Extracellular Enzyme Activity in Anaerobic Bacterial Cultures: Evidence of Pullulanase Activity among Mesophilic Marine Bacteria

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The extracellular enzymatic activity of a mixed culture of anaerobic marine bacteria enriched on pullulan [α(1,6)-linked maltotriose units] was directly assessed with a combination of gel permeation chromatography (GPC) and nuclear magnetic resonance spectroscopy (NMR). Hydrolysis products of pullulan were separated by GPC into three fractions with molecular weights of $\geq$10,000, ~5,000, and $\leq$1,200. NMR spectra of these fractions demonstrated that pullulan was rapidly and specifically hydrolyzed at α(1,6) linkages by pullulanase enzymes, most likely type II pullulanase. Although isolated pullulanase enzymes have been shown to hydrolyze pullulan completely to maltotriose (S. H. Brown, H. R. Costantino, and R. M. Kelly, Appl. Environ. Microbiol. 56:1985–1991, 1990; M. Klingeberg, H. Hippe, and G. Antranikian, FEMS Microbiol. Lett. 69:145–152, 1990; R. Koch, P. Zablowski, A. Spreinat, and G. Antranikian, FEMS Microbiol. Lett. 71:21–26, 1990), the smallest carbohydrate detected in the bacterial cultures consisted of two maltotriose units linked through one α(1,6) linkage. Either the final hydrolysis step was closely linked to substrate uptake, or specialized porins similar to maltoporin might permit direct transport of large oligosaccharides into the bacterial cell. This is the first report of pullulanase activity among mesophilic marine bacteria. The combination of GPC and NMR could easily be used to assess other types of extracellular enzyme activity in bacterial cultures.

Bacteria must initially hydrolyze macromolecules to smaller substrates outside the cell, since the size of substrate which can be transported into the bacterial cell is severely restricted. In gram-negative bacteria (a designation that includes most marine bacteria), a lipopolysaccharide outer membrane surrounds the cell wall. Porins, trimeric proteins spanning the outer membrane, form the channels through which hydrophilic substrates are transported into the cell. The transportable limit in most cases is a molecular weight (MW) of ~600 (35). Larger substrates must be hydrolyzed outside the cell by cell surface or extracellular enzymes prior to transport into the cell. Numerous studies of isolated enzymes have demonstrated that bacterial cell surface and extracellular enzymes are specific for particular structures and structural features (32). Enzymes also have specificities for particular linkages, MWs, and structural configurations. Among the polysaccharide-degrading enzymes, one example is isoamylase, which can hydrolyze α(1,6) linkages in branched glucose polysaccharides but does not hydrolyze the α(1,6) linkages in pullulan, a linear glucose polysaccharide (2). The types and specificities of enzymes secreted by bacteria therefore are important determinants of organic matter degradation in marine environments.

A range of carbohydrate-degrading enzymes have been isolated from marine bacteria and characterized to yield detailed information on the thermal stability, catalytic activity, and substrate specificity of select enzymes (1, 9, 10, 16, 22, 25). These studies have focused primarily on enzymes of thermophilic and extremely thermophilic bacteria; however, the enzymes of marine mesophilic bacteria have not been extensively investigated. In addition, in vivo enzyme activity cannot be evaluated from studies of purified enzymes. Remineralization

of macromolecules, especially under anaerobic conditions, typically involves a variety of bacteria, each of which has specific metabolic capabilities and performs selected transformations of a substrate.

Studying bacterial enzymatic activity in situ is difficult, however, since there is a lack of specific information about the nature and concentrations of natural substrates and about the types of enzymes expressed by marine bacteria. The activity of carbohydrate-degrading enzymes in seawater and sediments has often been studied with fluorescent substrate analogs such as methylumbelliferyl glycosides (11, 17–19, 23, 29–31, 33, 34, 36). Hydrolysis of a methylumbelliferyl glycoside bond leads to an increase in fluorescence, providing a useful monitor of enzyme activity in seawater and sediments.

The substrate specificity of the enzymes themselves remains elusive, however, since methylumbelliferyl substrates are not necessarily adequate representatives of polysaccharides. Hydrolysis of methylumbelliferyl substrates can occur within the periplasmic space, as has been demonstrated by Martinez and Azam (27). Polysaccharides, however, must be initially hydrolyzed prior to transport into the periplasmic space; cell surface (or extracellular) enzymes responsible for initial hydrolysis of a polymer may have activities and substrate specificities different from those of periplasmic enzymes. In addition, because of their chemical nature and size, polysaccharides have tertiary structures (ribbons, loops, coils), which may aid (or impede) enzymatic access to hydrolytic sites and cannot reasonably be represented by a dimeric substrate.

The goal of this study was to directly assess the extracellular enzymatic activity of a mixed culture of anaerobic marine bacteria enriched on a single polysaccharide substrate. Specifically, we wanted to determine whether a polysaccharide with several linkage types and positions was specifically cleaved at selected sites or whether simultaneous action of several enzymes resulted in the production of a range of hydrolysis

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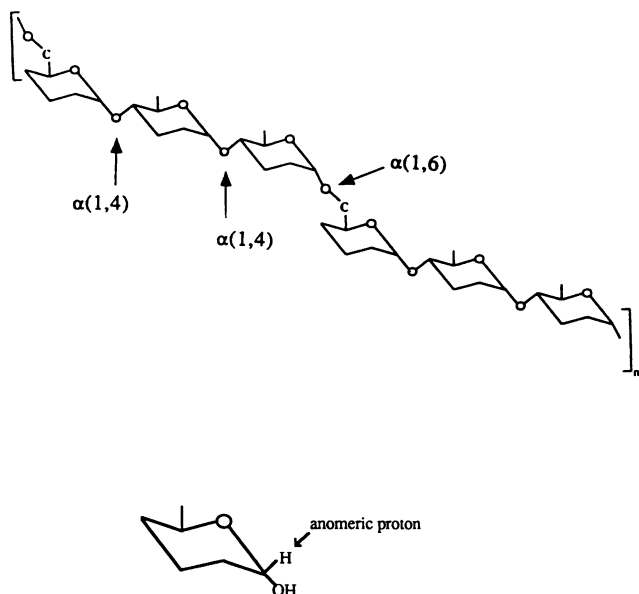
FIG. 1. Structure of pullulan substrate: $\alpha(1,6)$-linked maltotriose [$\alpha(1,4)$ linkages] units (MW, 200,000; $n = \sim 200$). The monosaccharide shows the position of the anomeric proton.

products. The techniques employed in our study are readily adaptable to studying the depolymerization of a variety of substrates by both aerobic and anaerobic bacteria.

As part of a study of structural characterization and bacterial degradation of carbohydrates in marine systems (3–6), we enriched cultures of anaerobic marine bacteria from sulfate-reducing sediments on a polysaccharide substrate. Using a combination of gel permeation chromatography (GPC) and nuclear magnetic resonance spectroscopy (NMR), we were able to monitor the course of polysaccharide degradation and the production and consumption of different MW classes of hydrolysis products. This study describes the precise location of enzymatic hydrolysis of a polysaccharide and, by extension, suggests the class of enzymes responsible for hydrolysis. The combination of GPC and NMR analyses is analytically tractable and provides unambiguous evidence of enzymatic cleavage patterns; neither substrate proxies nor isolation of a pure, active enzyme is required.

MATERIALS AND METHODS

Culture preparation. Replicate 125-ml serum vials were prepared with 50 ml of artificial seawater (4) under an N_2 headspace with Hungate anaerobic techniques (20). The medium was boiled vigorously for approximately 4 min and then was quickly cooled on an ice slurry, and the Na_2S solution was added to the medium. Medium was dispensed in 50-ml portions into 125-ml serum vials, which were capped with 1-cm butyl rubber stoppers, crimp sealed, and autoclaved. Vials in which the medium showed any pink coloration upon removal from the autoclave (i.e., incomplete reduction or the presence of oxygen) were discarded.

The substrate used was pullulan (U.S. Biochemicals), $\alpha(1,6)$-linked maltotriose [$\alpha(1,4)$] (Fig. 1), with a nominal MW of 200,000. After the culture vials had been filled and autoclaved, the pullulan was dissolved in deoxygenated water under N_2, filter sterilized, and injected into three replicate vials. Additional vials were prepared as blanks (see below). The final

substrate concentration after addition of the inoculum was 336 μg/ml (equivalent to 1.7 μM).

The inoculum source was sulfate-reducing, anoxic surface sediments from Salt Pond (Woods Hole, Mass.), a highly productive marine inlet (depth, 5 m; maximum summer temperature, 26°C; salinity, 31‰ [26a]) directly connected to Vineyard Sound (26). Approximately 27 cm^3 of sediment was slurried under N_2 with 70 ml of oxygen-free deionized water; 1.5 ml of the slurry was withdrawn by syringe and added to each vial. The amount of sediment added to a vial ranged from 80 to 100 mg (dry weight); variation in inoculum mass did not cause any observable differences between the triplicate cultures. Standard deviations of carbohydrate substrate measurements among the triplicate pullulan cultures ranged from 1 to 10% during the course of the experiment, while the standard deviation of replicate measurements from the same culture was 6%. The vials were incubated in the dark at 25°C for the course of the experiment (26 days).

Blanks. To ensure that changes in substrate composition and concentration were due to bacterial activity, a series of blank vials were also prepared and sampled with the pullulan cultures. Blank vials included vials with substrate and no inoculum, inoculated vials which were autoclaved prior to addition of substrate, and a vial which was inoculated without addition of substrate. Substrate recovery from the first two types of blank vials was complete 26 days after the beginning of the culture experiment, demonstrating that substrate was not lost through sorption to the walls of the vials or to sediment particles.

Culture sampling. Headspace gases, pH, and total CO_2 were measured during the course of the culture. A combination of GPC and NMR was used to separate, quantify, and characterize pullulan and its enzymatic hydrolysis products. Samples of culture medium (1.0 ml) were collected by syringe at intervals of 4 to 8 h for the first 70 h after inoculation. The samples were sterile filtered (0.2-μm-pore-size syringe filter) into clean vials, and stored frozen at $-40°C$ until analysis.

Sample analysis. GPC was carried out on a Sephadex G-50 column with a nominal exclusion of 10,000 Da and a 100- to 300-μm-mesh bead size. The column was 1.5 by 40.6 cm, with a void volume of 25.8 ml as calibrated with pullulan. The mobile phase was Millipore Q system deionized water (Q-H_2O), pumped at 1.1 ml/min with a peristaltic pump (Pharmacia P-1). Carbohydrates were detected with a refractive index detector (Showdex RI-71; detection limit, ca. 5 μg of carbohydrate), and data were acquired with a Hewlett-Packard 3396 II integrator. The column was also calibrated with laminarin [$\beta(1,3)$-linked glucose; nominal MW, 5,000 to 6,000] and maltoheptaose [$\alpha(1,4)$-linked glucose; MW, 1,153]. A mixed standard of pullulan, laminarin, and maltoheptaose injected onto the column was resolved into three overlapping peaks, with maxima at 23, 35, and 51 min. On the basis of the calibration standards, three fractions were collected for each sample: 20 to 29 min, 29 to 42 min, and 42 to 68 min. To separate the pullulan and its hydrolysis products by MW, samples were brought to room temperature, and 800 μl of sample and a small volume of rinse (Q-H_2O) were injected onto the GPC column.

The three fractions from each time point were individually collected, lyophilized, and redissolved in 0.5 ml of D_2O (100.0 atom% D; Aldrich). Lyophilization and dissolution in D_2O was repeated one to three times to exchange bound water. Proton spectra were acquired at 298 K with a Brucker AC3000 300-MHz spectrometer. Carbohydrate protons resonate in several distinct regions of the frequency spectrum. Anomeric protons, which are the protons attached to the no. 1 or

anomeric carbon of a monosaccharide ring, are well resolved from all other carbohydrate protons and typically resonate in the region from δ = 4.4 to 5.6. Exact resonance position and splitting (J coupling) depend on the size, chemical linkage, and linkage orientation of the carbohydrate structure. The resonance positions of most other carbohydrate protons overlap in the region between δ = 3.0 and δ = 4.0; deriving structural information from these protons can be difficult. The ^{1}H NMR spectra collected in this study included all of the pullulan protons, but the anomeric protons were the focus of interest, since changes in resonance intensity and position are unambiguous and are directly attributable to changes in the composition and concentration of the entire carbohydrate. At 298 K, the three anomeric protons of pullulan resonated at δ = 5.4 and 5.36 [the two α(1,4) anomers] and δ = 4.95 [the α(1,6) anomer]. Complete proton assignments for pullulan have been reported by McIntyre and Vogel (28).

Proton spectra were acquired for all pullulan fractions at all time points. Spectra of the first two fractions (20 to 29 min and 29 to 42 min) were acquired under identical conditions: solvent suppression, 1,024 scans, with a receiver gain of 400. Since the third fraction (42 to 68 min) contained nearly all of the salt and all of the buffer from the medium, the maximum receiver gain setting which could be used was 20; 2,048 scans were acquired. The spectra of the first two fractions were autoscaled to the zero time spectrum of the first fraction, so that changes in peak height and area should be proportional to changes in substrate concentration. Because the third fraction contained elevated carbon levels (buffer) and salt and was acquired under different spectrometer conditions, it was scaled by visual inspection to the same signal-noise level as the first two fractions.

The quantity of pullulan substrate in the fraction with a MW of ≥10,000 was determined from integration of the peak area from the gel permeation chromatogram, as determined by refractive index detection. The quantities of substrate in the fractions with MWs of ~5,000 and ≤1,200 were determined by integration of the NMR spectra, since coelution of salt (from the culture medium) dominated the response of the refractive index detector for these fractions.

RESULTS

The focus of this study was the pattern of enzymatic hydrolysis observed in the bacterial cultures; the complete time course of pullulan degradation and of the degradation of other polysaccharides is discussed elsewhere (3, 6). The zero hour and the 60-h samples illustrate the hydrolytic pattern observed in the pullulan cultures (Fig. 2 and 3). At zero hours, the pullulan substrate was found in the fraction with a MW of ≥10,000 (Fig. 2a), with three well-resolved anomeric resonances at δ = 4.95, 5.36, and 5.40, corresponding to the α(1,4)- and the two α(1,6)-anomeric protons, respectively. No substrate was observed in the fraction with a MW of ~5,000 (Fig. 2b). A weak anomeric resonance at δ = 5.4 in the fraction with a MW of ≤1,200 (equivalent to 2% of the total substrate) suggested that the pullulan contained a small amount of maltotriose as a contaminant (Fig. 2c).

At 60 h, both the size class distribution of substrate and the appearance of the anomeric resonances had changed (Fig. 3). The amount of pullulan in the fraction with a MW of ≥10,000 had decreased to 35% of the initial concentration (Fig. 3a). Thirty percent of the pullulan substrate had been hydrolyzed to a nominal MW of ~5,000 (Fig. 3b), and 17% was in the fraction with a MW of ≤1,200 (Fig. 3c). (Eighteen percent of the initial substrate had been converted to degradation products such as small organic acids and CO_2 [6]). The appearance
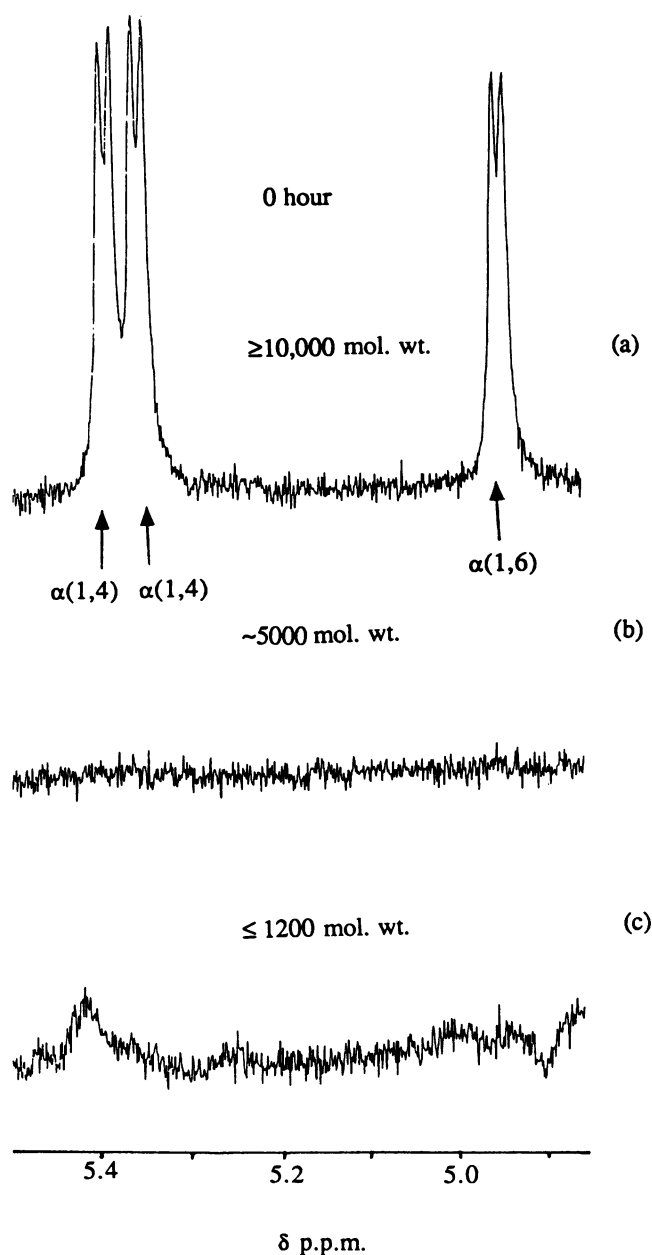

FIG. 2. ^{1}H NMR spectra of three GPC fractions of pullulan (zero time samples). (a) Fraction with MW of ≥10,000. (b) Fraction with MW of ~5,000. (c) Fraction with MW of ≤1,200. Nearly all of the substrate is in the fraction with a MW of ≥10,000 at zero hours; a low-intensity resonance at δ = 5.4 in the fraction with a MW of ≤1,200 suggests that a trace of maltotriose is also present.

of the anomeric proton resonances had changed as well. The anomeric resonance at δ = 4.95 was well resolved in all three MW fractions, while the anomers at δ = 5.36 and 5.40 were well resolved only in the fractions with MWs of ≥10,000 and ~5,000. In the fraction with a MW of ≤1,200, the anomeric resonance centered around δ = 5.4 became broader and less well resolved (in terms of the splitting observed in other size classes). Concurrently, the area of the resonance centered around δ = 5.4 became greater relative to the resonance at δ = 4.95. In pullulan, the ratio of the areas of the α(1,4) and
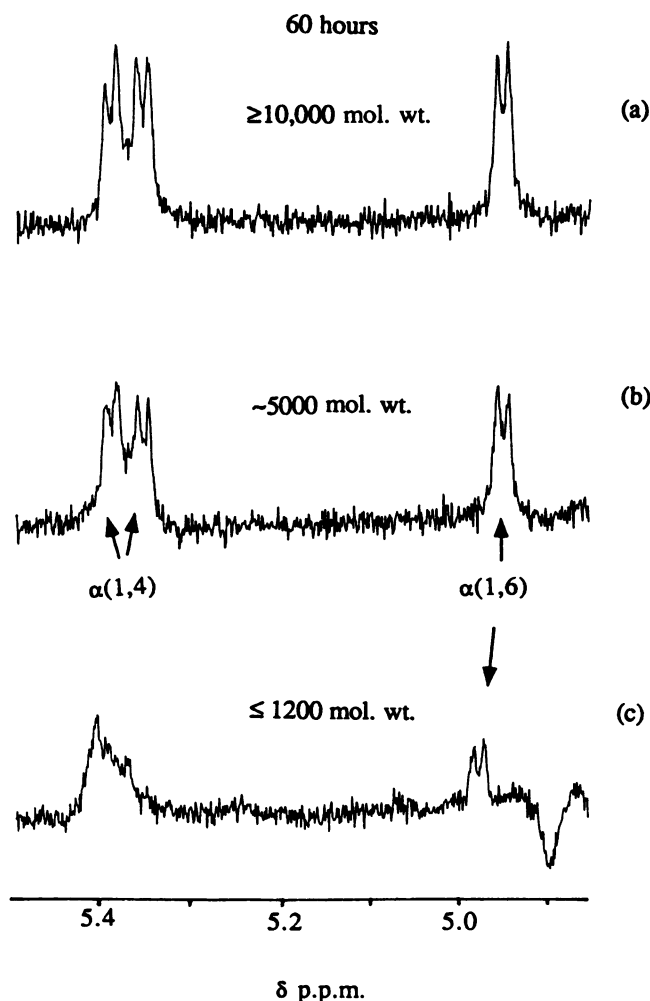
60 hours

FIG. 3. ^{1}H NMR spectra of three GPC fractions of pullulan (60 h). (a) Fraction with MW of ≥10,000. (b) Fraction with MW of ~5,000. (c) Fraction with MW of ≤1,200. The quantity of pullulan in the fraction with a MW of ≥10,000 (a) is significantly reduced, and the quantity of pullulan in the fraction with a MW of ~5,000 (b) is significantly increased. Note that the J couplings in panel b for both α(1,4) anomers and for the α(1,6) anomer are clearly resolved. The J coupling of the α(1,6) anomer is resolved in the fraction with a MW of ≤1,200 (c), but the α(1,4)-anomeric resonance is broad and shows no distinctive J coupling.

α(1,6) linkages is 2:1 [two maltotriose linkages to one α(1,6) linkage joining successive maltotriose units]. The same ratio is seen in the fraction with a MW of ~5,000, but in the fraction with a MW of ≤1,200, the area of the α(1,6) linkage is clearly less than half of the area of the α(1,4) linkages. A high-resolution ^{1}H NMR spectrum (12,800 scans, acquisition time of ~27 h) of the fraction with a MW of ≤1,200 at 60 h (Fig. 4) showed that the ratio of the α(1,4) to α(1,6) to free α anomers was 4.0:1.0:0.3.

DISCUSSION

The enzymatic hydrolysis of pullulan could occur by several different means. Either or both of the α(1,4) linkages could be hydrolyzed, or cleavage could occur preferentially at the α(1,6) linkage. If several bacteria excrete extracellular enzymes or

one type of bacterium secretes a range of saccharolytic enzymes, hydrolysis could occur simultaneously at all positions. Another possibility would be that one specific enzyme could hydrolyze pullulan to the oligosaccharide size range, at which point a range of enzymes might attack the oligosaccharide to produce a variety of smaller oligosaccharides.

Since cleavage of carbohydrate linkages produces distinct changes in NMR anomeric proton resonances, the pattern of extracellular hydrolysis of polysaccharides can be determined with NMR spectroscopy. Two aspects of the NMR spectra of the 60-h sample (Fig. 3), the changes in relative areas of the anomeric resonances and the change in the splitting of the anomeric resonances (J coupling), demonstrate that preferential hydrolysis occurs at the α(1,6) linkage. The 2:1 ratio of the α(1,4) to α(1,6) linkages in pullulan is seen in the fractions with MWs of ≥10,000 and ~5,000 (Fig. 3a and b). In the fraction with a MW of ≤1,200 (Fig. 3c), the relative area of the α(1,6)-anomeric proton is only one-fourth that of the α(1,4) linkages (Fig. 4). The 4.0:1.0:0.3 ratio of the α(1,4) to α(1,6) to free α anomers is exactly the ratio expected for a DP6 oligosaccharide consisting of two maltotriose units joined by one α(1,6) linkage (8). (DP is the degree of polymerization, and 6 is the number of monosaccharide units.)

The differences in appearance between the three MW fractions likewise show that the α(1,6) linkage is preferentially cleaved. In the fractions with MWs of ≥10,000 and ~5,000 (Fig. 3a and b, respectively), the J couplings of both the α(1,4) and α(1,6) linkages are well resolved and comparable in size, while in the ≤1,200-Da fraction, only the J coupling of the α(1,6) resonance is well resolved. In a DP6 oligosaccharide, as found in the fraction with a MW of ≤1,200, the magnetic environment of each of the four α(1,4) linkages differs slightly because of the difference in position within the oligosaccharide. These small differences are reflected in slight shifts in the resonance position of the α(1,4)-anomeric proton. The result is a net loss of resolution from the four overlapping, slightly different α(1,4) linkages. If both the α(1,4) and the α(1,6) linkages of pullulan were being hydrolyzed concurrently, the same broadening and loss of resolution would be apparent for the α(1,6)-anomeric proton.

The relative ratio of the α(1,4)-, α(1,6)-, and free α-anomeric protons in the fraction with a MW of ≤1,200 also precludes the presence of significant amounts of maltose or glucose in the fraction with a MW of ≤1,200, since free α anomers comprise 38 and 19% of the total anomeric protons for glucose and maltose, respectively (8); neither carbohydrate has α(1,6)-anomeric protons, and glucose additionally has no α(1,4)-anomeric protons. If significant proportions of maltotriose, maltotetraose, or maltopentose were present in solution, the relative proportions of free α anomers and α(1,4) anomers would be higher than observed in the spectrum, and the resolution of the α(1,6)-anomeric proton would be degraded. The NMR spectra also show that the hydrolysis of pullulan was specific through all substrate MW fractions. Even when substrate hydrolysis was well advanced, the remaining substrate had a well-defined linkage pattern.

Preferential cleavage of the α(1,6) linkage suggests an important point about the action of the enzymes hydrolyzing pullulan: they must be endohydrolases, capable of hydrolyzing linkages in the interior of the polysaccharide chain, and not exohydrolases, which can only hydrolyze the nonreducing terminal unit of a polysaccharide chain. Four different pullulanases and pullulan hydrolases, with different specificities, have been identified in bacteria: type I and II pullulan hydrolases and type I and II pullulanases. The enzymes in the pullulan cultures cannot be type I or II pullulan hydrolases,
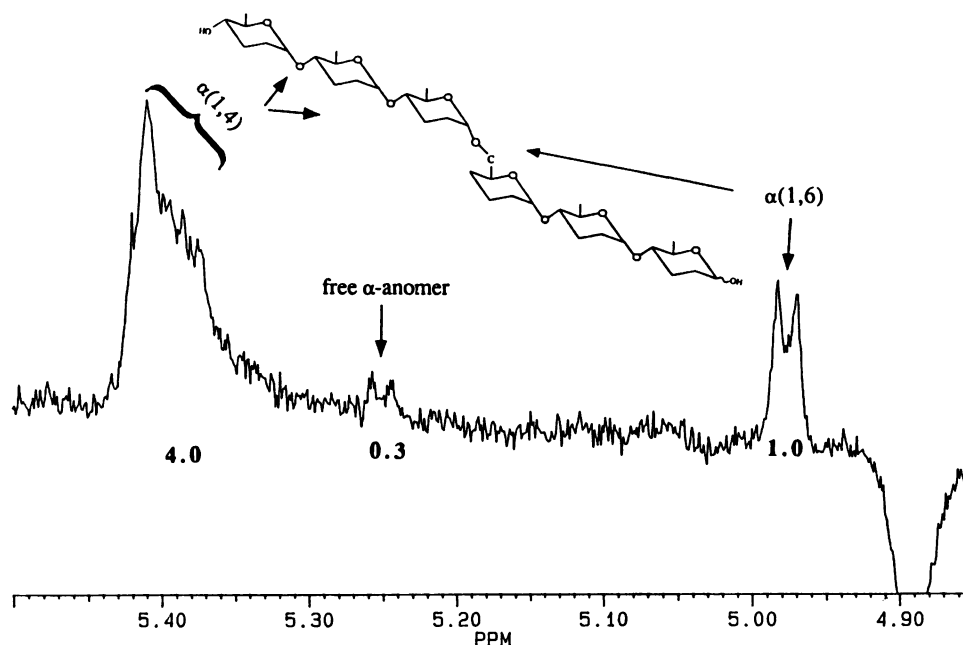
FIG. 4. High-resolution ^1H NMR spectrum of GPC fraction of pullulan with a MW of $\leq$1,200 (60 h [same fraction as Fig. 3c]). The ratio of the $\alpha(1,4)$ to $\alpha(1,6)$ to free α anomers exactly matches the ratio expected for two maltotriose units with an $\alpha(1,6)$ linkage (4.0:1.0:0.3). (The equilibrium ratio of free α to β anomers in glucose is 0.3:0.7; the free β anomer in this spectrum was obscured by spinning sidebands from the solvent peak.)

since these enzymes attack $\alpha(1,4)$ linkages in pullulan, yielding panose and isopanose, respectively (2). Type I and II pullulanases, however, specifically cleave $\alpha(1,6)$ linkages. Type I pullulanase cleaves both $\alpha(1,6)$ branches in branched polysaccharides such as amylopectin and $\alpha(1,6)$ linkages in pullulan. This endoenzyme typically produces maltotriose and a series of $\alpha(1,6)$-linked maltotriose oligosaccharides from pullulan. Type II pullulanase acts similarly to type I pullulanase but can hydrolyze $\alpha(1,4)$ linkages in addition to $\alpha(1,6)$ linkages (25). Hydrolysis of the $\alpha(1,6)$ linkage seems to be preferential, however, since type II pullulanases convert pullulan into a series of $\alpha(1,6)$-maltotriose oligosaccharides. Both pullulanases can only hydrolyze $\alpha(1,6)$ linkages which are in the vicinity of $\alpha(1,4)$ linkages (i.e., isomaltose and isomaltotriose are not hydrolyzed by pullulanase), which suggests that specific carbohydrate conformations are necessary for the enzyme-substrate complex (2).

The most active pullulan-degrading enzyme in the pullulan cultures must therefore be either type I or type II pullulanase. The evidence from the NMR spectra (Fig. 3 and 4) is consistent with either enzyme, since both hydrolyze pullulan to maltotriose and $\alpha(1,6)$-linked maltotriose oligosaccharides. Type I pullulanase, however, has been identified only in the aerobic bacteria *Klebsiella pneumoniae* and *Bacillus acidopullulyticus* (24) and in an anaerobic thermophilic *Fervidobacterium* species (2). Type II pullulanase, in contrast, has been found in a wide range of anaerobes. Because of their potential industrial utility, investigation of these enzymes has focused almost exclusively on thermophilic and extremely thermophilic bacteria, including thermophilic clostridia (24). Few studies have focused on marine bacteria; to the best of our knowledge, the only marine organisms reported to produce pullulanase were the hyperthermophilic marine archaebacteria *Pyrococcus furiosus* (10) and *Pyrococcus woesei* (2). The anaerobic bacteria enriched from sulfate-reducing sediments therefore more

likely produce type II pullulanase, which is found in other anaerobes as well.

A search of the literature has not revealed any other reports or investigations of pullulanase activity among mesophilic marine bacteria. In addition, the activity of pullulanase would not have been measured by previous studies of the enzymatic activity of bacteria in seawater or sediments (18, 23, 30, 34), because the fluorescent substrate analogs used in those studies do not conform to the structural specificity of pullulanase: pullulanase enzymes cleave only $\alpha(1,6)$ linkages which have $\alpha(1,4)$-linked monomers on either side (37). The ecological significance of pullulanase activity in marine sediments remains to be determined, since little is known about the structure of polysaccharides produced by marine organisms. Most carbohydrate analyses of marine waters, plankton, particles, and sediments have been restricted to determinations of monomer concentration and composition (12–15, 21).

This study is also a first resolution of the full range of pullulan hydrolysis, since the degradation of pullulan to smaller polysaccharides, as well as oligosaccharides, was directly monitored. In previous studies, pullulanase activity in pure cultures was determined by monitoring changes in the quantity of reducing sugars in the medium. Specific extracellular pullulanase activity was measured by incubating the cell-free medium with pullulan and monitoring production of glucose and DP2 to DP6 oligosaccharides with high-pressure liquid chromatography (HPLC) (24). In studies with isolated enzymes, the hydrolysis of starting substrate was determined by measuring reducing sugars colorimetrically, while production of oligosaccharides was determined by HPLC or thin-layer chromatography (10, 25).

The smallest size class of pullulan degradation products detected by NMR in our bacterial culture consisted of DP6 oligosaccharides. The DP6 oligosaccharides might have been taken up directly by bacteria, or the remaining $\alpha(1,6)$ linkage

might have been hydrolyzed to produce two maltotriose units. Additionally, the maltotriose units could have been hydrolyzed to maltose or glucose prior to uptake. Studies with enzyme mixtures isolated from bacterial cultures have shown that type I and II pullulanases ultimately convert pullulan almost entirely to maltotriose (10, 24, 25). In growing cultures, however, bacteria utilize substrate once it is hydrolyzed to a suitable size; the key is to determine the size at which the substrate can be transported into the cell.

Several lines of evidence suggest that, in our cultures, either the DP6 oligosaccharides were taken up directly, or hydrolysis of the DP6 oligosaccharides was so closely coupled to uptake that there was no measurable accumulation of free maltotriose, maltose, or glucose in the medium. As previously discussed, the NMR spectrum (Fig. 4) showing the relative proportions of the $\alpha(1,4)$-, $\alpha(1,6)$-, and free α-anomeric protons in the fraction with a MW of $\leq 1,200$ precludes the accumulation of significant amounts of maltose, glucose, or maltotriose in the medium. A related series of degradation experiments with DP2 to DP7 maltooligosaccharides as substrates also suggested that either maltooligosaccharides are transported intact through the bacterial membrane, or hydrolysis and transport are so closely coupled that no carbohydrate degradation products accumulated in detectable concentrations (3, 4).

The difficulty with direct bacterial uptake of DP6 oligosaccharides is that these oligosaccharides are nominally too large to be transported across the bacterial membrane, since the uptake limit of most porins is approximately 600 Da (35), which is the equivalent of a tri- or tetrasaccharide (DP3 or DP4). Maltooligosaccharides up to DP7, however, can be transported through the LamB porin (maltoporin), an inducible porin (i.e., expressed only in the presence of maltooligosaccharides) which has been extensively studied with Escherichia coli (see reference 7 and references cited therein). If maltoporin is also found in some species of marine bacteria, and if two maltotriose units connected with an $\alpha(1,6)$ linkage are similar enough to maltohexaose, then the DP6 oligosaccharide could be taken up directly without extracellular hydrolysis. In either case—whether uptake of maltohexaose is direct or is coupled to further enzymatic cleavage—the NMR spectra clearly demonstrate that significant amounts of carbohydrates smaller than maltohexaose do not accumulate in the cultures.

The results from the culture experiments unambiguously demonstrate pullulanase activity in mixed cultures of anaerobic mesophilic bacteria enriched from marine sediments. Pullulan was hydrolyzed to DP6 oligosaccharides, which were rapidly removed from the medium (6). To the best of our knowledge, this is the first report of pullulanase activity among mesophilic marine bacteria. A combination of GPC and NMR was used to analyze samples from growing bacterial cultures and to precisely determine the positions of extracellular enzymatic hydrolysis of pullulan. The same combination of GPC and NMR could be used to survey bacterial cultures for other types of extracellular enzymatic activity.

ACKNOWLEDGMENTS

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