Production of Refractory Dissolved Organic Matter by Bacteria

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Most of the oceanic reservoir of dissolved organic matter (DOM) is of marine origin and is resistant to microbial oxidation, but little is known about the mechanisms of its formation. In a laboratory study, natural assemblages of marine bacteria rapidly (in <48 hours) utilized labile compounds (glucose, glutamate) and produced refractory DOM that persisted for more than a year. Only 10 to 15% of the bacterially derived DOM was identified as hydrolyzable amino acids and sugars, a feature consistent with marine DOM. These results suggest that microbial processes alter the molecular structure of DOM, making it resistant to further degradation and thereby preserving fixed carbon in the ocean.

DOM is the largest reservoir of fixed carbon in the ocean and is approximately equivalent to the reservoir of atmospheric CO_2. The major bioelements (C, N, and P) in DOM occur in functional groups common to biopolymers found in marine organisms (*1–3*). Specific cellular components of bacteria have been identified in marine DOM (*4, 5*), indicating that bacteria are an important source of this material. These observations indicate the predominance of biomolecules in DOM, but <25% of marine DOM has been identified as specific biochemicals (*6, 7*), suggesting that its molecular structure has been modified. It appears that these molecular modifications also reduce the bioavailability of the DOM. Physicochemical reactions were proposed as the dominant mechanism for the formation of molecularly uncharacterized and refractory DOM (*8*), but a few studies reported that microorganisms also produce DOM that is resistant to decomposition (*9–11*). In a series of experiments, we examined the bacterial utilization of simple biochemicals and traced the production of fresh DOM by the bacterial community.

We used seawater cultures with natural bacterial assemblages to examine DOM production by marine bacteria (*12*). Culture media were prepared with organic-free artificial seawater and either glucose or glutamate as the sole C source. Incubations with glucose received ammonium and phosphate as N and P sources and an inoculum from Gulf of Mexico surface water. Incubations with glutamate received phosphate as a P source and an inoculum from Sagami Bay, Japan. Concentrations of free glucose and glutamate were measured using high-performance liquid chromatography (HPLC) (*13, 14*). Measurements of total organic carbon (TOC), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON), were made using high-temperature combustion (*15, 16*). Concentrations of total hydrolyzable neutral sugars (THNS), total hydrolyzable amino sugars (THAS), and total hydrolyzable amino acids (THAA) were measured by HPLC after acid hydrolysis (*17*). Both experiments were conducted at room temperature (22° to 28°C) with duplicate bottles under dark conditions.

Glucose (208 μM C) was rapidly consumed and undetectable after 2 days (Fig. 1). Within 2 days, TOC concentrations decreased by 78% due to respiration, and 7% of the initial glucose C was converted to particulate organic carbon (POC, i.e., bacterial biomass), whereas 15% was converted to DOC (Table 1). Similarly, glutamate was completely consumed within 2 days, and 66% of the added C was respired. Compared to the glucose incubations, glutamate incubations had a greater yield of bacterial biomass (22% of initial glutamate C), but a similar yield of DOC (13%). After 2 days, bacterial growth yields were 8% in the N-limited glucose incubations and 25% in the glutamate incubations, if bacterially derived DOC was excluded from yield calculations. Inclusion of bacterially derived DOC results in growth yield estimates of 22 and 35%, respectively, in the glucose and glutamate incubations.

Concentrations of bacterially derived DOC decreased gradually during the next week of decomposition (Stage II in Table 1). Assuming first-order kinetics, the average decay constant during Stage II was reduced by a factor of 8 compared with the utilization of glucose and glutamate (Stage I). It is generally recognized that bacteria in natural waters rapidly utilize labile compounds, such as free amino acids and monosaccharides, even at low (nM) concentrations (*18, 19*). Therefore, it is unlikely that the concentrations of remaining bacterially derived DOC (20 to 30 μM C) would limit bacterial utilization. Neither ammonium nor phosphate was depleted after 1 week of incubation, suggesting nutrients were not limiting for decomposition. These results demonstrate that marine bacteria rapidly consumed labile DOM and produced DOM that was relatively resistant to decomposition.

The experiments were continued to examine the long-term persistence of bacterially derived DOM. Consequently, 10.5 and 9.0 μM DOC were measured after 1 and 1.5 years, respectively, of incubation in the glucose and glutamate experiments, corresponding to 5 and 7% of the initial DOC added in each experiment and to 37 and 50% of the bacterially derived DOC concentrations at the end of 2 days. Although the degradation of DOC continued throughout the incubation (Stage III in Table 1), the degradation rate was remarkably low, and the decay constants were up to 100 times lower than those in Stage II. These decay constants were similar to previous estimates from long-term degradation experiments with marine DOC from oligotrophic waters (*20*) and corresponded to residence times of 1.2 to 2.3 years. Thus, bacteria rapidly produced a major component of refractory DOM from labile substrates.

Chromatographic characterizations of major biogenic components of bacterially derived DOM indicated that THAA, THNS, and THAS accounted for 2.5 to 4.3%, 2.5 to 5.9%, and 0.7 to 1.5% of the DOC, respectively, during the first week in the glucose experiment (Table 2). Only 6 to 11% of the DOC was identified at the molecular level. Amino acids and amino sugars are also important nitrogenous components of DON. No DON was initially present in the glucose experiment, but concentrations of 0.6 to 1.0 μM DON were measured during the incubation. Marine bacteria produced DON from glucose and ammonium, and amino acids and amino sugars accounted for 22 to 29% and 3.4 to 6.6%, respectively, of the DON during the first week (Table 2). As with DOC, most (67 to 75%) of the DON was not characterized at the molecular level. The C:N ratio of the DOM was 28 to 32, indicating that bacterially derived DOM was C-rich relative to the initial substrates (glucose C:NH_4-N = 20:1). The C:N ratio for the molecularly characterized DOM (THAA+THNS+THAS) was 7.5 to 9.2. In contrast, the C:N ratio of the molecularly uncharacterized DOM was 37 to 41, indicating a N-poor composition.

In the glutamate experiment, only amino

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acids (THAA) were measured in bacterially derived DOM. The yields of THAA were 12 to 18% of the DOC and 15 to 33% of the DON during the first week of the incubation. The uncharacterized fraction composed most of the DOM (82 to 88% of C and 67 to 85% of N). The C:N ratio of the total DOM was 5.1 to 9.3, whereas the C:N ratios of the THAA and uncharacterized fractions were 3.1 to 3.5 and 5.4 to 12, respectively, suggesting that most bacterially derived DOM was depleted in N relative to the initial substrate (glutamate C:N = 5). The yields of amino acids in DOM were higher and the C:N ratios were lower in the glutamate experiment compared with the glucose experiment, reflecting the different N content of initial substrates. By 1.5 years, the yields of amino acids decreased (3.1% of the DOC and 11% of the DON) in the glutamate experiment, and the C:N ratio of the DOM increased to 13. In both experiments, bacterially derived DOM was largely uncharacterized at the molecular level and rich in C relative to N.

Most oceanic DOM is of marine origin (*21, 22*), refractory (*20, 21, 23*), molecularly uncharacterized (*6, 7*), rich in C relative to N (C:N = 10 to 25) (*16, 24*), and exists as relatively small molecules (*1, 15, 24*). The simple experiments described here demonstrate that the bulk properties of bacterially derived DOM are similar to those of marine DOM. The size distribution of DOM in the glutamate experiment was investigated using ultrafiltration (*15*). After 6 months, 84 ± 3% of the DOC was <10 kD in size, consistent with the size distribution of marine DOM (>90% of DOC is <10 kD) (*15*).

The amino acid composition of marine DOM is relatively rich in neutral amino acids (30 to 40 mole %) and depleted in basic amino acids (5 to 10 mole %) (*25, 26*) compared with marine organisms, including bacteria (neutral = 25 mole %; basic = 15 mole %) (*27*). During the incubations, the amino acid compositions of bacterially derived DOM rapidly shifted from a composition similar to that of microorganisms to a composition similar to that of marine DOM (Table 2). A similar transition was observed in amino sugar compositions of bacterially derived DOM in the glucose incubations. The initial amino sugar composition of DOM in the incubations was similar to that in bacterial

Table 1. Carbon balances and kinetic decay constants for DOM at different stages in seawater cultures with bacteria and glucose or glutamate as an initial substrate. The data are given as the mean ± (mean-replicate) of duplicate bottle incubations. Carbon balances were calculated at the end of each stage period, including remineralized % (i.e., the loss of TOC) and remaining % as DOC or POC relative to the initial TOC. The results of POC at Stage III were not available, because the difference between TOC and DOC were statistically insignificant or no measurement of TOC was made. The decay constants for DOC during each stage were calculated on the assumption of first-order decay kinetics.

Stage (days)	Remineralized %	Remaining %		Decay constant (day^{-1})
		DOC	POC	
	Glucose (208 ± 0 μM C)			
I (0–2)	78 ± 1	15 ± 0	7 ± 1	1.1 ± 0.0
II (2–7)	87 ± 1	8 ± 1	6 ± 1	0.13 ± 0.03
III (7–365)	95 ± 0	5 ± 0	–	0.0012 ± 0.0003
	Glutamate (132 ± 2 μM C)			
I (0–2)	66 ± 5	13 ± 7	22 ± 2	1.1 ± 0.1
II (2–9)	77 ± 2	10 ± 1	13 ± 1	0.14 ± 0.08
III (9–560)	93 ± 1	7 ± 1	–	0.0023 ± 0.0003

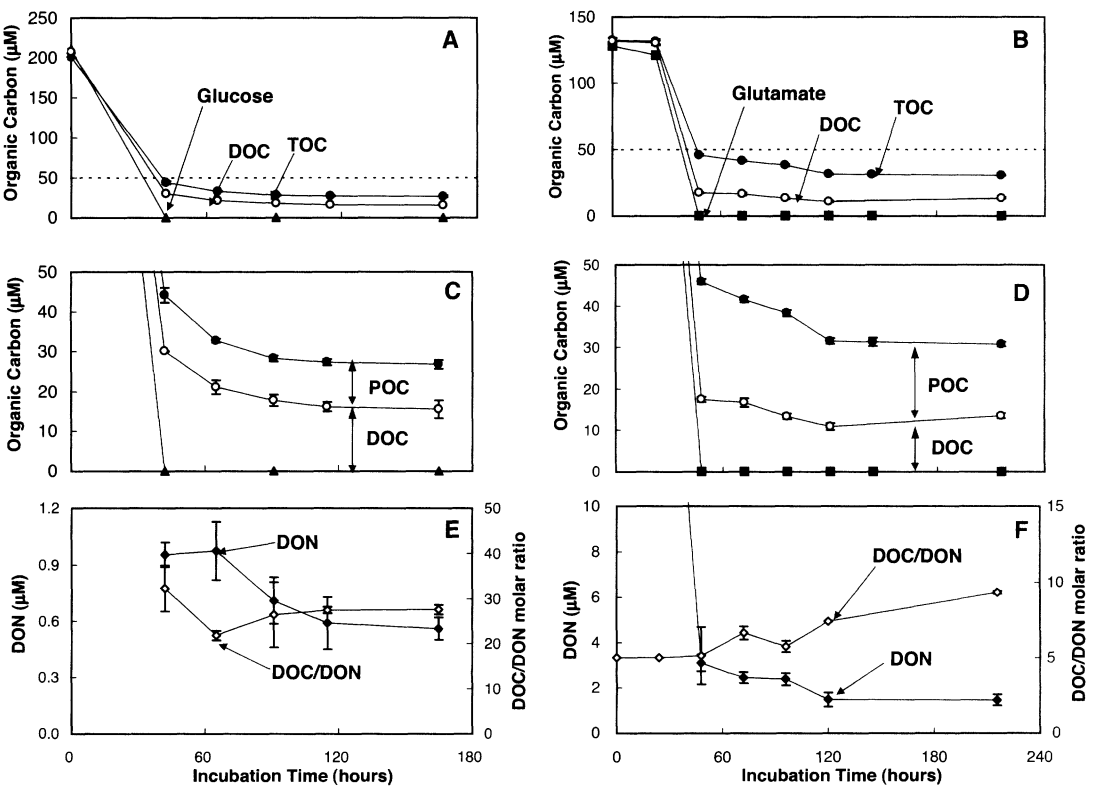

Fig. 1. Concentrations of free glucose (A), free glutamate (B), and TOC, DOC, POC, and DON in seawater cultures with natural bacterial assemblages and either glucose (**A**, **C**, and **E**) or glutamate (**B**, **D**, and **F**) as the sole carbon sources for bacterial growth. Panels (C) and (D) are reillustrations of panels (A) and (B) with magnified scales of the *y*-axis. Each point and error bar represents the average and range of duplicate-bottle experiments. Water samples were gravity-filtered through a glass fiber filter (GF/F, Whatman) in the glucose experiments for separation of the particulate and dissolved phases. In the glutamate experiments, water samples were passed through a 0.1-μm polytetrafluoroethylene membrane filter (Omnipore, Millipore) under reduced pressure. The DON concentration at the beginning of the glucose experiment was omitted because it was not significantly different from zero. Initial concentrations of DOC in glucose and glutamate experiments were accounted for in the measured concentrations of these compounds, indicating that no other sources of DOC were in the initial culture medium.

cells (*28*), whereas only glucosamine and galactosamine were detected at the end of the incubation (Table 2). The glucosamine:galactosamine ratio (2:1) in bacterially derived DOM at the end of the incubation was similar to the ratio of these amino sugars in seawater (*28*).

Recent studies have identified specific components of the cell envelopes of marine bacteria in DOM from the surface and deep ocean (*4, 5*). In addition to this evidence for a bacterial source of marine DOM, laboratory studies with radiotracers and marine bacteria noted the production of refractory DOM from simple compounds (*9–11*). Results from our study support these observations, and indicate that diagenetic processing by microorganisms is rapid and critical for shaping the composition and refractory nature of marine DOM.

The amino sugar muramic acid is uniquely found in the repeating disaccharide backbone of the bacterial cell wall polymer commonly known as peptidoglycan, and it is therefore an excellent biomarker for peptidoglycan in DOM (*28*). In the glucose experiment, muramic acid C concentrations of 35 to 89 nM were measured during the first week of incubation, and based on a typical muramic acid content in peptidoglycan (*29*), we estimate that peptidoglycan accounted for ~1% of the C and ~7.5% of the N in DOM. Muramic acid concentrations were below detection after 1 year of incubation (Table 2), indicating that peptidoglycan was absent in the refractory DOM derived from bacteria. This finding was unexpected given recent observations in marine DOM of high D/L enantiomer ratios in specific amino acids that are found in the peptide component of peptidoglycan (*5*). Overall, these results suggest that bacterial degradation sufficiently alters the structure of peptidoglycan so that its polysaccharide component is no longer recognizable at the molecular level.

Table 2. Chemical composition of bacterially derived DOM in the glucose and glutamate experiments. All are the mean of duplicate-bottle experiments, except for the data indicated by asterisks, which were obtained from single bottles. The mean deviation of replicates was 0.9 μM for DOC, 0.2 μM for DON, 2 for C/N ratio, and 1 to 2% of DOC, DON, THAS, and THAA compositions. The "uncharacterized" fraction in the glucose experiment is estimated as the difference between the total DOC and the DOC accounted for as THNS+THAS+THAA, and between the total DON and the DON accounted for as THAA+THAS, except for the data after 1 year, in which THNS was not included (data in parenthesis). The "uncharacterized" fraction in the glutamate experiment is based only on THAA, because THNS and THAS were not measured (indicated by "nm").

Chemical composition	Initial substrate							
	Glucose				Glutamate			
	Incubation time (days)				Incubation time (days)			
	2	4	7	365	2	4	9	560
	Concentration of the bulk DOM (μM)							
DOC	30	18	16	11	18	13	14	9.0
DON	1.0	0.7	0.6	1.2	3.1	2.4	1.5	0.7
	DOC composition (% as)							
THNS	2.5	4.9	5.9	nm	nm	nm	nm	nm
THAS	0.7	1.4	1.5	3.8	nm	nm	nm	nm
THAA	2.5	4.3	3.7	2.5	13	18*	12	3.1
Uncharacterized	94	89	89	(94)	87	82*	88	97
	DON composition (% as)							
THAS	3.4	5.5	6.6	5.6	nm	nm	nm	nm
THAA	22	29	27	6.4	15	31*	33	11
Uncharacterized	75	66	67	88	85	69*	67	89
	C:N molar ratio							
Bulk	32	26	28	8.9	5.1	5.7	9.3	13
Total characterized	7.5	7.9	9.2	(4.6)	3.5	3.1	3.3	3.6
Uncharacterized	41	37	37	(10)	5.4	6.3*	12	14
	THAS composition (mole %)							
GalN	20	26	31	33	nm	nm	nm	nm
GlcN	55	50	56	67	nm	nm	nm	nm
MA	26	24	12	0	nm	nm	nm	nm
	THAA composition (mole %)							
Acidic	26	27	21	30	26	12	17	28
Basic	12	10	11	11	8	6	7	8
Neutral	23	27	31	41	24	56	36	46
Hydrophobic	38	36	37	18	41	27	40	18

Our results are inconsistent with several mechanisms that have been proposed for the formation of refractory organic matter in the ocean. It is unlikely that abiotic condensation reactions or humification processes (*8*) were important for producing refractory DOM in our study, because the incubations were conducted in the dark at low temperature, concentrations of reactants were low, and the compositional changes were rapid (hours to days). Thus, it appears that biological processes were critical for the formation of refractory DOM. Specific biochemical components of cells can be selectively preserved during diagenesis (*30*), but these are relatively minor constituents of cells that should only slowly accumulate. Amino acids, neutral sugars, and amino sugars typically compose >70% of bacterial biomass (*29*), but these biochemicals were relatively minor components of bacterially derived DOM. It appears that the selective preservation of unusual biochemical components of cells could not, by itself, account for the rapid formation and accumulation of bacterially derived DOM.

Components of bacterial cells are released into the surrounding water as DOM through a variety of biological processes, including direct release (*11, 31*), viral lysis (*32*), and grazing (*33*). Exoenzyme activity is critical for the microbial utilization of this DOM, and it appears that enzymatic activity plays an important role in the formation of refractory DOM that is of small size (*34*). It is possible that nonspecific or promiscuous activities of enzymes, that occur with much lower efficiency than primary activities (*35*), occasionally produce fragments from macromolecules that escape recognition by bacterial enzymes and molecular-level chemical analyses. Given this scenario, the rate of formation of refractory DOM is dependent on the rate of microbial activity. This relatively simple mechanism could be responsible for much of the sequestration of fixed C in the ocean.

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12. An artificial seawater medium (0 ± 1 μM C) was prepared using precombusted salts (NaCl, KCl, $MgSO_4$, $CaCl_2$) with $NaHCO_3$ and organic-free, deionized water (Milli-RO and Milli-Q UV Plus). The filtrate (<0.7 μm pore size GF/F) from seawater samples was used as a natural inoculum (2% v/v) of marine bacteria. Based on the DOC content in the Gulf of Mexico seawater inoculum, the final concentration of DOC in the inoculated medium prior to the addition of glucose was 1 ± 1 μM. Based on the DOC content in the Sagami Bay seawater inoculum, the final concentration of DOC in the inoculated medium prior to the addition of glutamate was 2 ± 1 μM.
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17. THNS is the sum of seven neutral sugars (glucose, galactose, fucose, rhamnose, mannose, arabinose, and xylose), and THAS is the sum of three amino sugars [galactosamine (GalN), glucosamine (GlcN), and muramic acid (MA)], which were measured by HPLC after HCl hydrolysis (3 M, 5 hours, 100°C) (*13*, *28*). No samples had measurable mannosamine. MA was not detected after 1 year. THAA is the sum of 17 amino acids determined by HPLC (*14*) after vapor-phase hydrolysis (*36*). THAA composition is presented in Table 2 as four families of amino acids: acidic (aspartic acid, glutamic acid), basic (arginine, histidine, lysine), neutral (serine, glycine, threonine, tyrosine), and hydrophobic (β-alanine, alanine, γ-aminobutyric acid, methionine, phenylalanine, valine, isoleucine, leucine). The concentrations of individual amino acids are available as supplementary Web tables at www.sciencemag.org/cgi/content/full/292/5518/917/DC1
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REPORTS

Requirement of Phospholipase Cδ4 for the Zona Pellucida–Induced Acrosome Reaction

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Several phospholipase C (PLC) isoforms have been found in male and female mammalian gametes, and splicing isoforms of PLCδ4 are predominantly expressed in testis. Here we report that male mice in which the *PLCδ4* gene had been disrupted either produced few small litters or were sterile. In vitro fertilization studies showed that insemination with PLCδ4$^{-/-}$ sperm resulted in significantly fewer eggs becoming activated and that the calcium transients associated with fertilization were absent or delayed. PLCδ4$^{-/-}$ sperm were unable to initiate the acrosome reaction, an exocytotic event required for fertilization and induced by interaction with the egg coat, the zona pellucida. These data demonstrate that PLCδ4 functions in the acrosome reaction that is induced by the zona pellucida during mammalian fertilization.

Spatial and temporal activation of phosphoinositide turnover enables eukaryotic cells to induce various functions such as cell signaling, cytoskeletal remodeling, phagocytosis, membrane traffic, and ion channel activity (*1*, *2*). Phospholipase C (PLC) plays a crucial role in this turnover by hydrolyzing phosphatidylinositol-4,5-bisphosphate ($PtdInsP_2$) to generate two second messengers, inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$] and diacylglycerol. Eleven PLC cDNAs have been cloned from mammalian cells, and they can be divided into four types—β, γ, δ, and ε—on the basis of sequence homology and activation mechanisms (*3*). The delta type of PLC is thought to be evolutionarily the oldest form in the mammalian PLC family, and its pleckstrin homology (PH) domain is known to function in membrane association through $PtdInsP_2$. However, the physiological role and mechanism(s) of regulation of PLC delta types has remained unclear.

To understand the physiological importance of one of four isoforms of PLCδ, PLCδ4, we inactivated this gene by targeted disruption of exons 2 to 5, which encode the ATG translation start site (Fig. 1A) and the PH domain. Correct gene targeting of the mutant mice was verified by Southern blot analysis (Fig. 1B). Western-blot analysis of brain tissue revealed that neither 85-kD PLCδ4 nor fragments of the protein were expressed in homozygous mutant mice (Fig. 1C). Heterozygous PLCδ4$^{+/-}$ mice appeared phenotypically normal and fertile, and intercrosses between heterozygous mice produced pups with a Mendelian genetic distribution, indicating that disruption of this gene did not result in embryonic lethality. Homozygous adult PLCδ4$^{-/-}$ mice appeared healthy, and histological examination showed no obvious abnormalities in the brain, liver, kidney, or heart. However, fertility of PLCδ4 homozygous mutant males was severely impaired, whereas females seemed to be fertile. About 90.0% of intercrossed PLCδ4$^{+/-}$ females were fertile, and their average litter size was 7.6 pups. Similarly, 77.8% of PLCδ4$^{-/-}$ females became pregnant when mated with PLCδ4$^{+/-}$ males, and their average litter size was 5.8 pups. Conversely, when PLCδ4$^{-/-}$ females were mated with PLCδ4$^{-/-}$ males, only 35.2% of matings resulted in pregnancy, and the average litter size was 1.2 pups. Therefore, PLCδ4 affects primarily male fertility.

Histochemical analysis of testicular sections revealed that in PLCδ4$^{-/-}$ mice, spermatogenesis proceeded normally as indicated by the presence and production of mature sperm (Fig. 2, E and F). In wild-type testis, PLCδ4 was localized by using a specific antibody (*4*) to the outermost layer where the spermatogonia reside (Fig. 2, A and C), and it was also detected as a crescent-shaped domain in the developing

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