Characterization of marine bacteria and the activity of their enzyme systems involved in degradation of the algal storage glucan laminarin

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Abstract

The algal storage glucan laminarin is one of the most abundant carbon sources for marine prokaryotes. Its degradation was investigated in bacteria isolated during and after a spring phytoplankton bloom in the coastal North Sea. On average, 13% of prokaryotes detected by epifluorescence counts were able to grow in Most Probable Number dilution series on laminarin as sole carbon source. Several bacterial strains were isolated from different dilutions, and phylogenetic characterization revealed that they belonged to different phylogenetic groups. The activity of the laminarin-degrading enzyme systems was further characterized in three strains of *Vibrio* sp. that were able to grow on laminarin as sole carbon source. At least two types of activity were detected upon degradation of laminarin: release of glucose, and release of glucans larger than glucose. The expression of laminarinase activity was dependent on the presence of the substrate, and was repressed by the presence of glucose. In addition, low levels of activity were expressed under starvation conditions. Laminarinase enzymes showed minimal activity on substrates with similar glucosidic bonds to those of laminarin, but different sizes and secondary and/or tertiary structures. The characteristics found in these enzyme systems may help to elucidate factors hampering rapid carbohydrate degradation by prokaryotes.

Introduction

Microbial communities in marine ecosystems play a key role in the cycling of organic carbon and nutrients. An estimated 50% of the primary production is cycled as dissolved organic carbon (DOC) through the microbial loop to higher trophic levels (Azam, 1998). Most of the bioavailable DOC is present as high molecular weight (HMW) molecules (Amon & Benner, 1994, 1996), and has to be cleaved by extracellular enzymes prior to uptake, as bacteria can only transport substrates with a maximum molecular mass of *c.* 600 Da through their cytoplasmic membrane (Weiss *et al.*, 1991). On the basis of the location of the extracellular enzymes, two types of extracellular enzymes can be distinguished: 'free' extracellular enzymes, which occur dissolved in water or attached to surfaces other than the cell that produced them; and 'ecto-enzymes', which cross the cytoplasmic membrane and remain associated with the producing cell (Chróst, 1991).

Polysaccharides are important constituents of HMW organic matter produced by algae (Biddanda & Benner, 1997; Biersmith & Benner, 1998). They display remarkable structural diversity as a consequence of the wide variety of monosaccharides and the different glycosidic bonds between them. The primary structure is determined by the types of monosaccharide and their linkage, and leads to a secondary structure, determining the shape of the polysaccharides (for example, β-1,3-linked glucans form helices). Polysaccharides may be linked to each other by hydrogen bridges, determining a tertiary structure, e.g. a loose hydrogel, or a tightly packed network structure similar to cellulose.

Polysaccharides are degraded by glycoside hydrolases (EC 3.2.1.-), enzymes hydrolyzing the glycosidic bond between two or more carbohydrate moieties. On the basis of the site of cleavage, enzymes can be classified as exo-acting enzymes, which remove one or more sugar units from the end of a polysaccharide chain, and endo-acting enzymes, which randomly hydrolyze bonds within the chains, thereby producing more ends for the exoenzymes to act upon. Often a synergistic action of these different hydrolases is necessary for efficient degradation of polysaccharides (Driskill *et al.*, 1999). Therefore, degradation of a single substrate requires carefully coordinated expression of the different enzymes, referred to

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as a system (Warren, 1996). Although carbohydrates are usually considered to be labile substrates for prokaryotes, the high concentration of carbohydrates in DOC (Benner *et al.*, 1992), marine sediments and sedimentary pore water (Cowie & Hedges, 1984; Arnosti & Holmer, 1999) demonstrates that carbohydrates are not always rapidly metabolized.

The polysaccharide laminarin, the storage glucan found in most algae and phytoplankton (Meeuse, 1962; Painter, 1983), is one of the most abundant carbohydrates in the marine ecosystem (Painter, 1983). It is a soluble β-1,3-D-glucose polymer with some branching at positions C-2 and C-6, and is also known as laminaran or leucosin. The size typically ranges from 20 to 30 glucose residues, and some chains are terminated by mannitol end-groups (Meeuse, 1962; Painter, 1983; Read *et al.*, 1996). These mannitol groups are absent in chrysolaminaran, the type of laminarin that is the principal storage glucan in diatoms and in the cosmopolitan genus *Phaeocystis*, which are both important phytoplankton groups driving global geochemical cycles (Nelson *et al.*, 1995; Schoemann *et al.*, 2005). Photosynthesis by diatoms alone generates as much as 40% of the 45–50 billion metric tons of organic carbon produced each year in the sea (Nelson *et al.*, 1995). Glucan can account for up to 80% of the organic carbon of diatoms and *Phaeocystis* (Meeuse, 1962; Myklestad, 1974; Janse *et al.*, 1996; Granum *et al.*, 2002; Alderkamp *et al.*, 2006). Therefore, an estimated 5–15 billion metric tons of laminarin are produced annually. Laminarin is located intracellularly, in vacuoles (Chiovitti *et al.*, 2004). It may be released as DOC into the marine environment after algal cell lysis (Brussaard *et al.*, 1995), or 'sloppy feeding' by copepods (Møller *et al.*, 2003), where it is one of the most abundant substrates for marine bacteria. Laminarin seems to be rapidly degraded in the pelagic system (Keith & Arnosti, 2001; Arnosti *et al.*, 2005).

Very few studies have characterized the enzyme systems of marine bacteria degrading substrates that are relevant in marine systems. Hydrolyzing activity in the marine environment has mainly been determined using small substrate proxies, consisting of a monomer such as glucose linked to a fluorophore such as methylumbelliferyl (MUF), the fluorescence of which increases upon hydrolysis (Martinez *et al.*, 1996; Arrieta & Herndl, 2002). Because they lack the structural properties of real substrates, these substrate proxies will probably detect mainly exo-type activities. Therefore, in this study laminarin was used as a relevant carbohydrate substrate to study the enzyme systems of marine bacteria that are abundant during a phytoplankton bloom in the coastal North Sea.

Materials and methods

Sampling

Surface water samples from the coastal North Sea were collected from the 'Royal NIOZ jetty' in the tidal inlet of the Marsdiep, The Netherlands (53°00′18″N, 04°47′42″E) from April through July 2002, during the phytoplankton spring bloom. Samples were collected with a bucket, at high tide, twice a week. For chlorophyll *a* analysis, water samples were filtered though Whatman GF/F filters, extracted in 90% acetone, and subjected to fluorometric analysis. Phytoplankton abundance and species composition were determined on Lugol (nonacid) preserved samples (Utermohl, 1958) under a Zeiss inverted microscope, using 3-mL or 5-mL counting chambers, under 50×, 400× and 1000× magnification. Total bacterial numbers were counted under an epifluorescence microscope after staining with Hoechst dye no. 33258 (Paul, 1982) and by the Most Probable Number (MPN) technique in liquid marine medium and in laminarin medium (Clarke & Owens, 1983). Marine medium consisted of artificial seawater supplemented with 'minor salts', trace elements, vitamins, Tris buffer (pH 7.5) (Boehringer Mannheim), Na_2HPO_4 and NH_4Cl as in Janse *et al.* (1999), containing 0.01% yeast extract (w/v, Becton Dickinson) and 0.01% casamino acids (w/v, Difco) as carbon source. Laminarin medium contained no yeast extract or casamino acids, but 2 mM glucose equivalents of laminarin from *Laminaria digitalis* (Sigma) as carbon source. As laminarin is a natural substrate with variable polymer size, the substrate concentrations are expressed as glucose equivalents. All medium components were sterilized by autoclaving, except for the vitamins and the laminarin, which were filter-sterilized (0.2 μm). The MPN counts were performed in 200 μL of medium in 250-μL, 96-well microplates, with seven replicates, incubated at 12 °C for at least 3 weeks. Positive growth was determined by visual turbidity.

Isolation of bacterial strains

Bacterial strains were isolated from the lowest and the highest positive MPN dilutions on the laminarin medium of the 29 June sample and from the highest positive dilution of the 15 July sample, by plating on the marine medium described above solidified with 2% agar (w/v, granulated, Becton Dickinson), and incubating at 12 °C. Bacterial cultures were grown in cotton-plugged Erlenmeyer flasks (culture volume < 20% of the maximum Erlenmeyer volume), under continuous aeration (200 r.p.m.), in the medium described above, at 25 °C.

Sequencing of 16S rRNA gene

Single colonies from plates were resuspended in sterile MilliQ water and used as templates in a PCR reaction using the universal 16S rRNA gene primers B8F (5′-AGAGTTTG ATCCTGGCTCAG-3′) and U1406R (5′-GACGGGCG GTGTGTRCA-3′) (Sambrook *et al.*, 1989). The amplified 16S rRNA gene was sequenced on an ABI automated DNA sequencer (PE Applied Biosystems) with primer U1406R.

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Sequence similarities for at least 500 bp of the 16S rRNA gene sequence were determined by BLAST analysis (Altschul *et al.*, 1997) of the National Center for Biotechnology Information database. Phylogenetic analysis of the obtained sequences and their close relatives was performed using the neighbor-joining method with 1000 bootstrap replicates using MEGA version 3.0 software (Kumar *et al.*, 2004).

Preparation of extracellular and crude enzyme extracts

Laminarin-degrading activity was examined in pure cultures of bacteria grown on 2 mM laminarin as sole carbon source. Two hundred milliliters of culture was harvested in mid-exponential growth phase, by centrifugation at 3500 ***g*** for 30 min at 4 °C. To obtain extracellular enzymes, the supernatant from the harvested cultures was transferred to clean tubes and centrifuged again at 3500 ***g*** for 30 min at 4 °C. The supernatant was stored on ice until the activity was assayed on the same day. To obtain 'free' cellular enzymes, including ectoenzymes, cell pellets were washed twice with ice-cold artificial seawater buffered with Tris (pH 7.5), and resuspended in 50 mM sodium phosphate buffer (pH 7.5). Cells were disrupted by French press (9000 bar) and debris was removed by centrifugation at 20 000 ***g*** for 10 min at 4 °C. As the cell debris interfered with the laminarinase assay, and the supernatant contained more than 90% of the laminarin-degrading activity, the supernatant was used as a crude extract of cellular and ectoenzymes. Extracts were stored on ice until the activity was assayed on the same day.

Laminarinase assays

Extracellular enzymes and crude cell extracts were tested for their capacity to hydrolyze laminarin. To the supernatant containing the extracellular enzymes, 10 mM glucose equivalents of laminarin (final concentration) was added, and triplicate samples were incubated at 25 °C. Crude cell extracts containing cellular and ectoenzymes were diluted 1 : 10 in 50 mM sodium phosphate buffer (pH 7.5), and 20 mM glucose equivalents of laminarin (final concentration) was added. Two controls were incubated: 20 mM glucose equivalents of laminarin in 50 mM sodium phosphate buffer, and crude cell extract in 50 mM sodium phosphate buffer. Triplicate samples were incubated at 25 °C. After 3 h and after overnight incubation, a sample was taken, heat inactivated (3 min at 80 °C) and stored at − 20 °C. The release of glucose was measured using the Boehringer D-glucose test combination (Boehringer, Mannheim). This method selectively measures the D-glucose concentration, with a lower detection limit of 18 μM. The release of reducing sugars was measured using the alkaline ferricyanide reaction, using the reagent 2,4,6-tripyridyl-*S*-triazine (Sigma) (Myklestad *et al.*, 1997). This sensitive method was originally developed to determine carbohydrate concentrations in natural seawater, with detection levels as low as 2.5 μM glucose equivalents. It involves hydrolysis of polysaccharides prior to colorimetric detection of each reducing sugar molecule by the alkaline ferricyanide reaction. For the purpose of this study, hydrolysis of polymers was omitted, and the enzymatic release of reducing sugars was determined. The release of glucans larger than glucose was determined by subtraction of the glucose concentration from the total reducing ends. The protein concentration was measured using the Bradford method (Bradford, 1976). To test the effect of the buffer, incubations were also carried out using GF/P-filtered and autoclaved natural seawater that was buffered with Tris (50 μM final concentration; pH 7.5).

Kinetic analyses: apparent K_m and V_{max} determinations

Apparent K_m and V_{max} were determined for crude extracts from each strain. Total activity and glucose release were determined at different substrate concentrations (0.1–20 mM glucose equivalents). At least eight substrate–activity data pairs were fitted according to Michaelis–Menten kinetics using the nonlinear regression program TABLECURVE (Jandel Scientific, AISN Software).

Substrate specificity

The substrate specificity of crude extracts was determined using the enzyme activity assay described above using 0.5% (w/v) of the following substrates: curdlan from *Alcaligenes faecalis* (Sigma), β-glucan, dietary fiber control (Sigma), β-D-glucan from barley (Sigma), lichenan from *Cetraria islandica* (Sigma), β-1,3-glucan from *Euglena gracilis* (Fluka), and 20 mM glucose equivalents of pullulan from *Aureobasidium pullulans* (Sigma). To determine the solubility of these substrates, solutions were incubated at room temperature for 1 h and mixed several times, before centrifugation (14 000 ***g***, 10 min). Total carbohydrate concentration was determined in the supernatant by the phenol–sulfuric acid method (Liu *et al.*, 1973).

Results and discussion

Isolation of bacterial strains from MPN dilution series growing on laminarin as a sole carbon source

The phytoplankton bloom of 2002 was dominated by the colony-forming haptophyte *Phaeocystis globosa* from 6 June until 27 June. Prokaryote numbers varied between 2.3×10^9 and 3.3×10^9 cells L^{-1} over the period April through July (Fig. 1). On average, 13% of the prokaryotes detected by epifluorescence microscopy were able to grow in the MPN

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dilution series, both on marine medium and on laminarin medium. The percentage of prokaryotes that were able to grow on the marine medium increased from 5–6% during the wax of the *P. globosa* bloom to 37% during the wane of the bloom (29 June). The percentage of prokaryotes able to grow on laminarin was highest (33%) on 5 June and varied between 2% and 14% in the other samples.

The fraction of culturable prokaryotes was high in comparison to other studies (Ferguson *et al.*, 1984; Button *et al.*, 1993; Eilers *et al.*, 2000), especially following the *P. globosa* bloom. A similar result was obtained by Noordkamp *et al.* (2000), following the same MPN procedure used in this study. The liquid, marine medium with relatively low carbon concentrations (Janse *et al.*, 1999) seems suitable for culturing a high fraction of the marine prokaryotes present in the coastal North Sea. Over the whole study period, there was no difference between the fraction of culturable prokaryotes on marine medium and that on the medium containing laminarin as a sole carbon source. This suggests that a high fraction of prokaryotes had laminarin-degrading enzymes. Laminarin degradation seems to be a common feature in marine microbial communities, as it was degraded in all of the various marine microbial communities tested (Keith & Arnosti, 2001; Arnosti *et al.*, 2005). As laminarin is the principal storage glucan of the haptophyte *Phaeocystis globosa*, which dominated the phytoplankton bloom, and also of the diatoms that were abundant prior to the *P. globosa* bloom, it is not surprising that a large fraction of the prokaryotes could use laminarin as a carbon source during the investigated period.

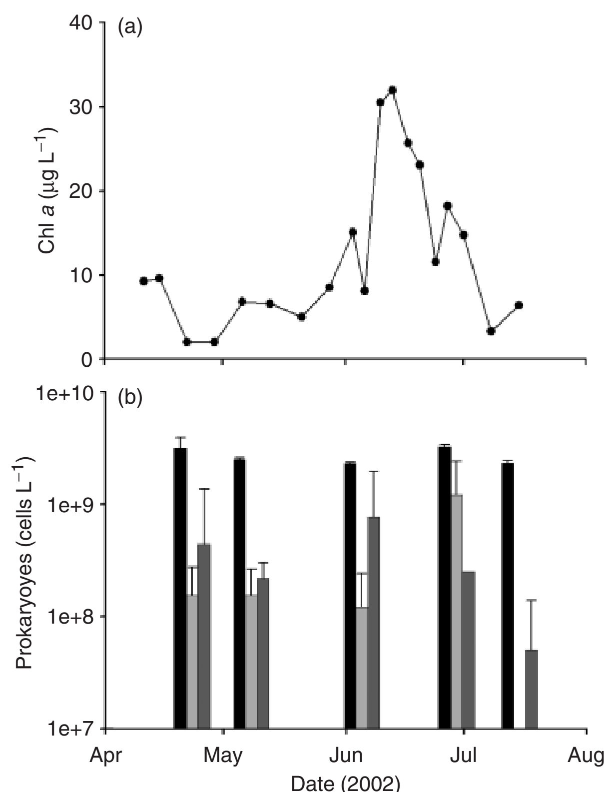

Fig. 1. (a) Temporal dynamics of chlorophyll *a* during spring and summer 2002. (b) Total prokaryote numbers counted by epifluorescence microscopy (black bars) and the Most Probable Number (MPN) technique on marine medium (light gray bars) and medium containing laminarin as sole carbon source (dark gray bars). Data for MPN counts on marine medium on 15 July are missing due to an infection. Error bars indicate a standard deviation of at least 10 counted fields (microscopy), or a 95% confidence interval of seven replicates (MPN).

Nineteen different bacterial strains were isolated from several dilutions of the MPN series on laminarin as a sole carbon source and subjected to phylogenetic analysis (Table 1, Fig. 2). Strains isolated from the same dilution with identical 16S rRNA gene sequences were considered to be the same (numbers in parentheses in Table 1). These strains were able to grow on either laminarin as a sole carbon source, or on byproducts of laminarin hydrolysis by other strains. The strains that were isolated from the highest dilutions were the most abundant of the isolates. An estimate of their abundance in the original sample is given in Table 1.

The isolates belonged to different phylogenetic groups that are known to be abundant in coastal waters, such as *Roseobacter*, *Bacteroidetes*, *Pseudoalteromonas*, and *Vibrio* (Eilers *et al.*, 2000; Pinhassi *et al.*, 2004). Members of *Roseobacter* and *Bacteroidetes* have previously been isolated from the coastal North Sea, and culture-independent analysis showed their abundance in marine systems (Eilers *et al.*, 2000a, b). In addition, they were detected during and after a *Phaeocystis* bloom in a mesocosm (Brussaard *et al.*, 2005), and in stable microbial enrichments degrading *Phaeocystis* carbohydrates (Janse *et al.*, 2000). *Gammaproteobacteria* such as *Pseudoalteromonas* and *Vibrio* have also frequently been isolated, but usually comprise $< 1\%$ of the total prokaryote population (Eilers *et al.*, 2000a, b). However, bacteria from the genus *Vibrio* are ubiquitous and have long served as models for heterotrophic processes. They play an important role in coastal seas and estuaries, owing to their widespread abundance and high metabolic activities. They are present both as free-living bacteria and attached to particles, algae, copepods and fish (Huq *et al.*, 1990; Heidelberg *et al.*, 2002). They are capable of growing rapidly under nutrient-rich conditions, and surviving prolonged periods of starvation (Oliver *et al.*, 1991; Nyström *et al.*, 1992; McDougald *et al.*, 2002), and are known to grow on complex substrates such as chitin (Li & Roseman, 2004; Meibom *et al.*, 2004). As members of the genus *Vibrio* were isolated both from enrichments and from the highest dilution, we chose three *Vibrio* strains for further characterization of enzymes involved in the degradation of laminarin.

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Table 1. Bacterial strains isolated from the MPN dilution series on laminarin as sole carbon source inoculated with surface samples from the Marsdiep, The Netherlands

Strain	Sample date	Sample dilution	Presence in original sample (cells L^{-1})*	Closest phylogenetic match†
B1A	29 June	1 : 10	5×10^4	*Vibrio splendidus*
B1B (4)‡	29 June	1 : 10	5×10^4	*Cobetia marina*
B4C	29 June	$1:10^4$	5×10^7	*Vibrio splendidus*
C4B	29 June	$1:10^4$	5×10^7	*Vibrio* sp. PMV19
C4C	29 June	$1:10^4$	5×10^7	*Vibrio* sp. PMV19
C4E	29 June	$1:10^4$	5×10^7	*Vibrio splendidus*
ABE3A (2)	15 July	$1:10^3$	5×10^6	*Pseudoalteromonas tetradonis*
ABC3C (2)	15 July	$1:10^3$	5×10^6	*Pseudoalteromonas tetradonis*
ABC3A (5)	15 July	$1:10^3$	5×10^6	*Sulfitobacter pontiacus*
AB F3A	15 July	$1:10^3$	5×10^6	Uncultured member of *Bacteroidetes*

*These are conservative estimates, based on the presence of a single cell from the isolate in the dilution.
†All isolates were more than 99% similar to their closest match.
‡Numbers in parentheses are the number of strains isolated from the same dilution sample with identical 16S rRNA gene sequences.

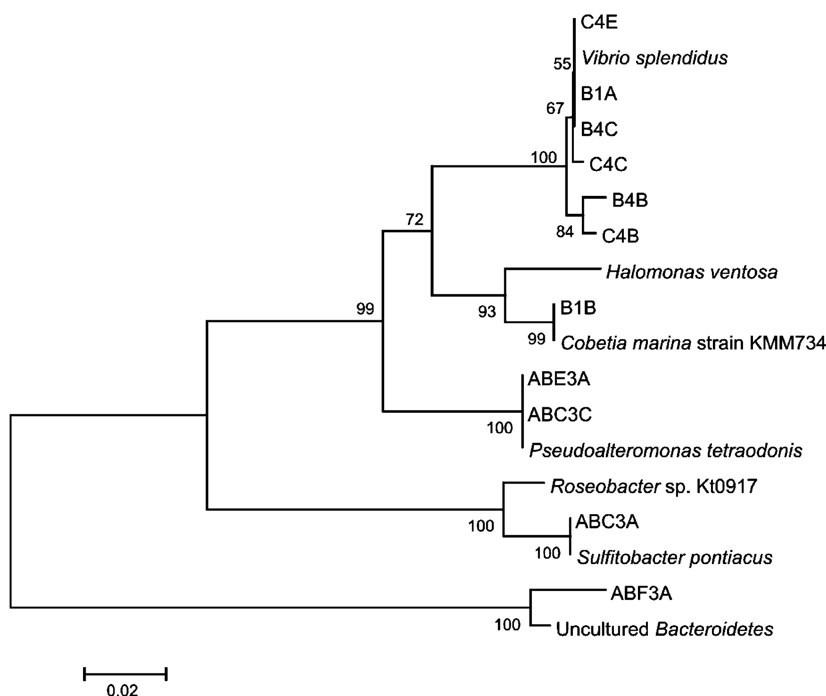

Fig. 2. Neighbor-joining tree based on partial 16S rRNA gene sequences derived from bacterial isolates and close relatives (identified via a BLAST search). The scale bar indicates 2% of sequence variation.

Laminarinase activity in three strains of *Vibrio* sp.

The laminarinase activity was not affected by the use of either Tris-buffered, filtered seawater or sodium phosphate buffer; therefore, the sodium phosphate buffer was used in the assays. Laminarinase activity was detected both in the medium and in crude cell extracts of the three *Vibrio* strains, indicating the presence of both extracellular enzymes and ectoenzymes. As more than 90% of the activity could be detected in the crude cell extract, this was used for further characterization of the enzyme systems degrading laminarin. These extracts probably include intracellular enzymes, periplasmatic enzymes and/or ectoenzymes. The total laminarinase activity closely followed Michaelis–Menten kinetics in each of the three strains ($r^2 = 0.978$, 0.943 and 0.944, respectively). At least two types of activity were detected: release of glucose, and release of glucans larger than glucose (Table 2). These activities are consistent with the presence of a gene encoding an endo-β-glucanase of glycoside hydrolase family 16 (http://afmb.cnrs-mrs.fr/CAZY/fam/acc_GH.html) (Henrissat, 1991) (EC 3.2.1.39) and genes encoding exo-β-1,3 glycosidases of family 17 (EC 3.2.1.58) in the genome sequence of *Vibrio vulnificus* (Kim *et al.*, 2003). As substrate cleavage by an endo-β-1,3-glucanase yields a new free end

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Table 2. Apparent kinetic parameters for laminarinase activity of crude cell extracts of *Vibrio* sp. strains B1A, B4C, and C4B. One unit of activity is expressed as μmol reducing ends released per hour per gram protein at pH 7.5 and 25 °C. The standard deviation (SD) for at least eight independent measurements is given in parentheses

Strain	Total activity, V_{max} (U)	Total activity, K_m (mM)	V_{max}/K_m values for total activity	Glucose-releasing activity, V_{max} (U)
B1A	34.17 (1.84)	4.50 (0.73)	7.6	0.83 (0.03)
B4C	10.26 (0.67)	0.78 (0.16)	13.1	0.90 (0.02)
C4B	8.53 (0.36)	0.57 (0.12)	15.0	0.81 (0.03)

that the exo-β-1,3 glycosidase can act upon, the synergistic interaction of these enzymes is likely to be responsible for efficient degradation of laminarin. Upon prolonged incubation, laminarin was degraded by more than 95% to glucose by the crude extracts of each of the three strains.

Both the total laminarinase activity (V_{max}) and the affinity constant (K_m) were highest in strain B1A and similar in B4C and C4B (Table 2). The V_{max}/K_m ratio, which represents the slope of the Michaelis–Menten equation at low substrate concentrations, is an indicator of the ability of the strain to achieve high hydrolysis rates at low substrate concentrations (Healey, 1980). This ratio was higher for strain B1A than for strains B4C and C4B. With similar rates of uptake and metabolism of the reaction products, strains B4C and C4B would be better competitors at low substrate concentrations, whereas strain B1A would be a better competitor at higher substrate concentrations.

The glucose-releasing activity was *c.* 10% of the total laminarinase activity at saturating substrate concentrations (Table 2). The higher rate of glucan release than of glucose release resulted in the accumulation of glucan intermediates during degradation at high substrate concentrations (Table 3). At lower substrate concentrations, however, the proportion of reducing ends released as glucose increased, suggesting a lower K_m value for the glucose-releasing activity than for the total activity. Release of glucans was also reported during microbial degradation of high concentrations (2% w/v) of complex carbohydrates in *Laminaria* thallus (Uchida, 1995). In the sea, bacterial hydrolysis of polymers of aggregates and uptake of low molecular weight compounds are often uncoupled processes, resulting in release of free polymers from particles into the surrounding water mass (Cho & Azam, 1988; Smith *et al.*, 1992, 1995; Unanue *et al.*, 1998; Azúa *et al.*, 2003). If the differences in kinetic properties between the release of glucan and glucose found in this study are general to other marine endohydrolase and exohydrolase activities, this may explain part of the mechanism. In aggregates, the carbohydrate concentrations are high (Azúa *et al.*, 2003), leading to high substrate concentrations for glycosidases. The higher V_{max} of endohydrolases than of exohydrolases will thus lead to the accumulation of polymer and/or oligomer intermediates. If these poly/oligomers are too large to be taken up by the prokaryotes, they will be released into the surrounding water. Accumulation of intermediates is unlikely to occur in the environment outside aggregates, where substrate concentrations are much lower, because the glucose/glucan release ratio is higher at lower substrate concentrations (Table 3).

Table 3. The ratio of glucose formation to glucan formation after overnight incubation of crude cell extracts of *Vibrio* sp. strains B1A, B4C and C4B with different concentrations of laminarin

Strain	Laminarin concentration		
	1 mM	5 mM	10 mM
B1A	0.85	0.18	0.11
B4C	0.85	0.15	0.14
C4B	0.67	0.18	0.11

Table 4. Relative laminarinase activity normalized to V_{max} rates of crude extracts of cells grown on laminarin and harvested at mid-exponential phase

Growth phase	Carbon source	B1A (%)	B4C (%)	C4B (%)
Exponential	Laminarin	100	100	100
	Pyruvate	ND	ND	ND
	Glucose	ND	ND	ND
	Pyruvate+laminarin	22	15	11
	Glucose+laminarin	ND	ND	ND
Stationary	Laminarin	78	132	104
	Pyruvate	3	3	2
	Glucose	9	3	2
	Pyruvate+laminarin	21	29	44
	Glucose+laminarin	12	32	34

ND, no activity could be detected, the detection limit being 0.5% of the activity on laminarin.

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To compare the kinetic parameters with those of different β-glucosidases present during and after a bloom of *P. globosa* in the coastal North Sea, determined using the fluorogenic substrate analogue MUF–β-D-glucoside (Arrieta & Herndl, 2002), we express the K_m values per mol of substrate, using an average size for the laminarin molecule of 25 glucose units. This leads to K_m values for the total activity of 180, 31.2 and 22.8 μmol L^{-1} laminarin for strains B1A, B4C, and C4B, respectively. These values are in the range of 12.1 to over 282.7 μmol L^{-1} MUF–glucose detected using MUF-β-D-glucoside (Arrieta & Herndl, 2002).

Expression of laminarinase activity in three strains of *Vibrio* sp.

When cultures were grown to exponential phase on glucose or pyruvate as a sole carbon source, no laminarinase activity was detected in crude extracts (Table 4). When cultures were grown on a mixture of laminarin and pyruvate as carbon sources, low levels of laminarinase activity were detected. Therefore, we conclude that during the exponential growth phase of the *Vibrio* strains, expression of laminarinase activity was dependent on the availability of laminarin. However, when cultures were grown on a mixture of laminarin and glucose, no laminarinase activity was detected. This suggests that synthesis of laminarinase is repressed in the presence of glucose. In the stationary growth phase, laminarinase activity was detected in all cultures. Stationary cultures grown on either pyruvate or glucose expressed low levels of laminarinase activity, whereas cultures grown on a mixture of laminarin and glucose, or laminarin and pyruvate, expressed intermediate activity.

Enzyme synthesis triggered by the presence of a suitable substrate and inhibition by monomeric compounds is a common feature of β-glucosidases in marine bacteria (Chróst, 1991; Middelboe *et al.*, 1995; Chin *et al.*, 1998). The expression of low levels of activity upon carbon starvation resembles the expression of extracellular chitinase activity upon starvation in *Vibrio furnisii* (Bassler *et al.*, 1991; Li & Roseman, 2004). The explanation put forward by Li & Roseman (2004) is that secreted chitinase from starving cells comes into contact with the insoluble chitin in the microenvironment of the organism and generates a disaccharide and/or oligomer gradient. The organism senses the soluble oligomer intermediates and swims up the gradient towards the chitin. In addition, oligomers induce the expression of the full chitin degradation system. Although laminarin is a soluble substrate, and may therefore directly be sensed by the organism, we speculate that expression of the laminarin degradation system may be regulated in a similar fashion. Thus, expression upon carbon starvation of different extracellular hydrolase enzymes may be a mechanism for the sensing of potential substrates in the *Vibrio* microenvironment.

Substrate specificity of the laminarinase enzymes

The activity of the laminarinase enzymes in the crude cell extracts of the *Vibrio* sp. strains grown on laminarin until mid-exponential phase was tested on several glucose polymers that differ from laminarin in size, solubility and structure (Table 5). Curdlan and glucan from *E. gracilis* are both β-1,3-glucans and thus have a similar primary and secondary structure to laminarin, but are much larger in size and are insoluble polymers. There was low activity on curdlan, but no activity was detected on the glucan from *E. gracilis* (Table 6). Barley glucan and lichenan have β-1,3-glucosidic bonds, connecting stretches of β-1,4-linked glucose, and consequently differ in secondary and tertiary structure from laminarin. Low activity was detected on both substrates. Pullulan is a repeating structure of three α-1,4-linked glucoses (maltotriose) connected by α-1,6-glucosidic bonds; it is a soluble substrate differing from laminarin in its primary, secondary and tertiary structure. No activity was detected with it. Each of the *Vibrio* strains, however, was able to grow on pullulan as sole carbon source (results not shown). The absence of pullulanase activity in strains grown on laminarin as sole carbon source shows that expression of

Table 5. Relevant information on the substrates used to determine the substrate specificity of crude cell extracts of *Vibrio sp.* strains B1A, B4C and C4B grown on laminarin to mid-exponential phase

Substrate	Backbone	Branches	Size	Solubility (%)	Source
Laminarin	β-1,3-Glucose	β-1,6	3.9 kDa	100	Food reserve in most algae
Barley glucan	β-1,3 Cellotriose and cellotetrose	No	49 MDa	21.6	Cell wall constituent in barley and other higher plants
Lichenan	β-1,3–1,4 Glucose	No	No information	15.7	Cell wall constituent of Irish moss
Dietary glucan	No information	No information	No information	5.5	
Curdlan	β-1,3-Glucose	No	100 kDa	0.15	Extracellular bacterial glucan
Euglena glucan	β-1,3-Glucose	No	500 kDa	0.12	Food reserve in yeast
Pullulan	α-1,6-Maltotriose	No	200 kDa	100	Extracellular polysaccharide in yeast, containing similar linkage types as amylopectin

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Table 6. Relative activity (%) of crude cell extracts of *Vibrio sp.* strains B1A, B4C and C4B normalized to V_{max} rates of crude extracts at mid-exponential phase grown on laminarin

Strain	Barley glucan	Lichenan	Dietary glucan	Curdlan	*Euglena* glucan	Pullulan
B1A	1.9	2.4	0.7	3.4	ND	ND
B4C	1.7	1.1	ND	1.2	ND	ND
C4B	0.7	1.5	ND	1.9	ND	ND

ND, no activity could be detected, the detection limit being 0.5% of the activity on laminarin.

pullulanase activity is likely to be dependent on the presence of pullulan; in a similar way, expression of laminarinase activity was dependent on the presence of laminarin.

The minimal activity of laminarinase enzymes on substrates similar to laminarin with respect to their primary and secondary structure may have important implications for polymer degradation in the marine environment. Polymers derived from algae are known to assemble spontaneously into hydrogels (Chin *et al.*, 1998), which may be the precursors of larger particles, such as transparent exopolymeric particles or marine snow (Verdugo *et al.*, 2004). Although particles are regarded as 'hot spots' of microbial abundance and activity (Azam, 1998), assemblage may influence the secondary structure of the polymers, analogous to the difference between laminarin and curdlan. If the difference in degradation potential between laminarin and curdlan is representative of the difference in degradation potential between 'free' polymers and polymers embedded in a gel structure, turnover times may be increased from days to years. This may be an additional explanation of why carbohydrates are usually regarded as labile substrates for marine microorganisms, but nevertheless form an important fraction of the DOC in the marine environment (Benner *et al.*, 1992), in marine sediments, and in sedimentary pore water (Cowie & Hedges, 1984; Arnosti & Holmer, 1999).

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