspired by this natural fine-scale microbial diversity, we show here that it can be used to develop hypotheses guiding biochemical experiments for establishing the role of these enzymes in nature. In this work, we investigated alginate degradation among closely related strains of the marine bacterium Vibrio splendidus. One strain, V. splendidus 13B01, exhibited high extracellular alginate lyase activity compared with other V. splendidus strains. To identify the enzymes responsible for this high extracellular activity, we compared V. splendidus 13B01 with the previously characterized V. splendidus 12B01, which has low extracellular activity and lacks two alginate lyase genes present in V. splendidus 13B01. Using a combination of genomics, proteomics, biochemical, and functional screening, we identified a polysaccharide lyase family 7 enzyme that is unique to V. splendidus 13B01, secreted, and responsible for the rapid digestion of extracellular alginate.

These results demonstrate the value of querying the enzymatic repertoires of closely related microbes to rapidly pinpoint key proteins with beneficial functions.

Polysaccharide degradation by microbes represents one of the largest heterotrophic transformations of carbon-rich organic matter in the marine environment. Every year, marine algae transform ~45 gigatons of carbon dioxide from the atmosphere into biomass (1). Key constituents of algal biomass are glycans, which function as intracellular carbon/energy stores (2), cell wall building components (3), or secreted exudates (4). Glycans play a central role in the marine carbon cycle because they represent 30–80% of the total carbon content in algal matter, yet measurements of residual algal biomass in sinking particles obtained with sediment traps indicate that >99% of the autotrophic biomass that is produced at the surface, including the glycans, disappears throughout the water column (5–7). These results suggest that microbes, key decomposers of organic matter, have the capacity to efficiently deconstruct the wide variety of glycans produced by algae in the sea. However, most of the enzymes involved in the marine glycan cycle remain biochemically uncharacterized.

Microbes require multiple enzymes to deconstruct even the simplest glycan polysaccharides into simple sugars. These enzyme systems typically involve hydrolytic glycoside hydrolases or lytic polysaccharide lyases (PLs)4 (www.cazy.org)5 (8). Such enzyme systems have recently been described for a variety of algal polysaccharides (9–11). Alginate is a linear, carboxylated polysaccharide consisting of the mixed 1,4-linked epimers α-L-guluronate (G) and β-D-mannuronate (M), that is readily consumed by coastal microbial communities (12). It is one of the most abundant coastal polysaccharides and is found in the

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4 The abbreviations used are: PL, polysaccharide lyase; CBM32, family 32 carbohydrate-binding module; $G$, α-L-guluronate; M, β-D-mannuronate; qPCR, quantitative PCR; DP, degree of polymerization; TSb, tryptic soy broth; PDB, Protein Data Bank; TEAB, triethylammonium bicarbonate; NSAF, normalized spectral abundance factor; LCR, ligase cycling reaction; ESI, electrospray ionization.

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cell walls of brown seaweeds, such as kelp, that dominate primary production plant growth along the coasts of temperate marine provinces (13). Alginate has also been proposed as a renewable source for the production of biofuels (14, 15).

The enzymes involved in alginate degradation are known as alginate and oligoalginate lyases. They use β-elimination of the glycosidic bond to cleave alginate into smaller oligosaccharides or monomeric sugars (16, 17). They can be classified based on whether they preferentially degrade poly-M, poly-G, or poly-MG regions of alginate using either an endolytic or exolytic mechanism. In addition, the lyases can be secreted, membrane-bound, or intracellular. Many bacteria employ multiple alginate lyases to degrade alginate. A prime example is Saccharophagus degradans 2–40, which has 13 predicted alginate lyase genes (18), as well as other proteobacteria, including the Alteromonadales (19) and marine Flavobacteria, such as Zoobellia galactanivorans (20).

A recent study investigated the ability of 55 closely related marine Vibronaceae bacteria to degrade alginate (21). These bacteria were able to degrade extracellular alginate to varying degrees, with some growing best on short-chain (DP ~ 3–4) and others growing best on long-chain (DP > 20) substrates. The behaviors were generally consistent among closely related strains except those within the Vibrio splendidus population, where significant diversity was observed. In particular, whereas all of the V. splendidus strains were able to grow well on short-chain substrates, only some grew well on long-chain substrates, and their growth rates and lag phases varied on these long-chain substrates. The ability to rapidly grow on long-chain substrates was attributed to enzyme secretion; strains that secrete alginate lyases grew much better than those that do not. Interestingly, the V. splendidus strains all exhibited similar levels of intracellular and membrane-bound alginate lyase activity, suggesting that some strains acquired additional lyases with high extracellular activity. These results are in line with a previous bioinformatics study revealing very high genotypic microdiversity of extracellular CAZymes, even among very closely related microbes (14).

In this work, we sought to identify the enzymes responsible for high secreted alginate lyase activity within the V. splendidus population. One strain, V. splendidus 13B01, was found to have significantly higher activity than the others, although they are indistinguishable by 16S RNA or other single-marker gene phylogenies. Using a combination of genomics, proteomics, biochemical, and functional screening, we identified a PL7 alginate lyase that is critical for rapid extracellular alginate breakdown. Our results demonstrate that differences in the enzyme repertoires between closely related strains can be used to rapidly pinpoint key proteins with beneficial functions.

Results

Activity screening reveals the diversity of alginate degradation within closely related V. splendidus strains

The alginate lyases have only been characterized in V. splendidus 12B01, which exhibits low extracellular lyase activity (15, 22). Therefore, we sought to identify the alginate lyases with high extracellular activity. As a first step, we screened the eight V. splendidus strains examined in a study by Hehemann et al. (21) for their ability to broadcast alginate lyase activity. Briefly, we grew the cells as individual colonies on agar plates supplemented with 0.25% alginate and then screened for secreted alginate lyase activity through the formation of dark halos.

Among the eight strains tested, V. splendidus 13B01 had the highest secreted activity, whereas the previously characterized V. splendidus 12B01 had the second lowest secreted activity (Fig. 1). Therefore, we focused on V. splendidus 13B01 due to its high extracellular alginate lyase activity. We next compared the growth of V. splendidus 12B01 and 13B01 on 1% alginate in liquid culture (supplemental Fig. S1). V. splendidus 13B01 grew 24% faster on alginate than V. splendidus 12B01 (0.51 h\(^{-1}\) versus 0.41 h\(^{-1}\)). These results confirm that V. splendidus 13B01 is better at catabolizing alginate than V. splendidus 12B01.

Comparative genomics reveals differences between alginate lyases in V. splendidus strains

Previous studies have suggested that rapid microbial adaptation to new environmental conditions or resources can result from gene transfer (21). Reconciliation of species and gene tree phylogenies revealed that V. splendidus 13B01 acquired an additional PL7 enzyme, which is lacking in 12B01 and which may account for the improved performance of 13B01 on long-chain alginate. To test this hypothesis, we compared the genomes of V. splendidus 12B01 and 13B01 to identify other genetic properties related to alginate metabolism that may account for the observed differences in alginate usage.

Both strains contain a homologous genetic island involved in alginate metabolism. V. splendidus 12B01 has four PL7 alginate lyases (PL7A, PL7B, PL7D, and PL7E) that are also present in V. splendidus 13B01. Phylogenetic analysis indicates that the four PL7 enzymes common to both V. splendidus 12B01 and V. splendidus 13B01 are closely related orthologs (sequence similarity >98%) (Fig. 2A). The PL7B, PL7D, and PL7E lyases contain one PL7 domain (Fig. 2B), whereas the PL7A lyase con-
contains two PL7 domains, which are more distantly related to the other enzymes (16, 17).

V. splendidus 13B01, however, also has several unique features. First, it contains a PL7 alginate lyase, PL7G, not present in V. splendidus 12B01 and containing a single catalytic domain. This lyase is nested in a clade surrounded by the lyases PL7D, PL7B, and PL7E. Both PL7B and PL7G contain a family 32 carbohydrate-binding module (CBM32) in addition to their PL7 domain (23). Second, V. splendidus 13B01 also has a putative alginate lyase with a PL6 domain (16, 24). Both alginate lyases unique to V. splendidus 13B01, PL6F and PL7G, have the catalytic residues typical for PL6 and PL7 enzymes, suggesting that both are functional (24, 26, 27). These bioinformatics results suggest that PL6F, PL7G, or both may be responsible for the high extracellular lyase activity of V. splendidus 13B01.

Alginate-induced expression of PL7 and PL6 alginate lyase genes in 13B01

To ascertain the physiological response of the alginate lyase genes to alginate, we measured the expression of the alginate lyases in V. splendidus 13B01 using quantitative PCR (qPCR). Briefly, we grew cells in M9 minimal salt medium supplemented with 0.2% (w/v) glucose, 0.1% (w/v) alginate, or 1.0% (w/v) alginate and then harvested the mRNA during late exponential phase.

Growth on alginate induced expression of all alginate lyase genes relative to growth on glucose (Fig. 3). In particular, lyase gene expression at both alginate concentrations is induced >2-fold, with pl7G being induced >10-fold. Growth on 0.1 and 1.0% alginate yielded no discernable difference in gene expression for any of the six lyases. The lyases in V. splendidus 12B01 are also induced by alginate (22); however, they are expressed at a lower levels than their orthologues in V. splendidus 13B01. In addition, their expression is more tightly regulated because less expression occurs in the absence of alginate. Their expression also exhibits a more graded response to alginate, with less expression observed at lower alginate concentrations. Increased expression of the lyases at low alginate concentrations may explain why V. splendidus 13B01 grows better in liquid culture than V. splendidus 12B01.
We first determined the optimal conditions for each lyase (supplemental Fig. S4). The lyases were found to have optimal activities over a range of pH values varying from 7.5 to 10. The optimal temperatures were between 20 and 25 °C, and the optimal NaCl concentrations were between 250 and 1000 mM. We also determined the enzymatic function of each of the two PL7A domains by producing each domain separately as a recombinant protein. We found that domain 2 of PL7A had 85% activity in comparison with the full PL7A enzyme, whereas domain 1 of PL7A had negligible enzymatic activity (supplemental Fig. S5), suggesting that it may have other functions, such as sugar binding. Similar results were reported for the two domains of PL7A from V. splendidus 12B01 (22).

We next determined the kinetic parameters for each lyase by measuring the activity at different alginate concentrations under optimal salt and pH conditions. All five alginate lyases exhibited Michaelis–Menten type kinetics (supplemental Fig. S6). The associated kinetic parameters are provided in Table 1. We observed some differences among the lyases common to V. splendidus 12B01 and 13B01. In general, the shared lyases are more active in V. splendidus 13B01, as determined by the enzymatic turnover number. However, these differences in the enzyme kinetics are not significant enough to explain the phenotypic differences (e.g. high secreted activity) between V. splendidus 12B01 and 13B01.

In the case of the alginate lyases unique to V. splendidus 13B01, we found that PL7G exhibits much stronger activity than PL6F. In fact, PL7G exhibits the highest turnover number among the six lyases. However, these differences in enzymatic turnover number are not significant enough to explain the phenotypic differences (e.g. high secreted activity) between V. splendidus 12B01 and 13B01.

In addition to measuring the kinetic parameters, we also determined specificity of the lyases. Alginate is a polysaccharide consisting of the mixed 1,4-linked epimers, G and M. Different lyases can preferentially cleave the G–G, M–M, G–M, or M–G bonds in alginate. To determine the specificity of the V. splendidus 13B01 lyases, we measured the degradation products of

We next tested the possibility that increased activity or altered specificity provides another source of physiological variation among the two V. splendidus strains. To examine the biochemical properties and substrate specificities of the alginate lyases, we produced the recombinant PL6 and PL7 enzymes from V. splendidus 13B01 in E. coli except PL7D, because it is identical to its previously characterized ortholog in V. splendidus 12B01 (22). Among the five enzymes, PL6F was the only soluble protein and could therefore be purified under native conditions, whereas the PL7 enzymes were found to be insoluble in inclusion bodies. To yield functional enzymes, we produced and purified the PL7 enzymes under denaturing conditions and then refolded them (supplemental Fig. S2). All of the lyases were stable and eluted as monomeric proteins in size exclusion chromatography (supplemental Fig. S3).
alginate, mannuronate-enriched alginate, and guluronate-enriched alginate using $^1$H NMR (Fig. 5). The results are summarized in Table 1. Among the shared lyases tested, no differences in specificity were detected. Among the unique lyases, PL6F exhibited G–M specificity, and PL7G exhibited M–G specificity. We also analyzed the degraded alginate lyases, PL6F exhibited G–M specificity, and PL7G exhibited M–G specificity. We also analyzed the degraded alginate lyases, PL6F exhibited G–M specificity, and PL7G exhibited M–G specificity. We also analyzed the degraded alginate lyases, PL6F exhibited G–M specificity, and PL7G exhibited M–G specificity. We also analyzed the degraded alginate lyases, PL6F exhibited G–M specificity, and PL7G exhibited M–G specificity. We also analyzed the degraded alginate lyases, PL6F exhibited G–M specificity, and PL7G exhibited M–G specificity.

**Table 1**

| Alginate lyases of V. splendidus 13B01 |
|--------------------------------------|
| **Optimal environmental conditions and enzymatic kinetics of studied alginate lyases** |
| The data and curve fits used to determine the V. splendidus 13B01 lyase kinetic parameters are found in supplemental Fig. S6. The V. splendidus 12B01 lyase parameters were previously determined (22). The kinetic parameters for PL7D in V. splendidus 13B01 were not determined because the enzyme is 100% identical to its homolog in V. splendidus 12B01. |
| **13B01** | **12B01** |
| pH | 8.5 | 8.5 |
| Temperature (°C) | 25 | 20 |
| $k_{cat}$ (nmol/mg) | 5000 | 750 |
| $k_m$ (mM) | 90 ± 7 | 90 ± 7 |
| $V_{max}$ (µmol/s) | 0.13 ± 0.01 | 0.34 ± 0.02 |
| Turnover (s$^{-1}$) | 0.06 ± 0.02 | 1.9 ± 0.1 |
| Specificity | G-M | G-M |

**PL7B**

| pH | 7.5 | 10.0 |
| Temperature (°C) | 20-25 | 25 |
| NaCl (mM) | 400 | 500 |
| $k_{cat}$ (µmol/mg) | 22 ± 5 | 150 ± 30 |
| $V_{max}$ (µmol/s) | 0.66 ± 0.06 | 0.76 ± 0.05 |
| Turnover (s$^{-1}$) | 3.7 ± 0.3 | 5.2 ± 0.3 |
| Specificity | G-M | G-M |

**PL7D**

| pH | 7.5 | 10.0 |
| Temperature (°C) | 25 | 20 |
| NaCl (mM) | 400 | 1000 |
| $k_{cat}$ (µmol/mg) | 60 ± 2 | Identical |
| $V_{max}$ (µmol/s) | 0.52 ± 0.06 | 150 ± 30 |
| Turnover (s$^{-1}$) | 4.5 ± 0.5 | 1.50 ± 0.07 |
| Specificity | G-G | 158.5 ± 0.7 |

**PL7E**

| pH | 7.5 | 10.0 |
| Temperature (°C) | 25 | 20 |
| NaCl (mM) | 400 | 1000 |
| $k_{cat}$ (µmol/mg) | 123 ± 6 | 170 ± 30 |
| $V_{max}$ (µmol/s) | 0.83 ± 0.02 | 1.50 ± 0.07 |
| Turnover (s$^{-1}$) | 7.1 ± 0.2 | 158.5 ± 0.7 |
| Specificity | G-G | G-G |

**PL6F**

| pH | 7.5 | 10.0 |
| Temperature (°C) | 25 | 20 |
| NaCl (mM) | 400 | 250 |
| $k_{cat}$ (µmol/mg) | No Homolog | No Homolog |
| $V_{max}$ (µmol/s) | 40 ± 6 | 0.99 ± 0.002 |
| Turnover (s$^{-1}$) | 0.6 ± 0.1 | 0.6 ± 0.1 |
| Specificity | G-G | G-G |

**PL7G**

| pH | 7.5 | 10.0 |
| Temperature (°C) | 25 | 20 |
| NaCl (mM) | 500 | 500 |
| $k_{cat}$ (µmol/mg) | No Homolog | No Homolog |
| $V_{max}$ (µmol/s) | 300 ± 30 | 2.6 ± 0.1 |
| Turnover (s$^{-1}$) | 18.0 ± 0.7 | 18.0 ± 0.7 |
| Specificity | M-G | M-G |

**Discussion**

Bacteria employ systems of complementary enzymes to degrade polymeric glycans into monosaccharides. Differences in the number of enzyme, due to loss, duplication, and transfer, in these glycan-degrading pathways have been documented even among closely related strains, suggesting that these changes reflect metabolic specialization. Indeed, a previous study was able to correlate the presence of different enzymes involved in alginate degradation in closely related coastal marine vibrios with their ability to grow on alginate substrates of varying chain length (21). Based on these results, the authors concluded that coastal vibrios have undergone an adaptive radiation that minimizes competition among closely related strains and creates an alginate degradation cascade involving three groups specialized for growth on substrates of different chain lengths and solubility (pioneers, harvesters, and scavengers).

In this work, we sought to identify the specific enzymes enabling this substrate specialization within the V. splendidus group. We focused specifically on the high extracellular lyase activity exhibited by some strains but not others. Those with high extracellular lyase activity are presumably specialized to grow on high–molecular weight substrates, such as alginate gels, whereas those with low extracellular activity are specialized for smaller–molecular weight substrates. As a first step, we compared the secreted lyase activity of eight related V. splendidus strains (Fig. 1). V. splendidus 13B01 exhibited the highest secreted lyase activity. Therefore, we sought to identify the specific lyases in V. splendidus 13B01 that enabled its high secreted lyase activity. This bacterium has six alginate lyases (Fig. 2). Four are shared with the previously characterized V. splendidus 12B01 (22), which exhibits low secreted lyase activity. Based on this difference, V. splendidus 12B01 was used as a reference point to develop hypotheses regarding the high secreted lyase activity of V. splendidus 13B01.

One question is whether the shared lyases are the same in these two strains. To address this question, we characterized the shared lyases. Whereas they exhibit higher activity in V. splendidus 13B01 than in V. splendidus 12B01 (Table 1), the increased activity was insufficient to explain differences in the ability to degrade extracellular alginate. We found instead, using a combination of genomic, proteomic, and biochemical approaches, that a single lyase, PL7G, present in V. splendidus 13B01 but not in V. splendidus 12B01, enables the high extracellular activity (Fig. 7). Removing PL7G from V. splendidus 12B01 by genetic manipulation created a knock-out strain that had the identical alginate lyase secretion phenotype as V. splendidus 12B01 when measured on our plate-based alginate lyase secretion assay. This result highlights the value of naturally occurring microdiversity to assign unknown functions to enzymes and/or other genes.

PL7G contains an N-terminal CBM32 domain (Fig. 2B). This domain is also present in PL7B. It is known to bind galactose, N-acetylgalactosamine, lactose, and N-acetyl-β-lactosamine (28). Because it is present in some alginate lyases, it may also bind to alginate. Previous work demonstrated that removing a homolog of this domain from PL7B in V. splendidus 12B01
eliminated the activity (22), indicating that it is necessary for enzymatic function. However, others have found that removing this domain from an alginate lyase in *Vibrio alginovorus* reduces but does not eliminate enzymatic activity (29). Although we did not explore the role of this domain in the present study, we suspect that it contributes to the ability of PL7G to degrade alginate gels. Indeed, carbohydrate-binding modules are known to enable enzymes to bind to insoluble glycans and modulate the specificity and activity of the cognate enzyme (30). Presumably, the CBM32 domain in PL7G serves a similar purpose by enabling the enzyme to attach to the alginate gels.

An additional question concerns the role of PL6F, the other lyase present in *V. splendidus* 13B01 but not in *V. splendidus* 12B01. This lyase exhibits relatively weak activity as compared with the other lyases. It is, however, expressed at a relative high level (Fig. 4), which could presumably compensate for its low activity. In addition, it appears to preferentially degrade lower-molecular weight alginate, unlike the other lyases. In particular, the average degree of polymerization (DP) of its products, as determined by NMR, is 50 (supplemental Table S3). The other lyases yield products with significantly smaller DPs (all are <10 except PL7A, whose products have an average DP of 20). Because PL6F is preferentially localized to the membrane, this would suggest that it is used to degrade large alginate molecules, proximal to the cell, into smaller fragments accessible by the other membrane-localized enzymes, such as PL7B and PL7E. Assuming that this hypothesis is correct, then these results would suggest that the acquisition of PL6F further enables *V. splendidus* 13B01 to preferentially grow on high-molecular weight alginate substrates.

In addition to its high extracellular activity, *V. splendidus* 13B01 also exhibits a higher growth rate on alginate than *V. splendidus* 12B01. Whereas increased growth may be partially attributable to PL7G, we suspect that other factors are also involved. For one, *V. splendidus* 13B01 also exhibits a higher growth rate on glucose (supplemental Fig. S1), suggesting that its growth rate is intrinsically higher. However, the shared lyases also exhibit higher expression and activity, suggesting that this bacterium is better adapted to grow on alginate. Moreover, the additional lyases seem to be especially adapted to liquefy alginate gels because PL7G can rapidly penetrate into the gel medium during the alginate screening assay (Fig. 1). This may be attributable to its relatively high affinity constant, which indicates adaptation to the high substrate concentrations found in alginate gels (~1–2%, w/v). The use of such an enzyme supports the idea that *V. splendidus* 13B01 is a pioneer strain within the *Vibrio* group (21), enabling it to initiate the degradation of not yet colonized alginate-rich nutrient patches. Although such pioneers have an advantage on not yet colonized alginate gels, they may be outcompeted by strains like *V. splendidus* 12B01 when alginate particles become saturated with alginate lyases of *V. splendidus* 13B01

Figure 5. $^1$H NMR (400-MHz) spectra of alginate and alginate-derived substrates following degradation with PL7A, PL7B, PL7D, PL7E, PL6F, and PL7G. G and M, signals from internal G and M residues, respectively; G-beta and M-beta, signals from reducing G and M residues, respectively; $\Delta$, signal from 4-deoxy-l-erythro-hex-4-enepyranosyluronate non-reducing end residue. Non-underlined residues are the neighboring residues to those generating each signal. Protons (H) are numbered to indicate which particular proton causes the signal. A, alginate; B, poly-G–enriched alginate; C, poly-M–enriched alginate.
secreted lyases that solubilize the polymer. Under these conditions, the cost of secreting alginate lyases may decrease the fitness of strains like *V. splendidus* 13B01, suggesting niche differentiation according to the degradation state of alginate gels.

The results from the present study may have application in the productions of fuel and other chemical production using macroalgae. In particular, a recent study engineered *Escherichia coli* to produce ethanol from alginate using the alginate degradation pathway from *V. splendidus* 12B01 (15). To break down extracellular alginate into smaller oligomers capable of being taken up by the cell, the *E. coli* strain was also engineered to secrete a PL7 lyase from *Pseudoalteromonas* sp. SM0525. The native PL7 lyases from *V. splendidus* 12B01 were not considered. The results from the present study may aid in the design of bacteria capable of producing different fuels and chemicals from alginate by identifying the new lyases, PL6F and PL7G, that enable growth on high–molecular weight alginate substrates.

In conclusion, our results demonstrate the value of exploiting the evolutionary process for enzyme identification. In par-
ticular, evolution creates an array of closely related microbes, which behave like the mutants that one constructs in the laboratory when determining the function of unknown proteins. Our study shows that the analysis of closely related microbes in combination with suitable activity screens can reveal the function of unknown proteins, with the potential of leapfrogging the classical suite of molecular biology experiments.

**Experimental procedures**

**Bacterial strains, media, and growth conditions**

All cloning was performed in *E. coli* strain DH5α. Protein expression was performed in *E. coli* strain BL21(DE3). Conjugation was performed by mating with *E. coli* strain WM3064. *E. coli* was grown at 37 °C in Luria–Bertani (LB) medium (5 g/liter yeast extract, 10 g/liter tryptone, and 10 g/liter NaCl). Kanamycin was added at a concentration of 30 μg/ml. Diaminopimelate was supplemented at a concentration of 0.3 mM for experiments involving *E. coli* strain WM3064. *V. splendidus* 12B01 and 13B01 were isolated from particulate material obtained by filtering coastal waters (31). The strains were cultured in rich medium conditions on tryptic soy broth (TSB) (Difco) supplemented with 2% NaCl unless noted otherwise. Chloramphenicol was used at a concentration of 5 μg/ml. *V. splendidus* was grown at 20 °C in M9 minimal salt medium (32) (per liter of tap water: 11.28 g of M9 minimal salts (Sigma-Aldrich), 2 g of casamino acids, and 18 g of NaCl) unless noted otherwise. M9 minimal salt medium was then supplemented with 1 mM MgSO_4_, 0.1 mM CaCl_2_, and either 0.2% glucose, 0.1% alginate, or 1% alginate. Alginate was purchased from Sigma-Aldrich. Growth rates were determined by growing *V. splendidus* 12B01 and 13B01 overnight in M9 minimal salt medium and then subculturing to an *A*_600 of 0.05 in fresh minimal medium. The increase in absorbance at 600 nm was then monitored.

**Enzyme secretion**

Cells, precultured for 24 h in 2216 marine medium (Difco), were plated in triplicate onto 2216 marine broth agar plates containing 0.25% (w/v) low-viscosity alginate (Sigma). The colonies were grown for 36 h at 20 °C and then imaged for colony size measurements. The colonies were removed by scraping the plate surface and washing with deionized water two times for 10 min. Lyase activity was determined by overlaying the agar plate with 10% (w/v) cetylpyridinium chloride (Sigma) in water for 20 min (33). The cetylpyridinium chloride solution was decanted, and the plate was washed twice with deionized water for a total of 20 min at 20 °C (34). The water was decanted, and lyase activity was revealed by the formation of cleared halos on an opaque background.

**Phylogenetic analysis**

An X-ray crystal structure-guided sequence alignment of the PL7 proteins was calculated with the previously published crystal structures of PL7 alginate lyases from *Sphingomonas* sp. A1 (PDB code 2CWS) (26) and *Alteromonas* sp. 272 (PDB code 1J1T) using ClustalW/T-coffee (35) as part of the Strap program (36). The alignment was calculated using default parameters. We included all protein sequences of *V. splendidus* 13B01 and 12B01 with PL7 domains. Because PL7A of *V. splendidus* 13B01 and 12B01 contained two PL7 domains, the domains were separated and treated as individual domains in the phylogenetic analysis. The structural alignment was plotted with Endscript to show the secondary structural elements of the associated PDB entries (37). Long inserts and N-terminal extensions, such as the CBM32 domains present in sequences of PL7B and PL7G, N-terminal signal peptides, and the C-terminal His tag of the sequence for 1J1T were manually removed from the alignment with Bioedit (38). This trimmed alignment, without the sequence 2CWS, was subsequently used to calculate the phylogenetic tree in Mega6 using the maximum likelihood method based on the JTT matrix-based model with default parameters and with 100 resamplings of the data set (39). The sequence of 1J1T, a PL7 alginate lyase from *Alteromonas* sp. 272, was used to root the tree.

**Gene expression of alginate lyases**

*V. splendidus* 13B01 was grown overnight in M9 minimal salt medium and then was subcultured 1:50 in fresh minimal medium. Samples were harvested at an *A*_600 of 1.0. Total RNA was isolated using the RNeasy minikit (Qiagen). cDNA was then generated using the QuantiTect reverse transcription kit (Qiagen) and then subcultured to an *A*_600 of 1.5 (after 7 h) at 93/84 °C, 15 min). The cDNA was then amplified using the RNeasy minikit (Qiagen). cDNA was then generated using the QuantiTect reverse transcription kit (Qiagen). The primers listed in *supplemental Table S2* were then used to perform qPCR in triplicate. The primers were designed using Primer3Plus (www.bioinformatics.nl/primer3plus) (40). qPCRs were performed using HotStar-IT SYBR Green qPCR Master Mix with UDG (Affymetrix) and a Bio-Rad MiniOpticon real-time PCR system (Bio-Rad). Serially diluted *V. splendidus* 13B01 genomic DNA was used to construct gene-dosing standards, and the 13B01 gene *rpoA* was used to control for differences in total mRNA (41).

**Protein extraction for proteomic analysis**

*V. splendidus* strains 12B01 and 13B01, respectively, were grown in triplicates in M9 minimal salt medium with 0.2% alginate as the carbon source (20 °C, 180 rpm). Bacterial cells were harvested at an *A*_600 of 1.5 (after 7 h) at 93/84 °C, 15 min), washed twice in Tris-EDTA buffer (10 mM Tris-HCl, 10 mM EDTA), and stored at −80 °C until analysis. The supernatant, containing those *V. splendidus* proteins that were excreted into the extracellular medium (i.e. the extracellular protein fraction), was precipitated with 10% trichloroacetic acid (4 °C, overnight). Extracellular proteins were pelletized (93/84 × g, 4 °C, 70 min), washed twice in 99% ethanol, and then resuspended in 50 mM triethylammonium bicarbonate (TEAB) buffer. *V. splendidus* cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, Roche Completer protease inhibitor), and the cells were disrupted by sonication (Bandelin Sonopuls ultrasonic homogenizer, Berlin, Germany; 4 °C, 30% power, 0.5-s cycle, 3 × 30 s, 30-s pause). Crude cell debris was removed by centrifugation (8000 × g, 4 °C, 15 min), and the resulting raw protein extract was subjected to ultracentrifugation (100,000 × g, 4 °C, 70 min) to collect cellular membranes and associated proteins (i.e. the membrane fraction). Membrane proteins were extracted from the pellet by repeated solubilization and ultracentrifugation as described by Eymann et al. (42) and resuspended in 50 mM...
Alginate lyases of V. splendidus 13B01

TEAB buffer. Protein concentrations in the extracellular protein fraction and in the membrane fraction were determined according to Bradford (43) using the Nanoquant protein assay (Carl Roth, Karlsruhe, Germany).

**LC-MS/MS analysis**

Protein extracts (100 μg) were subjected to in-solution digestion, as described by Muntel et al. (44), at a final concentration of 1 μg/μl in 50 mM TEAB, 0.1% RapiGest (Waters, Milford, MA). After reduction (tris(2-carboxyethyl)phosphine, 5 mM final concentration) and alkylation (10 mM iodoacetamide), proteins were digested with trypsin (Promega, Madison, WI) for 6 h at 37 °C. RapiGest was removed by acidification and repeated centrifugation, and peptide mixtures were desalted using StageTips (Proxeon), according to the manufacturer's recommendations. Peptides were separated using reversed phase C18 column chromatography on a nanoACQUITY UPLC (Waters) before MS and MS/MS in an online-coupled LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) (45). The SEQUEST® Sorcerer1<sup>TM</sup> platform (Sage-N) was used for database searches of MS data against target-decoy protein sequence databases, containing the V. splendidus 12B01 and 13B01 sequences, respectively, and common laboratory contaminants. Peptide and protein identifications were validated in Scaffold version 4.2 (Proteome Software, Portland, OR) and filtered as described by Heinz et al. (46). Protein false discovery rates were 0.0% throughout all data sets. For label-free protein quantification, normalized spectral abundance factors (NSAFs) were calculated (47) from total spectrum counts as a measure of protein abundance. Only proteins that were identified in at least two of three replicates were included in the quantification. Relative protein abundance (i.e. a protein’s proportion of all proteins in the same sample) was calculated as average NSAF% from the individual replicate NSAF% values (n = 2).

**Plasmid construction**

The alginate lyases from V. splendidus 13B01 were expressed from a T7 promoter using the overexpression plasmid pET-28(a). Each lyase was analyzed for the presence of a signal peptide using the SignalP version 4.1 server (http://www.cbs.dtu.dk/services/SignalP)/<sup>5</sup> (48) and the LipoP 1.0 server (http://www.cbs.dtu.dk/services/LipoP)/<sup>5</sup> (49). The signal peptides of PL7B, PL7E, PL6F, and PL7G were removed before cloning. The nucleotide sequence of each lyase can be found in supplemental Table S1. The overexpression vectors, pPL7A, pPL7B, and pPL7G, were constructed by amplifying the alginate lyase genes pl7A, pl7B, and pl7G, respectively, from V. splendidus 13B01 genomic DNA and then cloning these fragments into pET-28(a) using the restriction enzymes Ncol and Xhol. pPL6F was constructed by amplifying pl6F from V. splendidus 13B01 genomic DNA and cloning the fragment into pET-28(a) using the restriction enzymes Ncol and Eagl. pPL7E was constructed in two steps. First, pET-28(a) was digested with restriction enzymes Ncol and EcoRI, the overhangs were filled in with Klenow fragment, and the blunt ends were ligated together to create a modified pET-28(a) lacking the N-terminal His tag, thrombin cleavage site, and T7 tag. pl7E was then amplified from V. splendidus 13B01 genomic DNA and cloned into the modified pET-28(a) using the restriction sites EcoRI and Xhol. Each of the overexpression vectors contains the alginate lyase gene with a C-terminal His<sub>6</sub> tag driven by an isopropyl β-D-1-thiogalactopyranoside-inducible T7 promoter. The alginate lyase domains of PL7A were independently amplified to include 5'-CTT TCC AGC-3' upstream and 5'-TGT GGT CGT-3' downstream of domain 1 and 5'-TCA AAC GAT-3' upstream and 5'-GTT-3' downstream of domain 2 and then cloned into pET-28(a) using the restriction enzymes Ncol and Xhol. When necessary, 5'-TT-3' was included upstream of the cloned fragment to ensure that the fragment was in-frame with the C-terminal His<sub>6</sub> tag. Supplemental Table S2 contains the sequences for the oligonucleotides used in this work.

**Protein purification and determination of molecular mass**

Cells were grown overnight in LB medium and were supplemented with 1% glucose. Subcultures were then started in fresh medium and grown to an A<sub>600</sub> of 0.5. Cells were cooled to 25 °C, and protein overexpression was then induced by adding isopropyl β-D-1-thiogalactopyranoside to a concentration of 1 mM. PL7E was grown for an additional 19 h at 25 °C. PL7A, PL7B, PL6F, and PL7G were grown for an additional 24 h at 16 °C. PL6F-expressing cells were lysed by suspending the cell pellet in native binding buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0), and then lysozyme was added at 1 mg/ml and followed by incubation on ice for 30 min. The lysate was then sonicated six times for 10 s on ice. The PL6F lysate was clarified by centrifugation at 10,000 × g for 20 min and then passed through a 0.45-μm filter. PL6F was purified by loading the lysate onto two 5-ml HiTrap Chelating HP columns charged with 100 mM NiSO<sub>4</sub> and installed on an ÄKTA prime FPLC system (GE Healthcare). The columns were washed with 5 column volumes of wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, pH 8.0), and then PL6F was eluted from the columns with 5 volumes of elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole, pH 8.0). PL7A-, PL7B-, PL7E-, and PL7G-containing cells were lysed by suspension in Buffer B (8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, pH 8.0) and inverting the tubes at 25 °C for 1 h. The lysates were clarified by centrifugation at 9000 × g for 30 min and then passage through a 0.45-μm filter. The lysates were then loaded on two 5-ml HiTrap Chelating HP columns charged with 100 mM NiSO<sub>4</sub> installed on an ÄKTA prime FPLC system. The columns were then washed with 5 column volumes of Buffer C (8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, pH 6.3). Each protein was then eluted with 5 volumes of Buffer D (8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-Cl, pH 5.3). PL6F was dialyzed three times against TKMD-G (50) (50 mM Tris-Cl, pH 8.0, 5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1 mM dithiothreitol, 10% glycerol, pH 8.0) at 4 °C for 2 h followed by an overnight charge at 4 °C. PL7A, PL7B, PL7D, PL7E, and PL7G were refolded via dialysis against TKMD-G three times at 4 °C for 2 h followed by an overnight charge at 4 °C.

Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific) with albumin standards following the supplier’s protocol. The native sizes of each protein were determined by size-exclusion high-performance liquid chromatography using a Shimadzu high-performance
liquid chromatography system that utilized an SPD-10A UV-visible detector set at 280 nm. A Bio-Sil SEC-250 column (300 x 7.8 mm) was then used to determine the native protein size using a mobile phase (50 mM Na2HPO4, 50 mM NaH2PO4, and 150 mM NaCl, pH 6.8).

**Alginate lyase activity assay**

Alginate lyase reaction conditions were as follows. 0.25 μg of enzyme was added to 100 μl of APT buffer (20 mM sodium acetate, 20 mM monosodium phosphate, and 20 mM Tris base) containing 0.2% sodium alginate, unless noted otherwise. Each reaction was incubated for 20 min at the reported pH, temperature, and NaCl concentrations. The thioarbituric assay was used to determine enzymatic activity (51, 52). 0.125 ml of 0.025 N H2IO6 in 0.125 N H2SO4 was added to each reaction mixture and then incubated for 20 min at 25 °C. Next, 0.25 ml of a 2% sodium arsenite in 0.5 N HCl solution was added and then incubated for 2 min. Finally, 1 ml of thioarbituric acid (0.3%, pH 2) was added to the mixture and then heated at 100 °C for 10 min. The increase in absorbance at 548 nm was determined using a Shimadzu spectrophotometer. Activities were reported in 2-deoxy-D-glucose equivalent concentrations (53). The kinetic parameters were found by fitting the dose response activities to a Michaelis–Menten kinetic model. Enzyme calculations were performed with primers AB478F and AB478R by PCR. The upstream and downstream 1-kb regions of pl7G were amplified from the V. splendidus 13B01 genome with primers AB476F and AB476R and primers AB477F and AB477R, respectively. These three pieces were then phosphorylated with a T4 poly-nucleotide kinase reaction. The 55 ng/kb of each fragment was mixed with 5 μl of 20 mM ATP, 2 μl of 10× Ampligase buffer (Epigenic), 1 μl of T4 poly-nucleotide kinase (New England Biolabs), and H2O to 20 μl. The mixture was then incubated for 1 h at 37 °C, followed by a deactivation for 20 min at 65 °C. The phosphorylated fragments were then ligated in an LCR. The phosphorylated fragment mixture was mixed with 0.83 μl of 10× Ampligase buffer, 1 μl of Ampligase (Epigenic), 2 μl of 100% DMSO, 0.25 μl of betaine, 0.5 μl of 1.5 μM primers AB487F, AB490F, AB510F, and H2O to 25 μl. The second step of the knock-out vector construction was subcloning the fused 1-kb upstream and downstream regions into the vector pJC4 (62). This was accomplished by amplifying the fused region from the LCR using primers AB513F and AB513R and subcloning this fragment into the vector pJC4 using the Spel restriction site. The resulting vector, pPL7G-KO, contains the 1-kb pl7G upstream region fused to the 1-kb pl7G downstream region in the vector pJC4, which contains the oriT transfer origin, the oriR6k* origin of replication, and the sacB gene for counterselection.

The knock-out vector pPL7G KO was conjugated from the E. coli strain WM3064 to V. splendidus 13B01 by the methods of Cordero et al. (62) with modification. Conjugation was performed by mixing 100 μl of each overnight-grown culture and washing once with TSB with 2% NaCl. The cells were then resuspended in 10 μl of TSB with 2% NaCl and spotted on TSB with 2% NaCl and 0.3 mxi dianamopimelate solid medium. The plate was then incubated overnight at 30 °C. The cells were then removed and washed in TSB with 2% NaCl and plated on TSB with 2% NaCl and 5 μg/ml chloramphenicol. Colonies were then purified by streaking three times on TSB with 2% NaCl and chloramphenicol. The presence of the integrated vector was also verified. Integrants were then streaked once on TSB with 2% NaCl and then plated on fresh TSB with 1% NaCl and 5% sucrose to select for cells that have lost the integrated vector and sacB gene. The sucrose-resistant cells were then screened for loss of chloramphenicol resistance and loss of pl7G.

**Electrospray ionization mass spectroscopy**

Electrospray ionization mass spectroscopy (Waters Quattro Ultima) was used to analyze the mass/charge ratios of the alginate lyase-degraded products. Positive- and negative-ionization modes were used. A solution containing 0.4% alginate in phosphate buffer, pH 7.6, was mixed with purified enzyme and incubated at 20 °C for 3 h. Samples were dried using a refrigerated speed vacuum, and 6 mg of the dried sample was dissolved in 1:1 MeOH-H2O (10 pmol/ml) (58, 59) and injected into the electrospray. The mass range of the scans was from 100 to 1100 atomic mass units.
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