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Microbial degradation of peptidoglycan in seawater

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Abstract

A constituent of the bacterial cell wall, peptidoglycan, has been suggested to be a significant fraction of marine dissolved organic matter, but little is known about its turnover. We measured hydrolysis and remineralization rates of peptidoglycan in surface waters by using radiolabeled peptidoglycan that was extracted from a Gram-negative bacterium. Polysaccharide (*N*-acetyl-glucosamine and *N*-acetyl-muramic acids) and peptide (D-glutamate) components of peptidoglycan were specifically radiolabeled. Purity of the preparations and specificity of labeling were ascertained by high-performance liquid chromatography. First-order kinetic constants of peptidoglycan remineralization were 2–21 times lower than those of proteins. The turnover time of peptidoglycan was estimated to be 10–167 d, indicating its semilabile nature. The two main components of peptidoglycan are degraded differently; the remineralization rate of the peptide component was three times greater than that of the polysaccharide moiety. Chemically modified, low-molecular-weight material was produced during the degradation of the polysaccharide component, but not during degradation of protein. These results indicate that peptidoglycan is less degradable than proteins in marine environments and are consistent with observations that D/L-isomer ratios of amino acids increase during early diagenesis.

A substantial fraction of marine dissolved organic matter (DOM), one of the largest reservoirs of organic carbon on earth (Hedges 2002), might be derived from bacteria (reviewed by Nagata 2000; *see also* Ogawa et al. 2001); concentrations of, especially, bacterial cell wall (peptidoglycan; McCarthy et al. 1998) and outer membrane components (porin; Tanoue et al. 1995) are relatively high in high-molecular-weight DOM recovered from surface and deep waters. Cell wall and membrane constituents might be inherently harder to degrade because of their biochemical composition (McCarthy et al. 1998) or because of the matrix surrounding these macromolecules (Nagata and Kirchman 1992, 1997; Borch and Kirchman 1999). However, few studies have examined whether cell wall and membrane components are more refractory than other cellular constituents, such as protein, that contribute to the DOM pool (Nagata et al. 1998).

The hypothesis that the bacterial cell wall component, peptidoglycan, is less degradable than proteins is supported by two lines of indirect evidence: (1) concentrations of D-amino acids, fundamental components of peptidoglycan, are high relative to L-amino acids in DOM (Lee and Bada 1977; McCarthy et al. 1998; Dittmar et al. 2001), and (2) D/L ratios of amino acids in dissolved combined amino acids increase during short-term (5–10 d) degradation experiments (Jørgensen et al. 1999; Amon et al. 2001). However, the interpretation of these data, which is based on the assumption that D-amino acids occur only in peptidoglycan, is complicated by D-amino acids produced by nonbacterial (e.g., archaea) or geochemical sources, about which only limited information is available (Amon et al. 2001). Furthermore, recent studies have failed to detect high concentrations of muramic acids, an amino sugar that exclusively occurs in peptidoglycan, in marine DOM (Kaiser and Benner 2000; Benner and Kaiser 2003) and in a refractory component of DOM produced by bacteria (Ogawa et al. 2001). Concentrations of D-amino acids and muramic acids would be high and low, respectively, if the polysaccharide strand of peptidoglycan is selectively degraded while the peptide bridge is left behind. However, this hypothesis cannot be tested because of the lack of data on degradation rates and transformation processes of peptidoglycan.

Comparing peptidoglycan and protein turnover could provide insights into the chemical nature and turnover of oceanic DOM, because of differences in the biochemical composition and lability of these two macromolecules and because of the potentially large input, especially of protein, into the dissolved pool. Both macromolecules might be important examples and test cases for the size-reactivity continuum hypothesis that attempts to explain the lability and composition of DOM in marine environments (Amon and Benner 1996). In brief, this hypothesis states that as organic material decreases in size, it becomes more refractory to microbial degradation, and less of it can be chemically identified at the molecular level (Benner 2002). The end point of the size-reactivity continuum is refractory DOC, which turns over on millennial timescales, is $<$1,000 Da, and can-

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Table 1. Precursors and protocols that were used for preparing radiolabeled bacterial cellular constituents.

Targeted components	Precursors	Extraction method	References
Polysaccharide moiety of peptidoglycan	^{3}H- or ^{14}C-*N*-acetyl-glucosamine	Hot SDS	Hancock and Poxton 1988
Peptide moiety of peptidoglycan	^{3}H-L-glutamic acid	Hot SDS	Hancock and Poxton 1988
Soluble protein	^{3}H-L-leucine	Sonication-ultracentrifugation	Nagata et al. 1998
Membrane protein	^{3}H-L-leucine	Sonication-ultracentrifugation, chloroform-methanol extraction	Nagata et al. 1998; Fukuda 2000

not be chemically identified at the molecular level (Benner 2002). The size-reactivity hypothesis is supported by studies that chemically characterized various molecular size fraction of DOM (reviewed by Benner 2002) and that examined the lability of these size fractions in bioassays (Amon and Benner 1996). Fewer studies have shown experimentally that low-molecular-weight (LMW) DOM is produced during DOM degradation. Smith et al. (1992) demonstrated the release of LMW DOM during marine snow degradation, and LMW DOM is apparently produced during chitin (Kirchman and White 1999) and protein degradation (Keil and Kirchman 1993). It is less clear whether chemically unidentifiable (at the molecular level) LMW DOM is produced by microbial activity.

Here, we describe the application of radiotracer techniques to determine degradation rates of peptidoglycan and protein in seawater. Radiolabeled peptidoglycan and protein were extracted from a marine bacterium, characterized by high-performance liquid chromatography (HPLC), and used in degradation experiments conducted in estuarine, coastal, and offshore environments. Our purpose was to test the hypothesis that peptidoglycan is less degradable than proteins in seawater. We found that marine bacterial assemblages readily hydrolyze and remineralize both polysaccharide and peptide components of peptidoglycan, but the rates were low compared to proteins. Hydrolysis and uptake of peptidoglycan were uncoupled, resulting in the accumulation of LMW materials of polysaccharide origin.

Materials and methods

Table 1 summarizes the precursors and extraction methods that were used for preparing radiolabeled peptidoglycan and proteins. Purity and specificity of these preparations were examined by HPLC (*see below*).

Preparation of radiolabeled peptidoglycan—Radiolabeled peptidoglycan was extracted from a Gram-negative marine bacterium, *Vibrio proteolyticus*, grown on culture media containing radiolabeled precursors. Either [^{14}C]- or [^{3}H]-*N*-acetyl-glucosamine (Amersham) was used to radiolabel the glycan strand. Fifty milliliters of LM broth (Trypton 10 g, yeast extract 5 g, NaCl 20 g in 1 liter of Milli-Q water) were inoculated with *V. proteolyticus*, spiked with the radiolabeled precursor (14.8 kBq ml^{-1} and 370 kBq ml^{-1} for ^{14}C and ^{3}H precursors, respectively), and incubated on a shaker at room temperature (20–25°C) for 5–7 h. Cells were harvested (10,000 × g for 10 min), washed three times with 2% NaCl, and resuspended with Milli-Q water. To extract peptidoglycan, the cell suspension was added to boiling sodium dodecyl sulfate (SDS; final concentration 4%) and incubated at 97–100°C for 30 min (Hancock and Poxton 1988). The pellet was collected by ultracentrifugation (142,000 × g for 60 min at 20°C), washed repeatedly (at least four times with Milli-Q water), resuspended in Milli-Q water, and stored in a freezer (−20°C). We also prepared ^{3}H-amino acid–labeled peptidoglycan from *V. proteolyticus* grown on glucose media (glucose 2 g, NH_4Cl 0.32 g, K_2HPO_4 0.04 g, $FeCl_3 \cdot 6H_2O$ 0.01 g in artificial seawater; Nagata et al. 1998) containing L-[^{3}H]-glutamic acid (Amersham).

HPLC analysis of radiolabeled preparation—The purity and radio specificity of peptidoglycan preparations were examined by reversed-phase gradient HPLC with precolumn derivatization with *o*-phthaldialdehyde (OPA; Lindroth and Mopper 1979) as described by Keil and Kirchman (1993). Samples were acid hydrolyzed with 3 N HCl at 95°C for 4 h. The two sugars of peptidoglycan, *N*-acetyl-glucosamine and *N*-acetyl-muramic acids, are deacetylated by hydrolysis, leaving glucosamine and muramic acid in the hydrolyzate. These amino sugars and amino acids were separated on a C18 column with a gradient of phosphate buffer and methanol. The chirality of amino acids was not examined; L- and D-forms of amino acids eluted at the same retention time. To obtain radiochromatograms, the effluent of HPLC was collected every 30 s, and the radioactivity in each fraction was determined by liquid scintillation counting. We also used an anion-exchange HPLC (Dionex CarboPac PA1 column) with pulsed amperometric detection to separate monosaccharides using a 12 mmol L^{-1} NaOH isocratic eluant (Borch and Kirchman 1997). Radiochromatograms were obtained as described above.

Preparation of radiolabeled bacterial protein.—In order to compare the degradation of peptidoglycan with that of protein derived from bacteria, radiolabeled membrane protein was extracted from *Vibrio alginolyticus* grown on the glucose medium (*see above*) containing ^{3}H-leucine (Fukuda 2000). Briefly, after the disruption of ^{3}H-leucine–labeled cells by sonication, pellets were recovered by ultracentrifugation (Nagata et al. 1998). Membrane protein and phospholipids were separated by using a methanol–chloroform mixture (Nagata and Kirchman 1992); the protein fraction was recovered at the solvent–water interface. Fukuda (2000) found that the membrane protein isolated by this method is

Table 2. Sampling locations and concentrations of added substrates.

Experiment No.	Location (date of sampling)	Substrates	Added concentration (μg L^{-1})
1	Tokyo Bay, Japan, Johnan Island (11 Nov 98)	[^{14}C-polysaccharide]-peptidoglycan	10
		^{3}H-protein*	42
2	U.S. Atlantic coast, Indian River Inlet (9 Dec 98)	[^{14}C-polysaccharide]-peptidoglycan	32
		^{3}H-protein*	41
3	U.S. Atlantic coast, Indian River Inlet (18 Oct 99)	[^{3}H-polysaccharide]-peptidoglycan	314
		^{3}H-protein*	2,254
4	U.S. Pacific coast, California (11 Jun 99)	[^{3}H-polysaccharide]-peptidoglycan	104
		^{3}H-protein†	92
5	U.S. Pacific coast, Monterrey Bay (3 Jun 99)	[^{3}H-polysaccharide]-peptidoglycan	114
		^{3}H-protein†	40
6	Tokyo Bay, Japan, Johnan Island (10 Jan 00)	[^{3}H-polysaccharide]-peptidoglycan	117
		[^{3}H-D-glutamate]-peptidoglycan	1,163
		^{3}H-protein*	289
7	Tokyo Bay, Japan, Johnan Island (29 Feb 00)	[^{3}H-polysaccharide]-peptidoglycan	125
		[^{3}H-D-glutamate]-peptidoglycan	405
		^{3}H-protein*	253

* Bacterial membrane protein extracted by using chloroform-methanol mixture (Fukuda 2000).
† Soluble bacterial protein extracted by sonication and ultracentrifugation (Nagata et al. 1998).

degraded in seawater as fast as soluble protein, suggesting that protection by liposomes (Nagata et al. 1998) is minimal. Thus we used this radiolabeled protein preparation as a model of labile protein. We also prepared a ^{3}H-leucine–labeled soluble bacterial protein (Nagata et al. 1998) for use as another model of labile protein.

Degradation experiments—Surface-water samples were collected from estuarine (Tokyo Bay, Japan, Experiments 1, 6, and 7), coastal (U.S. Atlantic coast, Experiments 2 and 3; Monterrey Bay, Experiment 5), and offshore (off California, Experiment 4) environments (Table 2). The type and added concentrations of substrates varied among the experiment (Table 2). The added concentrations (10–2,250 μg L^{-1}) were comparable to dissolved protein concentrations previously reported in coastal environments (40–1,500 μg L^{-1}, Keil and Kirchman 1993). McCarthy et al. (1998) suggest that peptidoglycan could be as abundant as protein in oceanic DOM.

In the dual-label experiments (Experiments 1 and 2, Table 2), mixtures of ^{14}C-peptidoglycan and ^{3}H-protein were added to 3 ml of 40-μm prefiltered seawater contained in glass vials sealed with Teflon-lined rubber septum caps. The vials were incubated in the dark at 20–22°C. At each sampling time, one vial was fixed with neutralized formalin (final concentration 2%) and used for the determination of $^{14}CO_2$ (Nagata et al. 1998). Another vial was used for measurements of $^{3}H_2O$ and low-molecular-weight dissolved organic material (LMW materials). Trichloroacetic acid (TCA, final concentration 5%) and protein carrier (bovine serum albumin, final concentration 1 mg ml^{-1}) were added to the samples to precipitate high-molecular-weight material. After incubation (10–15 min) and centrifugation (14,000 $\times$ g for 15 min), supernatant was collected. A portion of the supernatant was radio-assayed by liquid scintillation counting to determine the radioactivity of $^{3}H_2O$ and ^{3}H-LMW materials. Another portion of supernatant was evaporated at 60°C overnight. The residue after the evaporation was redissolved with Milli-Q water to measure the radioactivity (^{3}H- and ^{14}C-LMW materials) by liquid scintillation counting using a ^{3}H/^{14}C dual mode. The radioactivity of $^{3}H_2O$ was calculated by subtraction.

In the single-label experiments (Experiments 3–7), 10–15 ml of sample water (prefiltered through a 40-μm screen in Experiments 2, 6, and 7, whereas unfiltered sample water was used in Experiments 3, 4, and 5) were contained in polypropylene tubes and spiked with ^{3}H-labeled substrates (Table 2). Subsamples (1–2 ml) were collected over time to determine $^{3}H_2O$ and ^{3}H-LMW materials (*see above*).

Characterization of ^{3}H-LMW material produced during the degradation of ^{3}H-peptidoglycan—Concurrent with Experiment 3, we examined the size distribution of ^{3}H-LMW material produced during the degradation of ^{3}H-peptidoglycan. The experimental protocol was similar to that of Experiment 3, except that a high amount of ^{3}H-peptidoglycan (3.7×10^{5} Bq ml^{-1}, 3.5 mg L^{-1}) was added to increase the detection limit of ^{3}H after the separation. The sample water (200 μl each) was centrifuged with Microcon microconcentrators with a molecular weight cut off (MWCO) of 3,000 (3K) (Microcon-3), 10K (Microcon-10), and 100K (Microcon-100) according to the manufacturer's manual. Filtrates were collected, dried, and redissolved with Milli-Q water to determine the radioactivity by liquid scintillation counting. To test whether ^{3}H associated with LMW material is recovered with glucosamine, the <3K MWCO fraction was collected after 123 h incubation, dried, acid hydrolyzed, and analyzed by reversed-phase HPLC (*see above*).

Results

Purity and specificity of radiolabeled peptidoglycan preparations—Radiolabeled peptidoglycan was acid hydrolyzed and analyzed by reversed-phase HPLC with OPA derivatization. Glutamic acid, alanine, glucosamine, and muramic

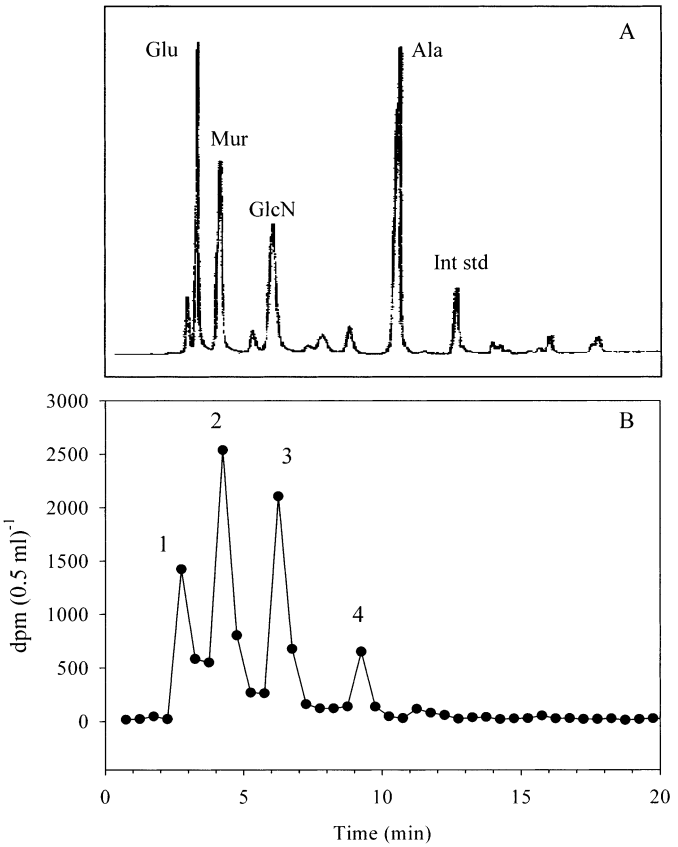

Fig. 1. HPLC analysis of the hydrolyzate of peptidoglycan radiolabeled with ^{3}H-*N*-acetyl-glucosamine. (A) A reversed-phase HPLC chromatogram and (B) the corresponding radiochromatogram are presented. Glu, glutamate; Mur, muramic acids; GlcN, glucosamine; Ala, alanine; Int std, internal standard (alpha-amino butyric acids).

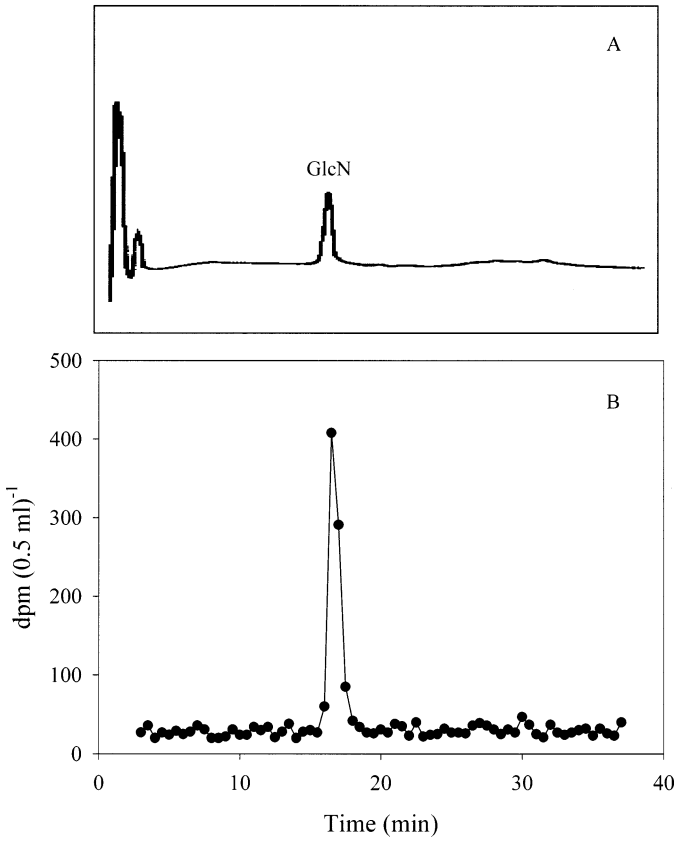

Fig. 2. HPLC analysis of the hydrolyzate of peptidoglycan radiolabeled with ^{3}H-*N*-acetyl-glucosamine. (A) A HPLC-PAD (pulsed amperometric detection) chromatogram and (B) the corresponding radiochromatogram are presented. GlcN is glucosamine.

acid, with an approximate molar ratio of 1 : 2 : 1 : 1, accounted for a major portion of primary amines recovered from the hydrolyzates (Fig. 1A). This composition of amino acids and amino sugars is consistent with that of peptidoglycan extracted from Gram-negative bacteria (Schleifer and Kandler 1972). According to anion-exchange HPLC pulsed amperometric detection (HPLC-PAD), the hydrolyzate contained only glucosamine (Fig. 2A). HPLC-PAD failed to detect muramic acid because this compound does not elute with 12 mmol L^{-1} NaOH (R. Benner pers. comm.). These data indicate the high purity of our preparations with minimal contamination by proteins and polysaccharides other than peptidoglycan.

Isotope recovery and specificity were examined for peptidoglycan preparations radiolabeled with ^{3}H- or ^{14}C-*N*-acetyl glucosamine. The radiochromatograms of ^{3}H- (Fig. 1B) and ^{14}C-labeled (data not shown) preparations were similar. A large portion of radioactivity coeluted with muramic acid (peak 2; recovery 33–38%) and glucosamine (peak 3; recovery 25–29%) (Table 3), indicating that the radiolabeled precursor, *N*-acetyl-glucosamine, was converted to *N*-acetyl-muramic acid to be incorporated into peptidoglycan. A significant fraction (18–23%) of radioactivity was recovered at a retention time shorter than that of glutamic acid (peak 1, Fig 1B; Table 3). This peak was probably associated with either glucosamine or muramic acid that had not derivatized with OPA; underivatized primary amines elute at the beginning of the chromatogram because of their hydrophilic nature (Fuhrman 1987). Two lines of evidence support this hypothesis. First, peak 1 was collected, dried with N_2 gas, derivatized with OPA, and analyzed by reversed-phase HPLC. A substantial fraction (40%) of total radioactivity associated with peak 1 coeluted with glucosamine and muramic acid, indicating that radiolabeled primary amines remaining in the eluent reacted with OPA. Second, ^{14}C-*N*-acetyl-glucosamine (Amersham) was acid hydrolyzed and

Table 3. Recovery of radioisotopes in HPLC elutions. Samples are acid hydrolyzates of peptidoglycan radiolabeled with ^{3}H- or ^{14}C-*N*-acetyl glucosamine. Peak numbers correspond to those indicated in Fig. 1B.

		Recovery (% of total injection)				
	Total injection (Bq)	Peak 1	Peak 2 (muramic acid)	Peak 3 (glucosamine)	Peak 4	Total*
^{3}H	175	18	38	29	8	93
^{14}C	11	23	33	25	5	86

* Sum of peaks 1, 2, 3, and 4.

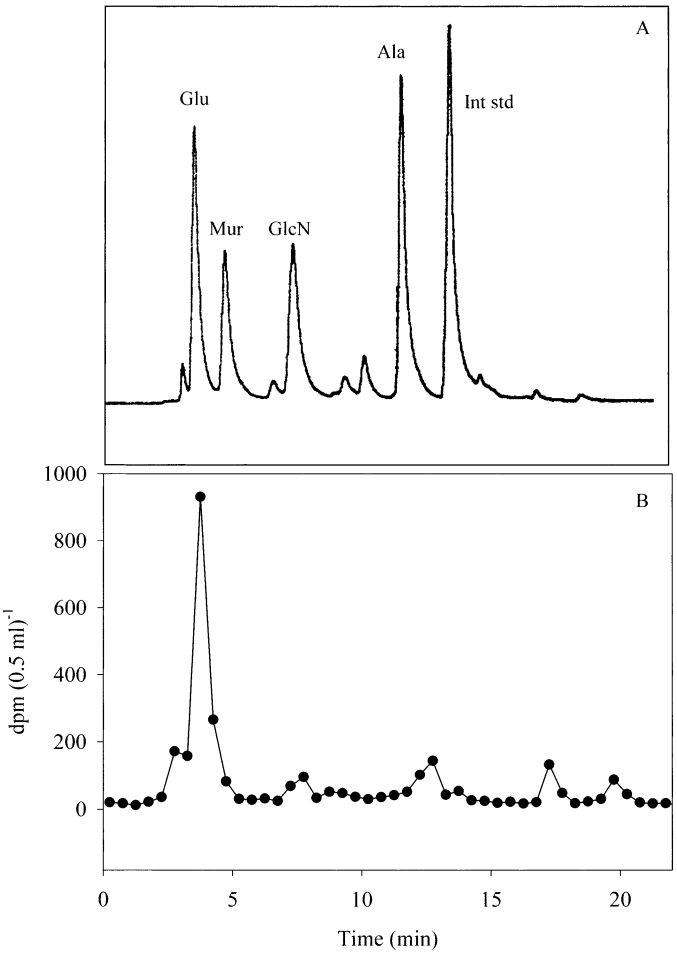

Fig. 3. HPLC analysis of the hydrolyzate of peptidoglycan radiolabeled with ^{3}H-L-glutamate. (A) A reversed-phase HPLC chromatogram and (B) the corresponding radiochromatogram are presented. See the legend of Fig. 1 for abbreviations.

analyzed by reversed-phase HPLC; 18% of total added radioactivity was recovered at a short retention time (2.5 min).

A small fraction (5–8%) of radioactivity eluted at a retention time of 9 min (peak 4, Fig. 1B, Table 3), which did not correspond to any primary amines detected by the OPA fluorescence. The identity of peak 4 was not clear. The overall recovery of radioactivity in HPLC elutions was 86–93% (Table 3). Specific activities were calculated to be 1.1×10^6 Bq mg^{-1} and 1.3×10^6 to 1.1×10^7 Bq mg^{-1} for ^{14}C- and ^{3}H-labeled preparations, respectively. With the anion-exchange HPLC, the radioactivity recovered in HPLC effluents was associated only with glucosamine (Fig. 2B), a further indication that the glycan strand of peptidoglycan was specifically radiolabeled.

The radiolabeled peptidoglycan prepared with ^{3}H-L-glutamic acid was acid hydrolyzed and analyzed by reversed-phase HPLC. A major fraction of radioactivity (67%) coeluted with glutamic acid (Fig. 3), suggesting that the added precursor, ^{3}H-L-glutamic acid, was substantially racemized to ^{3}H-D-glutamic acid and incorporated into peptidoglycan. The other fraction (33%) of ^{3}H was recovered either as part of the baseline or as three minor peaks (each peak accounts for <8% of total ^{3}H recovered) that did not correspond to any primary amines detected by the OPA fluorescence (Fig. 3B). The specific activity was 3.3×10^5 Bq mg^{-1}. The overall results of the HPLC characterization indicate that the purity and specificity of the radiolabeled peptidoglycan prepared with two precursors, ^{3}H- (or ^{14}C)-*N*-acetyl-glucosamine and ^{3}H-L-glutamic acid, were sufficient for this study. These peptidoglycan preparations were called [^{3}H- (^{14}C)-polysaccharide]-peptidoglycan and [^{3}H-D-glutamate]-peptidoglycan, respectively.

Degradation of radiolabeled substrates: release of LMW material and remineralization—Degradation of radiolabeled substrates in seawater was determined as the accumulation of ^{3}H- (^{14}C)-LMW material and radiolabeled end products of remineralization (^3H_2O or $^{14}CO_2$); Fig. 4 presents a typical time course. During degradation of [^{3}H-polysaccharide]-peptidoglycan (Fig. 4A), large amounts of ^{3}H-LMW material accumulated along with the production of ^3H_2O. After prolonged incubation, ^{3}H-LMW material tended to decrease, but a significant amount of ^{3}H-LMW material (18% of total addition) was recovered even at the end of incubation (200 h). With [^{3}H-D-glutamate]-peptidoglycan (Fig. 4B), LMW material initially increased slightly but then decreased, leaving only a small fraction (8%) of ^{3}H to be recovered as LMW material at the end of incubation. Degradation of ^{3}H-protein was characterized by no accumulation of LMW material, even though a substantial amount of ^3H_2O was produced during the same incubation period (Fig. 4C).

The difference in the release of LMW material and remineralization among different substrates was consistently observed in several experiments using seawater samples collected from diverse environments and with variable concentrations of added substrates (Table 2). To emphasize this, Table 4 compares the rates of the release of ^3H_2O (or $^{14}CO_2$) and of ^{3}H- (^{14}C)-LMW material during the initial 47–51 h of incubation. With [^{3}H- (^{14}C)-polysaccharide]-peptidoglycan, the rate of the release of ^{3}H- (^{14}C)-LMW material (1.6–8.4% d^{-1}) was equivalent to or exceeded ^3H_2O (or $^{14}CO_2$) release (2–5% d^{-1}), resulting in high ratios of LMW material release to ^3H_2O ($^{14}CO_2$) release (0.8 to >3.7). In contrast, the degradation of [^{3}H-D-glutamate]-peptidoglycan and ^{3}H-protein was not associated with the accumulation of LMW material; the ratio of LMW material release to ^3H_2O release was very low (<0.04–0.3).

Characterization of ^{3}H-LMW material accumulated during [^{3}H-polysaccharide]-peptidoglycan degradation—In Experiment 3, the size distribution of ^{3}H-LMW material accumulated during the degradation of [^{3}H-polysaccharide]-peptidoglycan was determined by ultrafiltration. During degradation, ^{3}H fragments of various sizes were produced; 7.0, 1.7, and 2.3% of ^{3}H were recovered as 10K–100K, 3K–10K, and <3K MWCO fractions, respectively, at 160 h (Fig. 5). At the end of incubation, 82 and 16% of ^{3}H were recovered as ^3H_2O and ^{3}H-LMW material (TCA-soluble, nonvolatile fraction) (data not shown). The recovery of ^{3}H in the TCA-soluble, nonvolatile fraction (16%) was close to that in the <100K MWCO fraction (11%), suggesting that peptidogly-

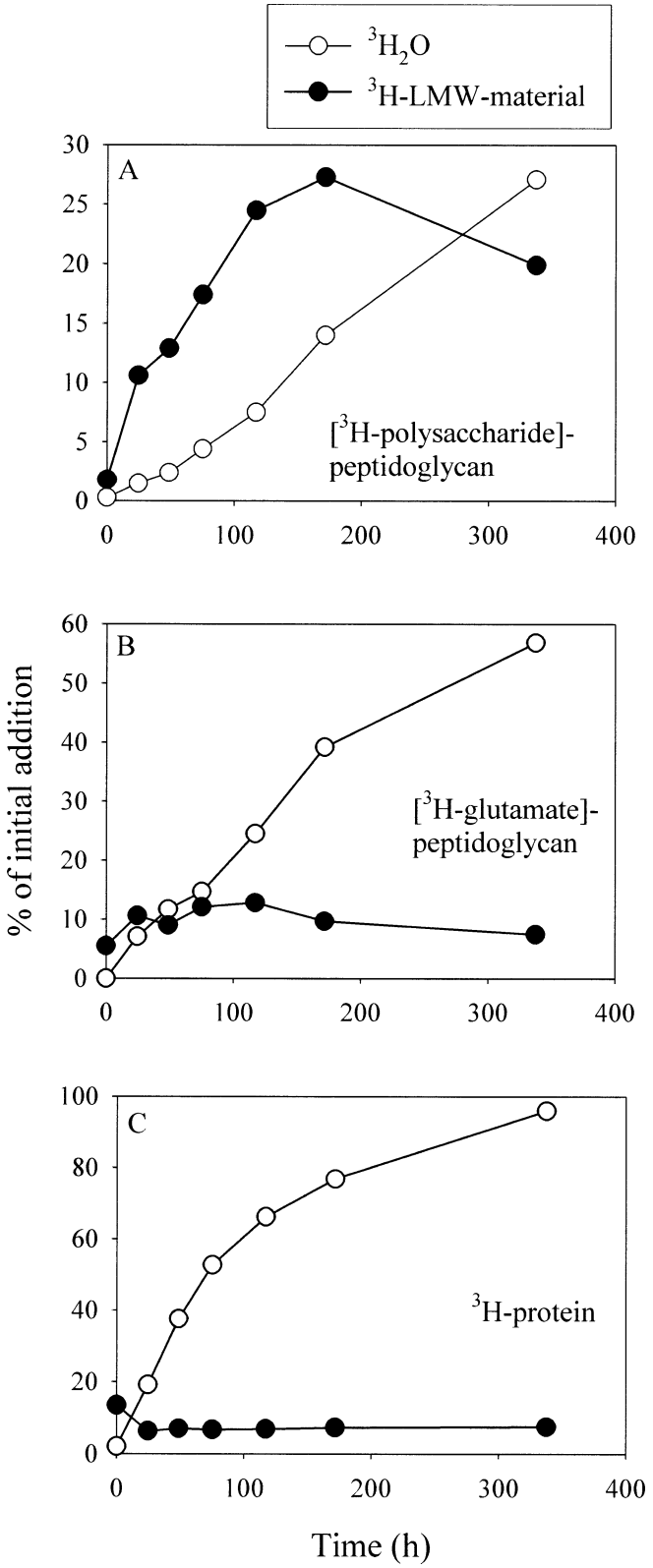

Fig. 4. Time-course of the increase in ^3H_2O and ^{3}H-LMW material during the degradation of (A) [^{3}H-polysaccharide]-peptidoglycan, (B) [^{3}H-D-glutamate]-peptidoglycan, and (C) ^{3}H-protein. Data are from Experiment 6 (*see Table 2*).

can fragments greater than 100K MWCO were precipitated with TCA.

Reversed-phase HPLC was used to analyze the <3K MWCO material accumulated during degradation. A major fraction of ^{3}H was associated with material that did not react with OPA; 80% of ^{3}H was collected at a retention time shorter than that of amino acids (data not shown). There was no peak of ^{3}H at retention times corresponding to glucosamine and muramic acids; these peaks should have been detected if the molecular structures of the amino sugars were preserved while peptidoglycan was reduced in size during the degradation process. These results suggest that sugar strands of peptidoglycan were not simply fragmented during degradation; rather, *N*-acetyl-amino sugars were largely modified to unidentified compounds.

First-order rate constants of remineralization—The remineralization rate of radiolabeled substrates was calculated from the release of ^3H_2O (or $^{14}CO_2$) by assuming a first-order reaction. The data generally fit well to the first-order model (Fig. 6), with $r^2 = 0.7$–0.9 (Table 5). An exception was Experiment 6, in which the degradation of ^{3}H-peptidoglycan was substantially enhanced during the incubation. In this experiment, remineralization rate constants of ^{3}H-peptidoglycan were calculated for the initial (0–96 h) and later (>96 h) incubation periods.

Remineralization rate constants of ^{3}H-protein varied in the range of 0.088–0.944 d^{-1}, with the maximum in the coastal Atlantic sample (Experiment 3) and the lowest in waters off California (Experiment 4) (Table 5). The rate constant of [^{3}H- (^{14}C)-polysaccharide]-peptidoglycan remineralization, which was 0.006–0.090 d^{-1}, was significantly (ANCOVA, $P < 0.05$) lower than that of ^{3}H-protein in six out of seven experiments (Table 5). In the California experiment (Experiment 4), the rate constant of [^{3}H-polysaccharide]-peptidoglycan mineralization did not differ significantly ($P > 0.05$) from that for ^{3}H-protein. The rate constant of ^{3}H-protein mineralization was not correlated significantly ($P > 0.2$) with that of [^{3}H- (^{14}C)-polysaccharide]-peptidoglycan. We also determined remineralization rates of [^{3}H-polysaccharide]-peptidoglycan and [^{3}H-D-glutamate]-peptidoglycan in Experiments 6 and 7. The remineralization rate constant of [^{3}H-D-glutamate]-peptidoglycan was significantly ($P < 0.01$) greater than that of [^{3}H-polysaccharide]-peptidoglycan in both Experiments 6 and 7, suggesting that the peptide component of peptidoglycan was more easily mineralized than the carbohydrate portion.

Discussion

Our results indicate that marine microbial assemblages readily degrade both polysaccharide and peptide components of peptidoglycan; degradation of both [^{3}H- (^{14}C)-polysaccharide]-peptidoglycan (Experiments 1–7) and [^{3}H-D-glutamate]-peptidoglycan generally started immediately after the addition of the substrate. This suggests that bacteria with effective lysozymes and peptidases, which cleave the β-1,4 glucosydic linkage between *N*-acetyl-glucosamine and *N*-acetyl muramic acid and peptide bonds of oligopeptides containing D-amino acids, are distributed widely in surface

Table 4. Accumulation rates (% d^{-1}) of respiration products (CO_2 or H_2O) and LMW material during the initial period (0 to 47–51 h) of incubation. If the amounts of CO_2 (or H_2O) and LMW material accumulated during this period are less than 3% of added substrate, the rates were below the detection limit (ND). The amounts of added substrates are given in Table 2.

Experiment No.	Substrate	Incubation period (h)	CO_2 or H_2O (% d^{-1} ± SE*)	LMW material (% d^{-1} ± SE*)	LMW : Resp
1	[^{14}C-polysaccharide]-peptidoglycan	47.5	ND	1.9±0.7	>1.2
2	[^{14}C-polysaccharide]-peptidoglycan	48.0	5.0±1.2	8.4†	1.7
3	[^{3}H-polysaccharide]-peptidoglycan	48.0	2.0±0.1	1.6±0.3	0.8
4	[^{3}H-polysaccharide]-peptidoglycan	47.5	ND	5.0±0.8	>3.3
5	[^{3}H-polysaccharide]-peptidoglycan	50.5	ND	ND	
6	[^{3}H-polysaccharide]-peptidoglycan	47.3	ND	2.0±0.0	>1.3
7	[^{3}H-polysaccharide]-peptidoglycan	48.7	ND	5.5±1.8	>3.7
6	[^{3}H-D-glutamate]-peptidoglycan	47.3	ND	ND	
7	[^{3}H-D-glutamate]-peptidoglycan	48.7	5.7±0.7	ND	<0.3
1	^{3}H-protein	47.5	14.5±0.7	ND	<0.1
2	^{3}H-protein	48.0	21.0†	1.6†	<0.1
3	^{3}H-protein	48.0	38.8±6.9	ND	<0.04
4	^{3}H-protein	47.5	5.2±2.8	ND	<0.3
5	^{3}H-protein	50.5	8.0±7.0	ND	<0.2
6	^{3}H-protein	47.3	7.8±1.4	ND	<0.2
7	^{3}H-protein	48.7	17.5±0.4	ND	<0.1

* SE, standard error of the linear regression slope, $n = 3$.
† $n = 2$.

waters. Also, the mineralization of [^{3}H- (^{14}C)-polysaccharide]-peptidoglycan and [^{3}H-D-glutamate]-peptidoglycan to $^{3}H_2O$ (or $^{14}CO_2$) suggests that sugar components (*N*-acetylglucosamine and muramic acid) and the D-isomer of glutamate were transported into the cell and catabolized by bacteria. First-order kinetic constants of peptidoglycan mineralization (0.006–0.097 d^{-1}) indicate that the turnover time of *V. proteolyticus* peptidoglycan in seawater is on the order of 10–167 d. These results suggest that peptidoglycan, in its isolated form, is a semilabile component of DOM that cycles on a timescale of weeks to months, rather than a refractory DOM component that persists over years to millennia (Carlson and Ducklow 1995).

We found that the remineralization rates of peptidoglycan were generally slower than those for proteins; first-order kinetic remineralization constants for peptidoglycan were 2–21 times smaller than those for proteins. These data support the hypothesis that peptidoglycan is less degradable than proteins in seawater. This difference in mineralization could explain previous observations that the D/L ratio of amino acids in dissolved combined amino acids increased during the degradation of DOM over 7–10 d (Jørgensen et al. 1999; Amon et al. 2001). In Experiment 7, remineralization rate constants for ^{3}H-D-glutamate-peptidoglycan and ^{3}H-protein were 0.061 and 0.223 d^{-1}, respectively. If we assume that the remineralization rate of ^{3}H-protein is equal to that of L-glutamate in proteins, the first-order kinetic model indicates

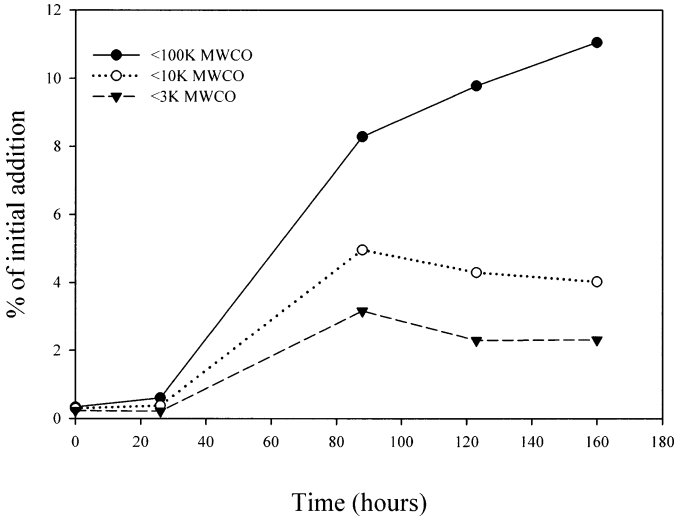

Fig. 5. Size distribution of the ^{3}H-LMW material released during the degradation of [^{3}H-polysaccharide]-peptidoglycan.

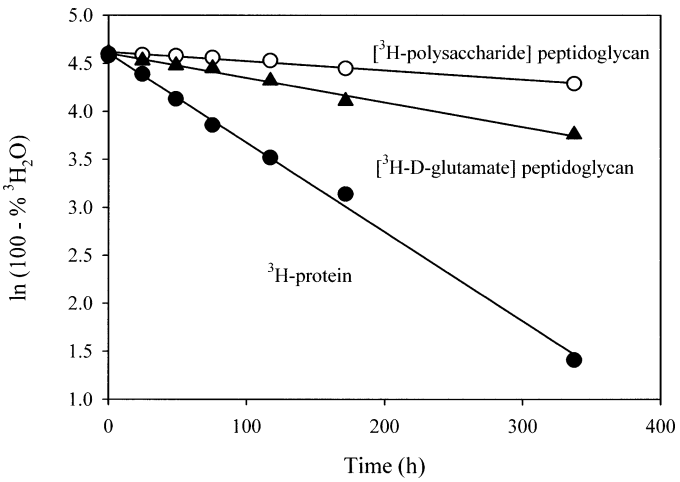

Fig. 6. First-order kinetic plots of the remineralization of [^{3}H-polysaccharide]-peptidoglycan, [^{3}H-D-glutamate]-peptidoglycan, and ^{3}H-protein. Data are from Experiment 7 (*see Table 2*).

Table 5. First-order kinetic rate constant of the remineralization of peptidoglycan and protein. The amounts of added substrates are given in Table 2.

Experiment No.	Substrate	Incubation period (h)†	*n*	r^2	Rate constant ± SE (d^{-1})	Protein : GlcN-PG‡	Protein : Glu-PG§	GlcN-PG : Glu-PG‖
1	[^{14}C-polysaccharide]-peptidoglycan	67	4	0.74	0.016±0.007	14.6*		
	^{3}H-protein	67	4	0.94	0.229±0.038			
2	[^{14}C-polysaccharide]-peptidoglycan	151	7	0.98	0.090±0.006	3.4**		
	^{3}H-protein	151	6	0.96	0.301±0.028			
3	[^{3}H-polysaccharide]-peptidoglycan	102	6	0.89	0.046±0.008	20.5**		
	^{3}H-protein	68	5	0.94	0.944±0.140			
4	[^{3}H-polysaccharide]-peptidoglycan	97	5	0.77	0.051±0.016	1.7$^{n.s.}$		
	^{3}H-protein	97	5	0.92	0.088±0.015			
5	[^{3}H-polysaccharide]-peptidoglycan	97	5		ND	—		
	^{3}H-protein	97	5	0.69	0.097±0.038			
6	[^{3}H-polysaccharide]-peptidoglycan	96	5	0.86	0.006±0.002			
		(96−510)	(6)		(0.046±0.003)			
	[^{3}H-D-glutamate]-peptidoglycan	96	5	0.93	0.021±0.003	20.5**	6.2**	0.30*
		(96−249)	(5)		(0.267±0.015)	(2.9)**	(0.5)**	(0.17)**
	^{3}H-protein	510	10	1.00	0.132±0.002			
7	[^{3}H-polysaccharide]-peptidoglycan	338	7	0.99	0.023±0.001			
	[^{3}H-D-glutamate]-peptidoglycan	338	7	0.99	0.061±0.003	9.7**	3.7**	0.38**
	^{3}H-protein	338	7	1.00	0.223±0.006			

Significant at * $P<0.01$, ** $P<0.001$. n.s., not significant at $P>0.05$.

† Rate constants were calculated from the start of the incubation to the indicated incubation time. In Experiment 6, rate constants were calculated for the initial (0–96 h) and the later period (>96 h, values in parentheses) because of the enhancement of remineralization.

‡ Ratio of the rate constant for ^{3}H-protein remineralization relative to that for [^{3}H-(^{14}C)-polysaccharide]-peptidoglycan. Asterisks indicate the level of the significance of the difference between the two rate constants (ANCOVA). In Experiment 5, remineralization of [^{3}H-polysaccharide]-peptidoglycan was not significant ($P>0.05$).

§ Ratio of the rate constant for ^{3}H-protein remineralization relative to that for [^{3}H-D-glutamate]-peptidoglycan. Asterisks indicate the level of the significance of the difference between the two rate constants (ANCOVA).

‖ Ratio of the rate constant of [^{3}H-(^{14}C)-polysaccharide]-peptidoglycan remineralization relative to that of [^{3}H-D-glutamate]-peptidoglycan. Asterisks indicate the level of the significance of the difference between the two rate constants (ANCOVA).

that 55% of D-glutamate (from peptidoglycan) and 11% of L-glutamate (from proteins) remain of the original addition after 10 d. Consequently, the D/L ratio of glutamate would increase by fivefold during this incubation period. However, our data also show that mineralization of [^{3}H-D-glutamate]-peptidoglycan was substantially enhanced and exceeded the mineralization rate of ^{3}H-protein during the prolonged incubation (>96 h, Experiment 6, Table 5), presumably because of the response of bacterial communities to the substrate addition. Thus, bacterial community composition and biochemical diversity could be important factors controlling differential degradation of peptidoglycan and proteins in seawater.

Production of LMW materials during the degradation of peptidoglycan—Our results indicate that large amounts of LMW material (TCA-soluble organic matter), relative to original additions, accumulated during the degradation of [^{3}H- (^{14}C)-polysaccharide]-peptidoglycan, whereas little increase in LMW materials was detected during the degradation of [^{3}H-D-glutamate]-peptidoglycan and ^{3}H-protein (Fig. 4, Table 4). Previous studies observed significant accumulation of LMW materials during the degradation of algal proteins (RuBPcase) and glucosidated-RuBPcase (Keil and Kirchman 1993), indicating an uncoupling between hydrolysis and uptake. The degree of uncoupling appears to vary among different substrates, even among proteins. In contrast with the results of Keil and Kirchman (1993), who examined algal protein degradations, the present study and Nagata et al. (1998) found little accumulation of LMW material during the degradation of bacterial proteins.

More importantly, we found that radiolabeled LMW compounds released during the degradation of [^{3}H- (^{14}C)-polysaccharide]-peptidoglycan were not recovered by HPLC at retention times of amino sugars, but they were recovered at the beginning of the chromatograph, where hydrophilic materials that do not react with OPA elute. This result suggests that LMW compounds were chemically modified (e.g., elimination of acetylated amino group) and released into the surrounding seawater. Although the mechanism of this alteration is not clear, our results indicate that not only size reductions but also diagenetic processes are involved in the production of LMW materials during the degradation of peptidoglycan. Rapid alteration of the polysaccharide component of peptidoglycan can explain recent results showing low or negligible concentrations of muramic acids in oceanic DOM (Kaiser and Benner 2000; Benner and Kaiser 2003) and in refractory DOM recovered from seawater culture after aging for more than 1 yr (Ogawa et al. 2001).

Preservation of peptidoglycan in marine environments—McCarthy et al. (1998) estimated, based on measurements of the D/L ratio of amino acids in oceanic DOM, that peptidoglycan might be as abundant as protein amino acids in

both surface and deep waters and could constitute a large pool of organic N in the oceans. However, production rates of protein amino acids by phytoplankton and bacteria should far exceed those of D-amino acids by bacteria in the oceans because peptidoglycan constitutes only 2.5% of bacterial biomass (Neidhardt et al. 1990), although cyanobacterial production can be as much as 90% of primary production in the open ocean (e.g., Li et al. 1983). These percentages indicate that peptidoglycan production accounts for <10% of primary production—much less than protein production, which should be at least 30% of primary production based on the amount of protein in phytoplankton (Loureno et al. 1998). The high D/L ratio but low input of peptidoglycan indicates that peptidoglycan fragments are strongly selectively preserved in seawater.

The accumulation and preservation of peptidoglycan or its remnants have been related to intrinsic biochemical properties of complex heteropolymeric structure and the presence of unusual peptide components (i.e., D-isomers), which might impede enzymatic hydrolysis (McCarthy et al. 1998). This model is supported by our data showing 2–21-fold lower remineralization rate constants for peptidoglycan compared to those for proteins (Table 3), but our estimate of peptidoglycan turnover (10–167 d) indicates that peptidoglycan is not as refractory as bulk DOM, which has a turnover time exceeding 1,000 yr (Williams and Druffel 1987). Instead, peptidoglycan could be classified as ''semi-labile'' DOM (Carlson and Ducklow 1995). If the above argument is correct, the peptidoglycan detected by McCarthy et al. (1998) in high-molecular-weight DOM in deep oceans (400–4,000 m) would not be as old as bulk refractory DOM transported by ocean circulation, but it is relatively fresh DOM presumably transported by sinking particles. In support of this notion, the data of McCarthy et al. show that D/L ratios of amino acids vary little between surface and deep waters, suggesting minimal diagenesis of amino acids over depth.

The peptidoglycan used here, which was purified and isolated from a Gram-negative marine bacterium, cannot model completely the complexity and diversity of peptidoglycan in the oceans. The peptidoglycan from different bacteria can vary, the most extreme example being the peptidoglycan from Gram-positive bacteria. Because of its thickness and high amounts of accessory compounds (Shleifer and Kandler 1972), peptidoglycan from Gram-positive bacteria might be less degradable than Gram-negative peptidoglycan, although Gram-negative bacteria appear to dominate marine bacterial communities (Giovannoni and Rappé 2000). The matrix surrounding peptidoglycan naturally released into seawater might also affect degradation. Peptidoglycan can be released to seawater during viral lysis and grazing as a part of high–organic matter complexes consisting of membrane and other cellular components; these complexes could substantially impede degradation of peptidoglycan and other material inside the complex (Nagata and Kirchman 1992, 1997, 1999). Also, other studies demonstrated that labile proteins associated with membrane and phospholipid vesicles (liposomes) are less degradable than the same protein freely dissolved (Nagata et al. 1998; Borch and Kirchman 1999). A similar mechanism could explain the preservation of peptidoglycan fragments in the oceans.

Although not all aspects of peptidoglycan degradation were probably captured by our experiments, they still appear to offer support for the size-reactivity continuum hypothesis (Amon and Benner 1996) and could provide insights into the refractory DOM pool. Peptidoglycan was degraded more slowly than protein in these experiments, but it was still mineralized much more rapidly than bulk DOM. Also, consistent with the size-reactivity continuum hypothesis, chemically modified LMW material was produced during hydrolysis of the high-molecular-weight peptidoglycan. This LMW material survived at least as long as the incubation period, unlike any LMW material that might have been produced during degradation of protein in these experiments. It remains to be seen whether or not the LMW fragments resulting from peptidoglycan degradation can be preserved for longer periods of time and actually contribute to the refractory DOM pool of seawater.

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