

Particulate Peptidoglycan in Seawater Determined by the Silkworm Larvae Plasma (SLP) Assay

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Peptidoglycan (PG) is a biopolymer found exclusively in the cell wall of bacteria. Recent chemical analysis of particulate organic matter suggests that a major amount of the muramic acid, an amino sugar present only in PG, could not be accounted for in terms of bacterial cells (Benner and Kaiser, 2003); however, data on particulate PG is quite sparse. In the present study, conducted in 1996, the PG was examined at 5 sampling sites in the northwestern Pacific Ocean, and in natural seawater cultures. Particulate PG, which was concentrated using a 96-well filtration plate equipped with Durapore filters (pore size, 0.22 μm), was measured by the silkworm larvae plasma (SLP) assay. The PG concentration generally decreased with depth and correlated significantly with bacterial abundance throughout the entire water column. However, the ratio of particulate PG to bacterial abundance varied with depth. The average ratio was 0.61 ± 0.53 (average \pm SD, $n = 40$) between 50 and 2000 m, which agreed with the bacterial cellular PG content from 0.63 to 1.1 fg cell^{-1} obtained in seawater cultures. On the other hand, the ratios of PG to bacteria from the surface to 50 m (3.7 ± 2.6 , $n = 29$) and below 2,000 m (2.1 ± 1.7 , $n = 7$) were significantly higher than that between 50 and 2,000 m. These results may suggest that, in the surface and deep layers, a significant fraction of particulate PG was present in bacterial detritus, whereas this fraction was reduced in the middle layer.

Keywords:

- Peptidoglycan,
- SLP assay,
- bacteria,
- detritus,
- particulate organic matter,
- cell envelope,
- cell wall,
- seawater.

1. Introduction

Peptidoglycan (PG) is a biomolecule found exclusively in bacterial cell walls. It consists of two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid, and a small group of amino acids, such as L-alanine, D-alanine, D-glutamic acid, and either lysine or diaminopimelic acid (Madigan *et al.*, 2003). Since muramic acid is an amino sugar present only in PG, this molecule has been used as a biomarker for quantifying bacterial biomass (Moriarty, 1983; Mimura and Romano, 1985), and for determining the source of amino sugars (Benner and Kaiser, 2003) in marine environments.

Several chemical characterizations of marine organic matter reported in this decade have revealed that specific biomolecules which are derived from the bacterial cell

envelope, including PG, are commonly present in a dissolved state in the sea (Tanoue *et al.*, 1995; Boon *et al.*, 1998; McCarthy *et al.*, 1998; Dittmar *et al.*, 2001; Wakeham *et al.*, 2003; Pérez *et al.*, 2003; Benner and Kaiser, 2003). On the other hand, recent chemical analysis of amino sugars indicated that a major amount of the muramic acid in a particulate state could not be accounted for in terms of bacterial cells (Benner and Kaiser, 2003); however, data on PG in a particulate state is very sparse (Mimura and Romano, 1985; Benner and Kaiser, 2003). Further examination of the particulate PG distribution would lead to an understanding of how this molecule exists in the oceans.

Although chemical analysis of the constituents of PG provides reliable data, it involves several time- and labor-consuming steps, including ultraconcentrating, hydrolyzing, and measuring samples. Tsuchiya *et al.* (1996) devised an enzymatic assay for determining PG using silkworm larvae plasma (SLP) derived from the

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Table 1. Location, date of the survey and position of sampling stations.

Location	Station	Sampling date	Position	Depth (m)	Cruise
Kuroshio Current Area	Sta. 5	2 August, 1996	33°34' N, 137°56' E	4,000	KT-96-14
Off Suruga Bay	Sta. 6	1 August, 1996	34°08' N, 138°32' E	3,900	KT-96-14
Suruga Bay	Sta. 7	1 August, 1996	34°32' N, 138°36' E	2,700	KT-96-14
Sagami Bay	Sta. 8	3 August, 1996	35°00' N, 138°19' E	1,500	KT-96-14
Off Bohso Peninsula	Sta. A	5 June, 1996	34°36' N, 140°27' E	3,100	KT-96-9

hemolymph of the silkworm, *Bombyx mori*, based on the pro-phenol oxidase cascade system that works as a self-defense mechanism in the insect (Ashida and Yamazaki, 1990; Ashida and Brey, 1995). In the assay, PG and (1 → 3)- β -D-glucan induce the production of melanin, which can be quantified spectrophotometrically. The technical procedure detected 62.5 pg of PG in one hour; thus one expects it to be a simple, and rapid technique for quantifying PG in the oceans.

In the present study, the concentration of PG in the particulate fraction of seawater (size, >0.22 μ m) was determined using the silkworm larvae plasma (SLP) assay. Seawater culture experiments were conducted to evaluate the bacterial cellular PG content. The assay was then applied to natural seawater samples from the northwestern Pacific Ocean, in order to determine the vertical distribution of the PG concentration, and its relationship with bacterial abundance.

2. Materials and Methods

Seawater samples were collected at 5 stations off of the east coast of Japan during the KT-96-9 (3–9 June 1996) and KT-96-14 (1–3 August 1996) cruises aboard the R/V *Tansei-Maru*, of the Ocean Research Institute, University of Tokyo (Table 1). Samples were collected using 12-liter Niskin bottles mounted on a CTD/rosette sampler. Surface seawater was also collected at Station 8 during the KT-96-14 cruise, and was used for a seawater culture experiment. Subsamples were transferred to 50 ml polypropylene tubes (Corning Inc., USA). A subsample for determining PG was fixed with 250 mM NaN₃, and another with 2% formalin for bacterial enumeration. Both were stored in the dark at 4°C until the analyses were done.

Seawater samples for the culture experiments were filtered through Nucleopore filters (pore size, 0.8 μ m) to remove bacterial grazers, and then glutamic acid was added producing final concentrations of 0, 5, or 20 μ g l⁻¹. The filtrates were incubated in the dark at room temperature (24 ± 2°C) in sterilized 1-liter glass bottles. Subsamples taken to determine the PG concentration and bacterial abundance were fixed as described above, and then stored at 4°C until the analyses were done.

The silkworm larvae plasma (SLP) assay (Tsuchiya *et al.*, 1996) was used to determine the PG concentration in the fixed samples, which was done within 2 weeks of sample collection. The seawater samples (0.25 to 2 ml) were filtered through Durapore filters (pore size, 0.22 μ m) which were sealed at the bottom of a 96-well plate (Millipore); the retained material, which was regarded as the particulate fraction, was analyzed. The filters were washed twice with 0.3 ml of distilled water (Ohtsuka Pharmaceutical Co., Ltd., Japan), 0.05 ml of distilled water and SLP reagent were placed in the well (Wako Pure Chemical Industries, Ltd., Japan), and the plates were incubated for 90 minutes at 30°C. During the incubation period their absorbance at 650 nm was continuously monitored by a THERMOMax microplate reader, (Molecular Devices, Co., USA), with the assistance of a commercially available software program (SOFTmax version 2.32; Molecular Devices, Co., USA). PG concentration was determined using a logarithmic plot calibration curve of standard PG (purified from *Micrococcus luteus*, Wako Pure Chemical Industries, Ltd., Japan) versus onset time, which was defined as the period of time the reaction continued until the absorbance reached 0.03. Each seawater sample was measured in six replicates.

Bacterial cells were stained with 4,6-diamino-2-phenylindole (DAPI) and counted under epifluorescence microscopy (BH-2, Olympus Co., Japan) (Porter and Feig, 1980). At least 400 cells in 20 randomly chosen fields were counted.

The average ratio of particulate PG to bacterial abundance data, as related to the three oceanic layers (see below), was analyzed by the Kuraskal-Wallis test, and the statistical significance ($p < 0.05$) was further examined by Dunn's multiple comparison test. Statistical analysis of the ratio of particulate PG to bacterial abundance from the seawater cultures was also performed using a repeated-measure analysis of variance (ANOVA). If the ANOVA was significant ($p < 0.05$), the data were further analyzed by Dunnett's multiple comparison test.

3. Results

Several preliminary experiments, which were conducted in order to optimize the protocol for applying the

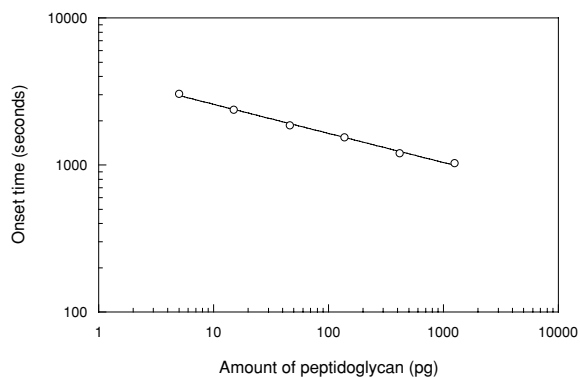


Fig. 1. Relationship between the amount of peptidoglycan and onset time. Onset time was defined as the period of time the reaction continued until the absorbance reached 0.03 (650 nm). Note that amount of peptidoglycan is shown as absolute value (pg).

SLP assay to seawater samples, revealed that fixation with NaN_3 (250 mM) gave consistent values if samples were washed twice with 0.3 ml of distilled water, and that the fixed samples could be stored at 4°C for up to 2 weeks without apparent loss of PG. A linear relationship between onset time and the amount of the standard PG ($r^2 = 0.98$, $n = 6$) was produced in the range of 5 to 1,250 pg of PG (Fig. 1). The minimum amount of PG, 5 pg, was detected within 60 minutes using our assay conditions. Abnormally high values in the natural seawater samples were omitted when the PG concentration was calculated.

Particulate PG measured at 5 sites in the northwestern Pacific Ocean generally decreased with depth, and was significantly correlated with bacterial abundance ($r^2 = 0.77$, $n = 76$, $p < 0.001$) (Fig. 2). Compared with bacterial abundance, PG concentration decreased sharply from the surface to 50 m (the surface layer), and increased slightly below 2,000 m (the deep layer). On the other hand, the PG concentration was generally low between 50 and 2,000 m (the middle layer), although high values were observed at 300 and 500 m at Station 8. Thus, these two values at Station 8 were omitted from the regression analy-

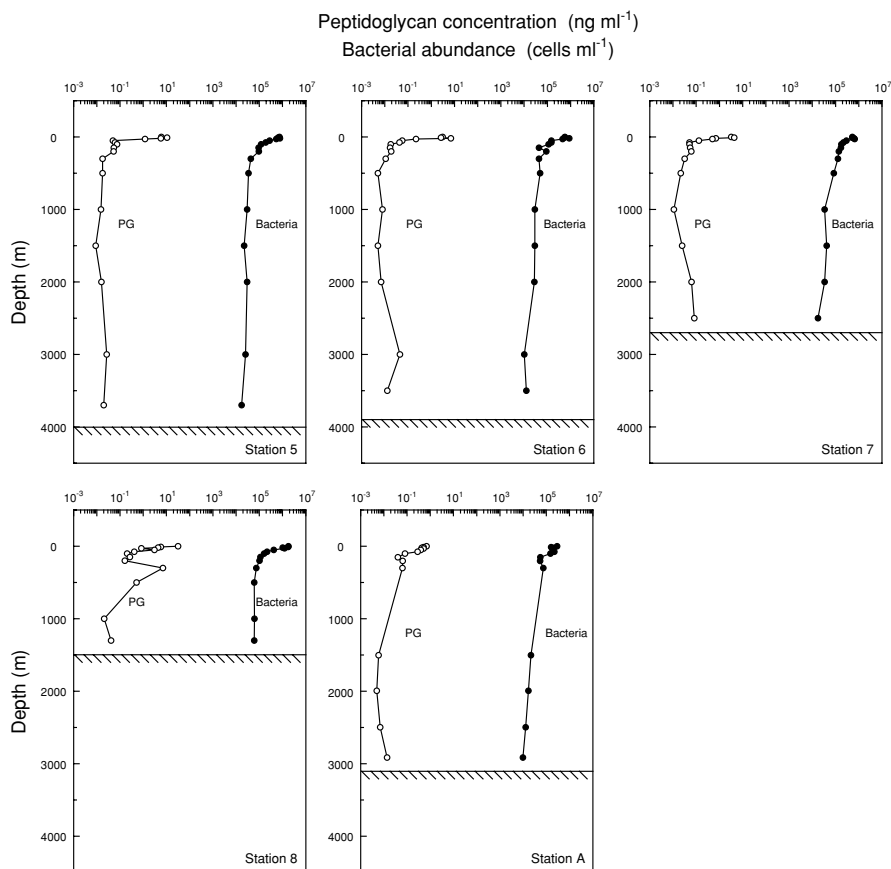


Fig. 2. Vertical profiles of particulate peptidoglycan concentration (○) and bacterial abundance (●) at Stations 5, 6, 7, 8, and A. Diagonal lines indicate the depth of the bottom.

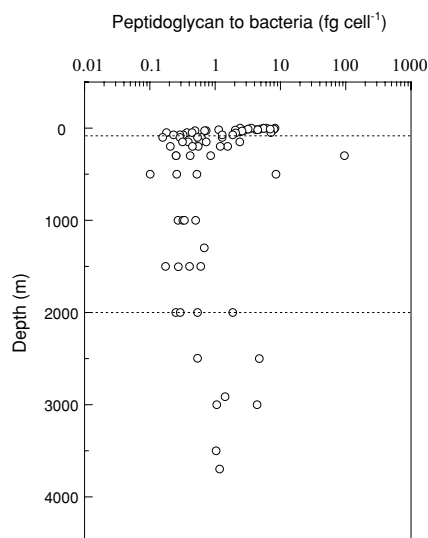


Fig. 3. Vertical profile of the ratios of particulate peptidoglycan to bacterial abundance obtained from all sampling stations. Dotted lines indicate depths of 50 and 2000 m. Note that ratio is expressed as fg cell^{-1} .

sis of the relationship between particulate PG and bacterial abundance, and from the calculation of the average ratio of particulate PG to bacterial abundance in the three layers (see below). These results indicated that the ratio of PG to bacterial abundance decreased sharply in the surface layer, and increased in the deep layer (Fig. 3). The average values were 3.7 ± 2.6 (average \pm SD, $n = 29$), 0.61 ± 0.53 ($n = 40$) and 2.1 ± 1.7 ($n = 7$) in the surface, middle, and deep layers, respectively. The ratios of PG to bacteria from the surface to 50 m and below 2,000 m were significantly higher than that between 50 and 2,000 m (Kruskal-Wallis analysis of variance, followed by Dunn's multiple comparison test; $p < 0.05$).

The cellular PG content in cultures with 0, 5, and 20 $\mu\text{g l}^{-1}$ glutamic acid was estimated from the apparent increase in particulate PG and bacterial abundance at each time point ($n = 9$) from time 0 throughout the 41.5 hour incubation; they had an average value of 0.63 ± 0.41 (average \pm SD), 0.83 ± 0.21 , and 1.1 ± 0.4 , respectively. The only significant difference between these averages was found in the values from the cultures augmented with 0 and 20 $\mu\text{g l}^{-1}$ glutamic acid throughout the incubation (repeated-measure analysis of variance, followed by Dunnett's multiple comparison test; $p < 0.05$).

4. Discussion

The SLP assay has never been applied to natural seawater samples; thus, we optimized the procedure including fixing, storing, concentrating, washing, and measuring the samples. Because this assay is based on an

enzymatic cascade system, salinity and fixatives added to the samples affect the reactions. However, washing the filters with distilled water immediately after sample filtration was effective in suppressing these problems. We recommend using NaN_3 (250 mM) for the fixation, and storing the samples at 4°C for a maximum of 2 weeks. Amongst all of the samples, the minimum concentration of particulate PG determined was 5 pg ml^{-1} (at 500 and 1500 m at Station 6 and at 2000 m at Station A), which required about 60 minutes for detection. Therefore the filtration protocol using 2 ml was sufficient for measuring PG in the samples collected from the middle and deep layers.

The cellular muramic acid content in pure cultures has been reported in a few previous studies; however, we do not know the cellular PG content of natural bacteria (Kirchman, 2000). Mimura and Romano (1985) determined the muramic acid content in a marine pseudomonad culture, from which we calculated the PG content of these cells to be between 1.7 and 1.9 fg cell^{-1} , which is about 2- to 3-fold higher than our values (Table 2). For growing cells of *Escherichia coli*, a cellular PG content of 7 fg cell^{-1} , which corresponds to c.a. 2.5% of their dry weight, is also high (Neidhardt *et al.*, 1990). Kirchman (2000) speculated that the PG content of natural bacteria made up about 4.1% of their total cellular dry weight. If we assume (1) that the carbon content of bacteria is 12 fg cell^{-1} (Fukuda *et al.*, 1998) and (2) that the carbon content represents half of the dry weight (Bratbak 1985), then the PG content is estimated to be 0.98 fg cell^{-1} in natural bacteria. The PG content obtained in our experiment (0.63 to 1.1 fg cell^{-1}) is in accord with the estimated value.

The average ratios of particulate PG to bacterial abundance in the northwestern Pacific Ocean were generally within the range of the ratio estimated from muramic acid determined in the other oceans (Mimura and Romano, 1985; Benner and Kaiser, 2003) (Table 3), suggesting that the SLP assay data is consistent with the other methods. On the other hand, the ratios of PG to bacterial abundance varied depending on the depth. Since the ratio in the middle layer (average, 0.61) agreed with the PG content obtained in the seawater cultures (0.63 to 1.1 fg cell^{-1}), it appears that the PG in this layer was mostly associated with intact bacterial cells. Interestingly, the lowest ratio obtained from 12 sampling sites in the northwestern Mediterranean Sea was 0.58 (Mimura and Romano, 1985), and the ratios from 2 sampling sites in the central equatorial Pacific Ocean were 0.55 and 0.73 (Benner and Kaiser, 2003). The ratios from these other studies were also very similar to the lowest PG content measured in the seawater cultures without substrate addition (0.63). Note that the ratio in the middle layer is possibly a somewhat conservative estimate. Karner *et al.* (2001) reported that the fraction of the total

Table 2. Ratio of particulate peptidoglycan to bacterial abundance obtained from culture experiments. The ratio shown here for Mimura and Romano (1985) was calculated based on the assumption that the molecular weight of peptidoglycan was about 3.6-fold that of muramic acid (Neidhardt *et al.*, 1990). Note that ratio is expressed as fg cell⁻¹.

Culture conditions	Muramic acid to bacteria (fg cell ⁻¹)	Peptidoglycan to bacteria (fg cell ⁻¹)	Reference
Marine pseudomonad culture			Mimura and Romano (1985)
0.2% of sodium lactate and 0.1% of yeast extract	0.47–0.53	1.7–1.9	
Natural seawater culture			Present study
0 μg l ⁻¹ of glutamic acid		0.63 ± 0.41 (n = 9)	
5 μg l ⁻¹ of glutamic acid		0.83 ± 0.21 (n = 9)	
20 μg l ⁻¹ of glutamic acid		1.1 ± 0.4 (n = 9)	

Table 3. Ratio of particulate peptidoglycan to bacterial abundance obtained from natural seawater. The ratios shown here for Mimura and Romano (1985) and Benner and Kaiser (2003) were calculated based on the assumption that the molecular weight of peptidoglycan was about 3.6-fold that of muramic acid (Neidhardt *et al.*, 1990). We used the bacterial abundance listed in Benner *et al.* (1997) to calculate the Benner and Kaiser (2003) ratio; the bacteria were recovered from seawater in the particulate fraction (size: 0.1 to 60 μm), and were derived from the same samples used for the muramic acid analysis in Benner and Kaiser (2003). Note that ratio is expressed as fg cell⁻¹.

Sampling location and depth	Size fraction (μm)	Muramic acid to bacteria (fg cell ⁻¹)	Peptidoglycan to bacteria (fg cell ⁻¹)	Reference
Northwestern Mediterranean Sea	>0.2			Mimura and Romano (1985)
0–100 μm		0.53 (n = 7)	1.9	
0.5 m		0.25 (n = 10)	0.90	
1–50 m		0.16 (n = 64)	0.58	
Central Equatorial Pacific Ocean	0.1–60			Benner and Kaiser (2003)
2–4000 m		0.15–1.8 (n = 9)	0.55–6.4	
Northwestern Pacific Ocean	>0.22			Present study
0–50 m			3.7 ± 2.6 (n = 29)	
>50–2000 m			0.61 ± 0.53 (n = 40)	
>2000 m			2.1 ± 1.7 (n = 7)	

bacterioplankton abundance composed of the *Archaea* gradually increased from depth to below the euphotic zone. The fact that the *Archaea* do not react with the SLP assay (data not shown), combined with the existence of these cells in the middle layer (Karner *et al.*, 2001), might lead to the apparent decrease in the ratio of particulate PG to bacterial abundance.

In contrast, compared with the middle layer, the ratios of particulate PG to bacterial abundance were significantly higher in the surface and deep layers (average, 3.7 and 2.1, respectively). Such high ratios in these layers were also observed in other studies (Mimura and Romano, 1985; Benner and Kaiser, 2003). Besides, the ratios obviously exceed values of the PG content obtained in the seawater cultures (0.63 to 1.1 fg cell⁻¹). Considering these facts, the results of the present study may suggest that, in the surface and deep layers, a significant fraction of particulate PG was present in bacterial detritus, whereas this fraction was smaller in the middle layer. If we assume that the cellular PG content is 0.63 fg cell⁻¹, the intact bacterial cells account for about 15 and 30% of the particulate PG in the surface and deep layers, respectively. Overall, only about 30% of the PG in all of the samples collected from the entire water column can be explained by that which was in the bacterial cells. Given that the abundance of *Archaea* equaled or exceeded the abundance of *Bacteria* below 1000 m (Karner *et al.*, 2001), the actual contribution of PG in intact bacterial cells to the particulate PG in the deep layer might be even lower. A considerable fraction of particulate PG in the water column seems to be attributed to bacterial detritus.

Our idea that the particulate PG was mostly associated with bacterial detritus is supported by recent chemical characterization of amino sugars (Benner and Kaiser, 2003). This study estimated that the contribution of muramic acid in intact bacterial cells to particulate muramic acid, which was recovered in the size class ranging from 0.1 to 60 μm , averaged about 30% in all samples collected from depths between 2 and 4000 m, but only 10 to 15% in the samples from the deep layer (depth at 4000 m); the estimate suggests that most of the muramic acid recovered in this size class was associated with nonliving particles. Therefore, our estimate of the contribution of PG in intact bacterial cells to the particulate PG content of the northwestern Pacific Ocean is most consistent with the estimate by Benner and Kaiser (2003) in the central equatorial Pacific Ocean, which used muramic acid for this determination. However, in order to produce a more reliable estimate of PG data throughout the water column, obtained using the SLP assay, the following subjects should be investigated in future. (1) The PG data should be compared with muramic acid data from other analytical methods, which may lead to an evaluation of the influence of (1 \rightarrow 3)- β -D-glucan on our data. (2) The

ratio of particulate PG to bacterial abundance should be corrected by determining what part of the total bacterioplankton abundance is composed of the *Archaea*. (3) A more detailed examination of the variation in PG content in marine bacteria and its natural assemblages would help in understanding the variation in the ratio of PG to bacteria.

In conclusion, we have used the SLP assay to determine the particulate PG concentration at 5 sampling sites in the northwestern Pacific Ocean and in natural seawater cultures. To our knowledge, this is the first report of the application of the SLP assay to natural aquatic samples. Considering the PG content of bacteria, obtained by investigating natural seawater cultures, the results of the oceanic observations may suggest that, in the surface and deep layers, a significant fraction of particulate PG was present in bacterial detritus, whereas this fraction was smaller in the middle layer. Our results would provide insight into the state of the POM derived from bacteria in the ocean.

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