New Family of Ulvan Lyases Identified in Three Isolates from the Alteromonadales Order*

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Ulvales is the main polysaccharide component of the Ulvales (green seaweed) cell wall. It is composed of disaccharide building blocks comprising 3-sulfated rhamnose linked to D-glucuronic acid (GlcUA), L-iduronic acid (IdoUA), or D-xylose (Xyl). The degradation of ulvan requires ulvan lyase, which catalyzes the endolytic cleavage of the glycoside bond between 3-sulfated rhamnose and uronic acid according to a β-elimination mechanism. The first characterized ulvan lyase was identified in Nonlabens ulvanivorans, an ulvanolytic bacterial isolate. In the current study, we have identified and biochemically characterized novel ulvan lyases from three Alteromonadales isolated bacteria. Two homologous ulvan lyases (long and short) were found in each of the bacterial genomes. The protein sequences have no homology to the previously reported ulvan lyases and therefore are the first representatives of a new family of polysaccharide lyases. The enzymes were heterologously expressed in Escherichia coli to determine their mode of action. The heterologously expressed enzymes were secreted into the milieu subsequent to their signal sequence cleavage. An endolytic mode of action was observed and studied using gel permeation chromatography and 1H NMR. In contrast to N. ulvanivorans ulvan lyase, cleavage occurred specifically at the GlcUA residues. In light of the genomic context and modular structure of the ulvan lyase families identified to date, we propose that two ulvan degradation pathways evolved independently.

Ulvales are green algae that proliferate in eutrophicated coastal waters. Members of this family are commonly grown and collected for food or feed, although currently most of the biomass is put to limited use (1, 2). Among the polymers synthesized by these algae, ulvan is the most abundant polysaccharide in the architecture of the cell wall (2). This complex watersoluble anionic polysaccharide represents up to 29% of the algae dry weight (3). Although the constituent ratio might vary according to algae growth conditions, it is mainly composed of 3-sulfated rhamnose (R3S), D-glucuronic acid (GlcUA), L-iduronic acid (IdoUA), D-xylose (Xyl), and D-xylose (2). Ulvan building blocks appear frequently as repeating disaccharides comprising either GlcUA or IdoUA linked to R3S, termed ulvanobiuronic acid A or B, respectively (2). In addition, disaccharide moieties made of R3S linked to Xyl occur in lower amounts.

Ulvan’s unique chemical and physicochemical properties make it an attractive candidate for several applications in the food/feed, agriculture, pharmaceutical, and biomaterials industries (2). In contrast to polysaccharides extracted from brown and red algae (e.g. alginate and agar, respectively), which are widely used in industry for their gelling and thickening properties, polysaccharides of green algae are less exploited. Expanding our understanding of ulvan structure and its enzymatic degradation would enable more extensive biomass utilization.

Thus far, only a few ulvan-degrading enzymes have been isolated from both marine and terrestrial microorganisms. Some of them, like the glucuronan lyases isolated from Sinorhizobium meliloti (4) and Trichoderma sp. GL2 (5), possess limited ulvanolytic activity. The first ulvan lyase activity was found in a marine bacterium by Lahaye et al. (1), who employed the newly discovered enzyme extract to degrade ulvan for structural analysis. More recently, several bacterial strains capable of metabolizing ulvan were isolated from the feces of a sea slug, Aplysia punctata, fed with Ulva. In this way, the Gram-negative Nonlabens ulvanivorans PLR was identified (6, 7) and its genome was sequenced (8). A novel ulvan lyase was purified from N. ulvanivorans batch culture, sequenced, and heterologously overexpressed in Escherichia coli, and the enzyme’s ability to depolymerize ulvan biochemically characterized (9). N. ulvanivorans ulvan lyase was reported to cleave ulvan at the β(1→4) glycosidic bond between R3S and GlcUA or IdoUA via the β-elimination mechanism. The proton at the C5 position is abstracted, regardless of its configuration (syn for IdoUA or anti for GlcUA) with the hydroxyl group at C4 (Fig. 1). The β-eliminative cleavage results in the formation of a reducing end on one fragment and an unsaturated ring (Δ, 4-deoxy-1-threo-hex-4-enopyranosiduronic acid) on the non-reducing end of the second fragment (10). Because the protein sequence of N. ulvanivorans ulvan lyase had no characterized homolog in the databases, it was considered the first representative of a new family of polysaccharide lyases.

Sequence encoding an unsaturated β-glucuronyl hydrolase belonging to the glycoside hydrolase family GH105 (based on the Carbohydrate-Active Enzymes (CAZy) database) was found in the vicinity of the ulvan lyase in the N. ulvanivorans genome. This enzyme was shown to cleave specifically the

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§The abbreviations used are: R3S, 3-sulfated rhamnose; GlcUA, D-glucuronic acid; IdoUA, L-iduronic acid; Xyl, D-xylose; CM, conditioned medium; GH, glycoside hydrolase.
unsaturated non-reducing end of the end products of the ulvan lyase (11). The spatial proximity within the genome of these two ulvan-degrading enzymes pointed to occurrence of polysaccharide utilization loci. With this premise in mind, we sequenced the genome of *N. ulvanivorans* and three additional ulvanolytic Alteromonadales isolates: *Alteromonas* sp. LOR, *Alteromonas* sp. LTR, and *Pseudoalteromonas* sp. PLSV (12, 13). Inspection of the genomes did not reveal genes encoding proteins homologous to *N. ulvanivorans* ulvan lyase. This led us to hypothesize that additional ulvan lyases that do not share sequence similarity with the known ulvan lyase may be encoded in the genomes of the three other ulvanolytic strains. Here, we report identification and biochemical characterization of four novel ulvan lyases, which belong to a new polysaccharide lyase family now being established4 and are unrelated to *N. ulvanivorans* ulvan lyase.

**Experimental Procedures**

*Strain and Plasmid Constructions*—The bacterial strains and primers used in this study are listed in Tables 1 and 2, respectively. Native ulvan-degrading isolates were grown in Marine Broth (Difco) at 25 °C, whereas *E. coli* strains were grown in Luria Bertani (LB medium, Difco) at 37 °C. When appropriate, cultures were supplemented with antibiotics as indicated in Table 1. Primers were designed for amplifying genes of interest from the corresponding genomic DNA, with (e.g. LOR_107) and without their native signal peptide (e.g. LOR_107d) (Table 2). Both the insert and the expression vector were digested using the relevant restriction enzymes, gel-purified, and ligated to form a C terminus His-tagged protein. Recombinant plasmids were used to transform T7-expressing-competent *E. coli* (New England Biolabs) (Table 1).

4 B. Henrissat, personal communication.

**Protein Expression and Purification**—Transformed *E. coli* strains (Table 1) were used for the expression and purification of the His-tagged ulvan lyases. Batch culture was inoculated 1:50 (v/v) with transformed cells grown overnight and incubated for 2.5 h at 37 °C to reach optical density (600 nm) of 0.6–0.8. Protein expression was induced with 0.01 mM isopropryl 1-thio-β-D-galactopyranoside for 18 h at 16 °C. The induced culture was fractionated by centrifugation to obtain the culture supernatant containing secreted enzymes (herein termed conditioned medium, i.e. CM) and cell pellet as two separated fractions. After centrifugation, the pellet was resuspended in 0.1 M Tris-HCl, 0.2 M NaCl at pH 7.5. Cells were lysed using a French press followed by centrifugation to remove bacterial debris. The resulting supernatant was applied to a nickel-Sepharose column charged with 100 mM NiSO₄ (GE Healthcare). After washing, the bound proteins were eluted with a linear gradient of imidazole ranging from 5 to 500 mM. The active fractions were pooled, and protein purification was verified using SDS-polyacrylamide gel electrophoresis stained with instant blue (Expedeon). Recombinant protein yields measured by NanoBlue (Thermo Scientific) were 2.03 mg/ml and 60 μg/ml purified from pET28a and pET22b constructs, respectively. Expression levels of His tagged proteins in CM and cell lysate fractions were analyzed by Western blotting using His-Tag monoclonal antibodies (Novagen) followed with ECL detection reagent (Thermo Fisher Scientific).

**Enzyme Assay**—Ulvan was provided by CEVA (Pleubian, France) and the polysaccharide was extracted from *Ulva rotundata* and was obtained as described by Nyvall Collén et al. (9). Screening for ulvan lyases by monitoring the formation of reducing ends was carried out using the ferricyanide method described by Lane and Lawen (14). Briefly, 2 g liter⁻¹ ulvan in 0.1 M Tris-HCl, 0.2 M NaCl at pH 7.5 was incubated in 1:1 (v/v) ratio with overexpressed cell lysate at 30 °C and sampled at

![FIGURE 1. Ulvan lyase mode of action. A and B, chemical scheme of *N. ulvanivorans* ulvan lyase (UL) cleaving ulvan at the β(1→4) glycosidic bond between R3S and GlcUA (A) or IdoUA (B) via β-elimination mechanism. The β-eliminative cleavage results in the formation of a reducing end on one fragment and an unsaturated ring (Δ, 4-deoxy-L-threo-hex-4-enopyranosiduronic acid) on the non-reducing end of the second fragment.](image-url)
various time points. Samples were mixed at 1:10 ratio (v/v) with ferricyanide solution composed of 300 mg liter⁻¹ K₃Fe(CN)₆ (Sigma-Aldrich), 28 g liter⁻¹ Na₂CO₃ (Sigma-Aldrich), and 1 ml liter⁻¹ 5 M NaOH dissolved in purified water (Milli-Q). The mixture was boiled and cooled, and the amount of reducing ends was monitored by measuring absorbance at 415 nm. Characterization of the ulvan lyases was also performed by measuring the formation of double bonds in the assay at 235 nm as described previously (15). The spectrophotometer (Synergy™ M4 Hybrid Microplate Reader, BioTek) was set to read at intervals (300 ng/ml) was added directly to the assay. The increase in absorbance at 235 nm was followed for up to 120 min. Degradation kinetics of ulvan was measured by gel permeation chromatography after various incubation periods with 6 ulvan in 0.1M Tris-HCl, 0.2M NaCl at pH 7.5.

Oligo-ulvan end products were purified using filtered digested ulvan (0.22-μm nylon Clarinert syringe filters, Agela Technologies). Samples were injected on three Superdex 30 columns (GE Healthcare, 2.6 × 60 cm) mounted in a series. Elution was conducted at a flow rate of 1.5 ml min⁻¹ with 50 mM (NH₄)₂CO₃ (pH 8) as the eluent. Detection of products was achieved with a Rapid Separation LC (RSLC) UV detector (Thermo Scientific, Dionex) operating at 235 nm, along with an IOTA 2 refractive index detector (Precision Instruments).

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NMR Spectroscopy—¹H NMR spectra were recorded at 298 K on a 400-MHz Avance DRX400 spectrometer (Bruker) in deuterated water as solvent and calibrated against the residual signal of the solvent. Prior to analysis, samples were exchanged twice in D₂O and re-dissolved in D₂O (99.97 atom % D). Chemical shifts are expressed in ppm in reference to an external standard (trimethylsilyl propionic acid). The HOD signal was not suppressed.

Results

Identification of Putative Ulvan Lyase Sequence in the Genome of Alteromonas sp. LOR—The genomes of the following four ulvan-degrading bacteria were sequenced and assem-
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FIGURE 2. Secretion of LOR_107 with and without signal peptide. A and B, ulvan incubation with CM (A) or cell lysate (B) derived from overexpressed LOR_107 (red), LOR_107d (green, i.e. without signal peptide), and empty vector (black) was followed by UV (235 nm). Ulvan lyase activity is indicated by the increase in 235 nm absorbance. Error bars indicate S.E. C, Western blot of the CM fractions (LOR_107 (lane 1), LOR_107d (without signal peptide) (lane 2), and empty vector (lane 3)), and of cell lysate fractions (LOR_107 (lane 4), LOR_107d (lane 5), and empty vector (lane 6)).
due (GlcUA-R3S-H1 4.82 and 4.9 ppm) decreased strongly. Similarly, the anomeric proton of the glucuronic acid residue (GlcUA-H1, 4.58 ppm) almost completely disappeared in contrast to the anomeric proton of the iduronic acid residue (IdoUA-H1), suggesting that the enzyme preferentially cleaves glucuronic acid residues.

Degradation kinetics of the ulvan lyase were followed at 235 nm to determine the optimal condition for activity. Initial velocity increased with temperature up to 40 °C; above this temperature, the enzyme began losing activity. The curve representing initial velocity as a function of pH showed a typical bell shape centered around pH 8. Variation of one pH unit led to a decrease of 8% in enzyme activity (data not shown).

Ulvan depolymerization catalyzed by LOR-107d ulvan lyase was monitored using gel permeation chromatography (Fig. 4). After the addition of enzyme, the molecular mass of ulvan, which eluted between 14 and 20 min of elution time, decreased rapidly, and intermediate oligo-ulvans appeared between 40 and 66 min of elution time (Fig. 4). After 24 h of incubation, the majority of the ulvan polysaccharides were converted into small-sized oligo-ulvans.

The ulvan lyase end products were purified and characterized by 1H NMR according to Lahaye et al. (1). The most abundant end products eluting at 63 min were disaccharides attributed to the 4-deoxy-L-threo-hex-4-enopyranosiduronic acid linked to sulfated rhamnose (Δ-R3S-H1/H3) decreased strongly.

**FIGURE 3**. 1H NMR spectra of ulvan degraded by LOR_107. A and B, ulvan polysaccharide NMR spectra (A) as compared with end products spectra of ulvan incubated with purified LOR_107 (B). In spectra B, signals ascribed to the anomeric protons of the sulfated rhamnose residues linked to glucuronic acid residue (GlcUA-R3S-H1/H3) decreased strongly.

**FIGURE 4**. Degradation kinetics of ulvan incubated with LOR_107 followed by gel permeation chromatography. The high molecular weight ulvan polysaccharide is converted into oligo-ulvans rapidly. The two most abundant oligo-ulvans are disaccharides (Δ-R3S) and tetrasaccharides (Δ-R3S-Ido-R3S).

Characterization of Proteins Homologous to LOR_107 ulvan lyase—Based on identification of LOR_107, inspection of the genome of Alteromonas LOR revealed a second ulvan lyase, LOR_61. The molecular mass of the protein encoded by LOR_61 was estimated as 110.9 kDa, and the structure was predicted to comprise two modules when aligned with the short ulvan lyase (Fig. 6). The first module of 55.2 kDa was observed to share 68% sequence identity with LOR_107. The second module of 54.7 kDa at the C terminus was noted to resemble a conserved type II dockerin repeat domain along with three repeating motifs of unclear function (p value < 0.0001) (19). The full protein was cloned and overexpressed successfully and, as expected, demonstrated ulvan lyase activity (Fig. 7).
Long and short ulvan lyases, homologous to LOR_61 and LOR_107, respectively, were found in Alteromonas LTR (LTR_2195, LTR_2241) and Pseudoalteromonas PLSV (PLSV_3925, PLSV_3875) (Table 3). Alteromonas LTR ulvan lyase sequences (LTR_2195, LTR_2241) are 100% identical to Alteromonas LOR ulvan lyases (LOR_61, LOR_107, respectively). The long protein similarly comprised an ulvan lyase catalytic module and a conserved type II dockerin. The proteins were overexpressed, and ulvanolytic activity was confirmed using gel permeation chromatography (Fig. 7). The end products were similar to those generated by LOR_107, suggesting common modalities of ulvan degradation.

Due to the low solubility of the purified overexpressed long ulvan lyases, biochemical characterization was performed on short ulvan lyases. The optimal temperature and pH for the three ulvan lyases were comparable, with only small variations observed. For example, temperature and pH optimum for PLSV_3875 was found to be 50 °C at pH 8, which differed only slightly from that observed for LOR_107. Notably, PLSV_3875 exhibited higher stability at elevated temperatures as compared with LOR_107, remaining 100% active after 24 h at 30 °C, whereas LOR_107 lost 90% of its activity in similar conditions.

The new ulvan lyase catalytic module sequence (Glu22–Leu495 in LOR_61) was utilized to screen for other homologous sequences in the database. Similarity searches were performed using BLASTp (20) against all non-redundant (nr) protein databases. Sequence-based similarity results showed 128 sequences with significant identity (E-value < 1 × 10−4). Homologous ulvan lyases were found in several bacterial strains, with the highest sequence similarities (up to 88% identity found in Pseudoalteromonas sp. PLSV, E-value = 0.0) and in certain marine strains. The majority of the marine strains belong to Gamma-proteobacteria, although surprisingly Flavobacteriaceae were found to possess putative proteins with 57% identity (E-value = 0.0). A few strains have multiple homologous proteins in their
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Discussion

Putative protein-coding genes located in a gene cluster likely involved in carbohydrate metabolism were cloned and overexpressed, and the resulting proteins were screened for ulvanolytic activity. This targeted screening strategy enabled identification of a new ulvan lyase, LOR_107, with a protein sequence unrelated to the previously characterized N. ulvanivorans ulvan lyase (9). 1H NMR analysis of the degradation end products, composed essentially of Δ-R3S and Δ-R3S-IdoUA-R3S, indicated that LOR_107 ulvan lyase cleaves specifically the β(1→4) glycoside bond between GlcUA and R3S residues according to a β-elimination mechanism. This substrate specificity is a unique feature, as the previously identified ulvan lyase in N. ulvanivorans cleaves both GlcUA and IdoUA residues by abstraction of the proton on the C5 position in both configurations, syn or anti, with regard to the hydroxyl group on C4 (9).

Protein sequences homologous to LOR_107 were found in Alteromonas sp. LOR (LOR_61) and in two recently sequenced genomes: Alteromonas sp. LTR and Pseudoalteromonas sp. PLSV. Each of these proteins was shown to be an endo-ulvan lyase, producing end products similar to LOR_107. Taken together, these proteins represent the first members of a new family of biochemically characterized polysaccharide lyases. A bioinformatic search using the newly identified protein sequence indicated that many other putative proteins could be members of this novel lyase family. Further, based on the BLASTp results, it seems that this new family is much more abundant in bacterial genomes than the first ulvan lyase family identified in N. ulvanivorans (128 as compared with 20 sequences, respectively, E-value < 1 × 10−6). A key difference between the two ulvan lyase families is the distinctive recognition modalities allowing the LOR ulvan lyase to cleave specifically the glycoside bond between GlcUA and R3S, as opposed to the N. ulvanivorans ulvan lyase that cleaves both GlcUA and IdoUA attached to R3S. Furthermore, the ulvan lyase end products are degraded by β-glucuronidase belonging to the GH88 family appears proximal to the long ulvan lyase of Alteromonas LOR, LTR and Pseudoalteromonas PLSV. This proximity suggests that different enzymes are involved in the degradation of oligo-ulvans. Taken together, these observations raise the possibility that at least two different pathways of ulvan degradation have evolved independently to perform saccharification of ulvan.

In Alteromonas LOR, Alteromonas LTR, and Pseudoalteromonas PLSV, we found two homologous ulvan lyases: one “short” (59 kDa) and one “long” (110 kDa). The “short” ulvan lyase comprised the catalytic module preceded by a signal peptide, suggesting that it is exported outside the bacterial cell. Interestingly, we were able to show that E. coli can identify the Alteromonas signal peptide and cleave it while secreting a mature and active enzyme to the milieu. The “long” ulvan lyase in addition to the ulvan lyase catalytic module (i.e. the “short” segment) had a conserved type II dockerin repeat domain of about 6.7 kDa at the C-terminal of the protein. Dockerin domains are involved in anchorage of proteins at the cell surface by binding to cohesin (21). A module of about 48 kDa of unknown function was inserted between the catalytic and

Table 3

| Strains                  | Short ulvan lyase | Long ulvan lyase | Pair identity (in %) |
|--------------------------|-------------------|-----------------|---------------------|
| Alteromonas sp. LOR      | LOR_107 (59.6 kDa) | LOR_61 (110.9 kDa) | 68                  |
| Alteromonas sp. LTR      | LTR_2241 (59.6 kDa) | LTR_2195 (110.9 kDa) | 68                  |
| Pseudoalteromonas sp. PLSV | PLSV_3875 (59.6 kDa) | PLSV_3925 (111.4 kDa) | 66                  |
| N. ulvanivorans PLR      | PLR_42 (59 kDa)   | PLR_48 (53.4 kDa)  | 74                  |

Pair identity refers to the amino acid similarity between the catalytic modules of pairs of ulvan lyases (short and long).
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dockerin module. In this regard, it is notable that two ulvan lyases were also documented in N. ulvanivorans. The short version is composed of a catalytic module with signal peptide, whereas the long ulvan lyase also comprises a Por secretion system (recently renamed type IX secretion system) C-terminal sorting domain sequence. The C-terminal sorting domain mediates protein secretion by the type IX secretion system, a system that is unique to the Bacteroidetes phylum (22–25). Flavobacterium johnsoniae genes corresponding to the type IX secretion system apparatus were also found in the N. ulvanivorans genome, the C-terminal sorting domain sequence in the “long” N. ulvanivorans ulvan lyase likely serves to ensure secretion to the medium.

Full enzymatic conversion of ulvan to monosaccharides requires several more enzymes in addition to ulvan lyase and β-glucuronidase, e.g. sulfatase, rhamnosidase, and xylanidase. Employment of a similar polysaccharide utilization locus screening strategy to that reported here may enable discovery of these enzymes. Further mining of the putative proteins may lead to the discovery of novel enzymatic activities and new GH families.

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