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The role of surface layer proteins in the degradation of a photosynthetic prokaryote, the cyanobacterium *Synechococcus* sp.

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ABSTRACT

The accumulation of refractory prokaryotic cell membranes has been suggested as a possible source of both dissolved and particulate organic matter in the deep ocean. A surface layer protein (S-layer) is widely found as part of the cell envelope in both Eubacteria and Archaea and is made up of a monomolecular layer of glycoproteins. This heavily glycosylated protein covers the outermost cell surface in a regularly ordered planar crystalline structure. With special attention to the possible geochemical importance of S-layer protein in seawater, we studied the degradation of two species of marine cyanobacteria, *Synechococcus* sp. CCMP2370 and CCMP1334, with and without an S-layer structure, respectively, after they had been treated with buffers that can strip the S-layer from the cell surface. Based on evidence from bulk chemical and molecular analysis as well as electron microscopy, stripped cells of CCMP2370 lost their cell membrane structure and degraded more rapidly and became more enriched in D-amino acids, while stripped CCMP1334 cells maintained their membrane structures, and degradation was not enhanced. These results suggest that S-layer glycoproteins have a limited contribution to selective preservation of proteinaceous materials during the degradation of the cyanobacterium *Synechococcus*, but like peptidoglycan, S-layer structure functions as a defensive barrier on the cell membrane to prevent cell lysis and slow degradation of cyanobacterial cellular materials. Our results imply that selective preservation of glycoprotein is not as important as physical protection by S-layer in regulating the degradation of cellular materials of cyanobacteria.

1. Introduction

Proteinaceous materials, a major component of the hydrolyzable amino acids, account for much of the organic nitrogen in marine organisms, and although they are generally considered to be labile to biogeochemical degradation, some of them are resistant to heterotrophic attack and remineralization. These resistant compounds may become preferentially enriched in seawater and particles during decomposition (Wakeham et al., 1997; Lee et al., 2000). This selective preservation mechanism substantially regulates the concentration and composition of proteinaceous materials in the oceans. Many past studies have shown that bacterial membrane proteins and peptidoglycan are selectively preserved in marine detrital organic matter (e.g., Tanoue et al., 1995; McCarthy et al., 1998; Kaiser and Benner, 2008; Nunn et al., 2010). For example, D-amino acids, enantiomers of essential cellular amino acids, are found in certain bacterial structures like peptidoglycan, teichoic acids, lipopeptides and siderophores (e.g., Schleifer and Kandler, 1972; Kaiser and Benner, 2008). The enrichment of D-amino acids relative to L-amino acids has been widely investigated as an indicator of bacterial biomass in the oceans (e.g., McCarthy et al., 1998). Although the lesser bioavailability of bacterial organic matter relative to phyto- and zooplankton derived organic matter has been demonstrated (Lomstein et al., 2009; Tremblay and Benner, 2009), the influence of this difference to early diagenesis of marine organic matter remains largely unknown.

Several mechanisms have been proposed to explain how proteinaceous materials are preserved at the early stage of diagenesis. Non-covalent interaction is one such mechanism. The surface of membrane proteins are relatively hydrophobic and can closely associate with other membrane structures through non-covalent binding. Membrane proteins are surrounded by relatively recalcitrant structures like lipid bilayers and peptidoglycans (Hedges et al., 2000). These structures can encapsulate membrane proteins and thus prevent the attack of hydrolytic enzymes or other chemical reactants such as hydroxyl radicals (Pogozelski and Tullius, 1998). Protection of proteins from enzymatic hydrolysis by hydrophobic interaction has been demonstrated to be important in preserving proteins from degradation (Borch and Kirchman, 1999; Nguyen and Harvey, 2003). Extensive post-translational modification after transcription is one of the characteristic features of prokaryotic membrane proteins (Schäffer et al., 2001), and glycosylation is the most common modification in vivo (Messner, 1997; Schäffer et al., 2001). Glycosylation is thought to stabilize protein conformation and protect proteins from enzymatic attack within the cell (Schäffer et al., 2001; Upreti et al., 2003), thus potentially preventing degradation of

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proteins in natural environments. This is supported in the marine environment by the finding that glycosylation reduces turnover of proteins in seawater by a factor of 100 (Keil and Kirchman, 1993).

Membrane proteins, however, are highly diverse in molecular structure, biological function and location in cells, as shown in molecular biological studies (Engelman, 2005; Barrera and Robinson, 2011). These differences indicate that individual membrane proteins may behave very differently after cell death, which could result in different degradation pathways. As an example, S-layer protein is a unique membrane glycoprotein found in many Eubacteria and almost all Archaea; it covers the outermost cell surface and consists of a regularly ordered, planar array of paracrystalline proteinaceous subunits of 1 or 2 proteins (Sleytr and Beveridge, 1999; Sleytr et al., 2014). S-layer proteins account for up to 15% of the total proteins in bacteria; they are glycosylated with an overall degree of glycosylation of 2–10% (w/w) (Schäffer and Messner, 2004; Messner et al., 2013). As the outermost layer of prokaryotic cells, S-layer plays a role in maintaining cell shape, behaving as scaffolding for enzymes, and excluding the transport of large molecules (Sleytr et al., 2014). The characteristic S-layer monomolecular ultrastructure is located on top of the outer membrane and directly beneath the polysaccharide sheath. Thus S-layer structures are among the first structures exposed to the ambient environment when a cell begins to degrade. S-layers have been found in many species of cyanobacteria (Smarda et al., 2002), among which is the marine cyanobacterium *Synechococcus*, CCMP2370 (Brahamsha, 1996; McCarren et al., 2005). But the geochemical importance of S-layer protein to organic matter degradation in marine environments remains unexplored.

To further our understanding of the lability of membrane proteinaceous materials in marine environments, we chose to use surface layer (S-layer) protein from *Synechococcus* sp. CCMP2370 (SynS) as a model to study the behavior of an individual membrane protein during the degradation of cyanobacterial cells. D-amino acids were specifically targeted for analysis to investigate the extent of bacterial activity during degradation. We compared degradation of two strains of cyanobacteria, one with S-layer structure, *Synechococcus* sp. CCMP2370 (SynS), and one without, CCMP1334 (SynNO). SynS (also known as WH8102) is motile, and favors strong light intensity. SynNO (also known as WH7803) is a non-motile species that is adapted to weak light (Brahamsha, 1996). *Synechococcus* S-layer structures have been relatively well characterized in previous studies (Brahamsha, 1996; McCarren et al., 2005). A glycosylated protein of 132 kDa is enriched in *Synechococcus* S-layer structures and present in the gene sequences of this species. Here we report results of degradation experiments using *Synechococcus* with and without S-layer structures (SynSintact and SynNOintact, SynSstripped and SynNOstripped, respectively) (see Table 1), using a stripping treatment that removes S-layer from cells but leaves other cellular components intact.

2. Methods

2.1. Culture preparation

Strains of *Synechococcus* sp. CCMP2370 and CCMP1334 (Bigelow Laboratory) were grown axenically in L1 medium prepared with 0.2-μm filtered coastal seawater from Stony Brook Harbor, New York (Guillard and Hargraves, 1993). The cultures were incubated in 500-mL sterile glass flasks at 20 °C with a 16:8 illumination cycle of ~10 μmol quanta m^{-2} s^{-1}. Cells of both strains were harvested at the end of the exponential growth stage or early stationary stage. The harvested cultures were centrifuged at 5250 ×*g* at 4 °C for 40 min, and the pelletized cells were frozen until use. Chlorophyll *a* concentrations in collected cultures were estimated from the whole cell spectra from 600 to 800 nm using a Perkin Elmer UV-VIS spectrophotometer (Collier and Grossman, 1992).

2.2. Isolation of S-layer and preparation of stripped cells

S-layer was stripped from frozen SynS cells by optimizing the method of Brahamsha (1996). SynNO cells without S-layer were treated in exactly the same way to make cell preparations of these two strains as similar as possible. Cell concentrations for both strains were estimated from chlorophyll concentrations, assuming a constant Chl/carbon ratio (Collier and Grossman, 1992), so that the initial cell concentrations in each flask were identical before the stripping treatment. Briefly, the frozen pellets of SynS and SynNO were thawed and washed with sterile artificial seawater, and the cell pellets resuspended in a stripping buffer (50 mmol L^{-1} Tris·HCl/50 mmol L^{-1} Na_2EDTA/15% sucrose, pH = 7.25). After immersion in the stripping buffer at 4 °C overnight, stripped cells were collected by centrifugation at 6300 ×*g* for 12 min, and rinsed twice with sterile artificial

Table 1
Experiment matrix of degradation incubations using stripped and intact *Synechococcus* CCMP2370 and CCMP1334.

	Medium	Cell addition	Membrane morphology	Cell treatment
SW	Coastal seawater	N/A		N/A
SynSstripped	Coastal seawater	*Synechococcus* sp. CCMP2370		Stripping treatment
SynSintact	Coastal seawater	*Synechococcus* sp. CCMP2370		N/A
SynNOstripped	Coastal seawater	*Synechococcus* sp. CCMP1334		Stripping treatment
SynNOintact	Coastal seawater	*Synechococcus* sp. CCMP1334		N/A

seawater. After stripping, the stripped cells were then used in the incubation experiments together with intact cells without stripping treatment as described in Section 2.2.

To collect the S-layer protein, the supernatant from stripped SynS cells after centrifugation was ultrafiltered using 3 kDa centrifugal ultrafiltration vials to separate stripping buffer from the S-layer components. The retentate (S-layer) was rinsed twice with sterile artificial seawater. The S-layer was analyzed by gel electrophoresis and amino acid analysis as described below.

2.3. Incubation of stripped and intact Synechococcus cells

Centrifuged pellets from the four cell treatments described above were washed twice with sterile artificial seawater, and then resuspended into filtered coastal seawater (200 mesh, 74 μm) collected from Stony Brook Harbor at high tide. Most zooplankton and large phytoplankton were removed, while the microbial community remained largely unchanged in the filtered seawater. Nanoflagellates smaller than 74 μm that remained in the filtered seawater may have influenced the incubation; however, no growth of nanoflagellates was observed during the 30-day incubation as observed by epifluorescence microscopy. All cells were incubated in the dark at 22–24 °C for 30 days, so that growth of phytoplankton was inhibited. A blank of 200 mL of 200-mesh filtered coastal seawater was incubated in parallel. Subsamples were collected from each incubation after 0, 2, 7, 15 and 30 days. Samples were centrifuged at 5250 $\times g$ for 40 min, and supernatants (surrounding seawater) and pellets (particles) were collected for analysis of proteins (gel electrophoresis) and amino acids (HPLC) as described below.

2.4. Amino acid analysis

Amino acids were measured in both particles and surrounding seawater from the incubations. Centrifuged pellets were analyzed for particulate amino acids (PAA). Surrounding seawater was analyzed for total dissolved amino acids (TDAA) and dissolved free amino acids (DFAA). Both PAA and TDAA were acid hydrolyzed before analysis to free the combined amino acids. Samples were diluted with Milli-Q water and acidified with an equal amount of 12 N HCl (99.999% trace metal basis, Sigma-Aldrich). Ascorbic acid (10 μM, final concentration; reagent grade, Sigma-Aldrich) was added to the acidified samples to prevent oxidation during heating (Robertson et al., 1987). In addition, samples were sparged with dry nitrogen gas for about 15 s before sealing in glass vials to remove oxygen. The prepared samples were hydrolyzed at 110 °C on a heating block for 20 h, and then dried by a stream of N_2. Dried samples were rinsed with drops of distilled water (DI) water and dried again to remove remaining acid. Dried samples were resuspended into 6:4 distilled water:methanol before derivatization and chromatographic analysis. DFAA samples were diluted with Milli-Q water and methanol added to a ratio of 6:4 sample:methanol before derivatization and chromatographic analysis.

After pretreatment, PAA, TDAA and DFAA were analyzed following the HPLC method of Fitznar et al. (1999). Amino acids were separated on a 5 μm Alltima C18 column (length: 250 mm; ID: 4.6 mm) with 25 mmol L^{-1} sodium acetate (pH = 7) and methanol: acetonitrile (13:1, v/v) as mobile phases. Amino acid enantiomers were identified using fluorescent detection (Ex/Em = 330/445 nm) after online chiral derivatization with 3 g L^{-1} o-phthaldialdehyde (OPA) (Sigma-Aldrich) and 5 g L^{-1} N-isobutyryl-L-cysteine (>97%, Fluka) in 0.8 mol L^{-1} boric acid buffer with a pH of 10.5. For quantification, we used mixed standards prepared by adding individual D-amino acids as well as β-alanine (BALA) and γ-aminobutyric acid (GABA) standards (Sigma-Aldrich) to a Pierce H L-amino acid standard. The analytical error of individual standards ranged from 1% to 16% with an average of 6%. Dissolved combined amino acids (DCAA) were calculated as the difference between TDAA and DFAA. The relative abundances of individual D-amino acids were estimated as

$$\%D = 100 \times \frac{[D-AA]}{[D-AA] + [L-AA]} \quad (1)$$

where [D-AA] is the concentration of individual D-amino acids. We corrected %D for hydrolysis-derived racemization using Table 1 from Kaiser and Benner (2005). Total %D was calculated using the following equation:

$$\text{Total}\%D = 100 \times \frac{[D-ASP] + [D-GLU] + [D-SER] + [D-ALA]}{[D-ASP] + [D-GLU] + [D-SER] + [D-ALA] + [L-ASP] + [L-GLU] + [L-SER] + [L-ALA]} \quad (2)$$

2.5. Gel electrophoresis

Proteins extracted from *Synechococcus* cells or the isolated S-layer were sorted by their size and charge using gel electrophoresis following the methods of Saijo and Tanoue (2005). Briefly, S-layer or cell pellets were resuspended in 62.5 mmol L^{-1} Tris/2% SDS/5% 2-mercaptoethanol and boiled for 3 min. After cooling to room temperature, urea was added to a concentration of 8 mol L^{-1}. The mixtures were stored at 4 °C overnight, and then mixed with Laemmli sample buffer (Bio-Rad) and boiled for 10 min. After cooling on ice, samples were loaded on 4–20% Ready Gel Tris-HCl gels (Bio-Rad) using a Bio-Rad Mini-PROTEAN system at 20 mA constant AMP. The S-layer isolated using stripping buffer was mixed with Laemmli sample buffer and loaded on a gel. Parallel gels were prepared at the same time; one was stained with Coomassie Brilliant Blue (CBB), and the other one with periodic acid-Schiff stain (PAS).

For CBB staining, gels were rinsed with Milli-Q deionized water (DW) and then stained for 30 min with a CBB G250 staining buffer that consisted of 100 gL^{-1} CBB/10% acetic acid/45% methanol in DW. The gel was then rinsed with a destaining solution of 10% acetic acid and 10% methanol in DW for another 30 min. For PAS staining, gels were stained using Glycoprotein Staining Kits (Pierce) as described in the manual protocol. The gel was first rinsed with 50% methanol in DW for 30 min on a shaker, and then rinsed twice with 3% acetic acid in DW for 10 min each. Oxidizing solution (25 mL) was added after the rinse and shaken for 15 min. The gel was then washed three times with 3% acetic acid for 5 min each time. Staining solution (25 mL) was then added to the gel, followed by 25 mL reducing solution. The gel was washed thoroughly with 3% acetic acid before photographed using a Canon digital camera.

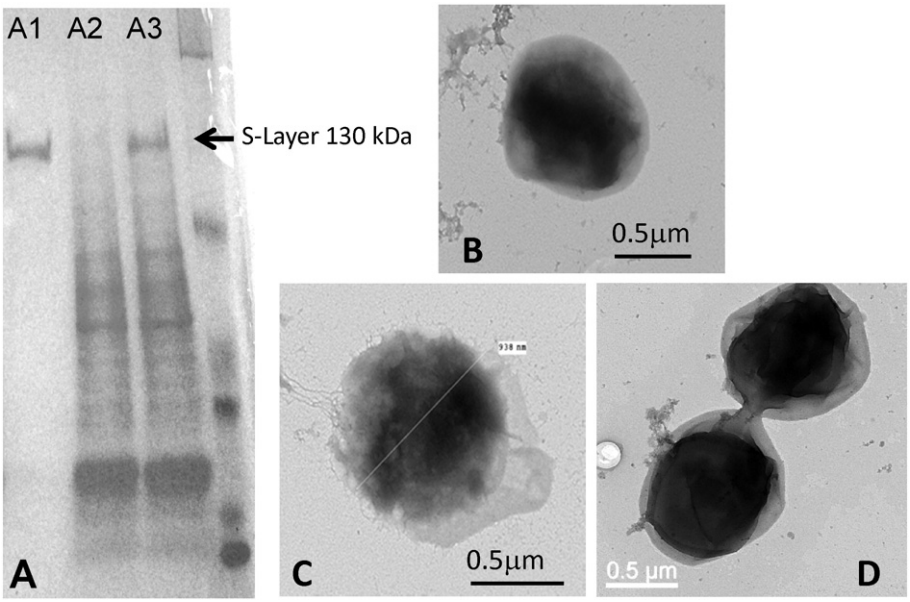

Fig. 1. CBB-stained SDS-PAGE of the stripping buffer with S-layer alone (A1), stripped cells (A2), and intact cells (A3); and transmission electron micrograph (TEM) of intact cells (B) and stripped cells (C) of CCMP2370 (a species with S-layer); stripped cells of CCMP1334 (a species without S-layer) (D).

2.6. Electron microscopy and bacterial abundance

To concentrate the sample, 1 mL subsamples were taken from incubations over time and centrifuged at 4000 × *g* for 20 min. The resulting pellets were resuspended in 100 μL supernatant. One drop (~25 μL) of this suspension was fixed on 300-mesh, carbon-coated copper grids for 60 s before being air-dried for transmission electron microscopy (TEM) analysis. Prepared grids were viewed on a JEOL JEM-1400 TEM or FEI BioTwinG2 TEM at an accelerating voltage of 120 kV. This work was conducted at the Center for Functional Nanomaterials, Brookhaven National Laboratory, Upton, New York.

Subsamples of bacterial abundance were stained with DAPI (4–6-diamindino-2-phenylidole) and filtered through 0.2 μm black polycarbonate filters; bacterial cells were counted under a 100× Nikon Eclipse E400 epifluorescence microscope. Ten fields were counted for each sample.

2.7. Degradation index

The degradation index (DI) of particulate amino acids in samples was calculated using their relative abundances and the equation described by Dauwe et al. (1999):

$$DI = \sum_i \left[\frac{v_i - AVG\ v_i}{STD\ v_i}\right] \cdot fac.coef._i \quad (3)$$

where *i* represents individual amino acids in subsamples, v_i is the mol% of individual amino acids in the subsample, *AVG vi* and *STD vi* are the mean and standard deviation of individual amino acids, respectively. The factor coefficients are from Table 1 in Dauwe et al. (1999).

3. Results

3.1. Isolation of the S-layer

The stripping treatment used in this study removed one major protein (about 130 kDa) from *Synechococcus* sp. CCMP2370 (SynS) as identified using polyacrylamide gel stained with Coomassie Blue (Fig. 1A). The protein distribution in stripped cells was identical to that in whole cells as seen in SDS-gels, except for the absence of the 130 kDa protein (compare Bands A2 and A3 in Fig. 1A). Meanwhile, only one protein of 130 kDa was identified in the stripping buffer after the treatment (Band A1 in Fig. 1A). This suggests that the 130 kDa protein was the only major protein removed from SynS by the stripping procedure; this protein was demonstrated by McCarren et al. (2005) to be a glycoprotein required for the generation of surface layer structure in SynS. It is possible that other membrane components, e.g., pigments or polysaccharide sheath, may also be removed with the S-layer protein; but these molecules cannot be detected by CBB after SDS-PAGE.

Extraction of the S-layer structure caused visible changes to the morphology of the cell surface. Under TEM, SynS cells had an intact and smooth surface (Fig. 1B). In comparison, the stripped SynS cells still maintained their shape, but the surface of the cell was rough and indistinct (Fig. 1C). The stripping treatment did not cause visible damage to the surface of the non S-layer containing SynNO as shown in Fig. 1D; the surface was still smooth after treatment, indicating that the buffer apparently only targets S-layer structures. Thus, the stripping treatment appears to be an efficient way to remove S-layer proteins from *Synechococcus* cells, while leaving other parts of the cells intact. This allowed us to compare degradation of cells with and without S-layer protection. Moreover, the integrity of cells after one freeze-thaw cycle was shown by the intact morphology of SynS and SynNO cells.

3.2. Changes in dissolved and particulate amino acids over time

Particulate amino acids (PAA) were measured in both intact and stripped SynS cell incubations (Table 2). PAA concentration was initially 447 μmol L^{-1} in seawater with intact cells, and decreased exponentially

Table 2
The change in L-and D-amino acid concentrations, total %D-amino acids and bacterial contribution estimated from D-GLU and D-ALA during the degradation incubations of stripped and intact *Synechococcus* CCMP2370 and CCMP1334. The ± values are standard deviations of replicate analysis. The analytical errors of TDAA, DFAA and PAA concentrations from the external standards are 10%, 18% and 6% respectively (n = 10, 6, 3).

	SynSstripped					SynSintact					SynNOstripped					SynNOintact				
Days	0	2	7	15	30	0	2	7	15	30	0	2	7	15	30	0	2	7	15	30
TDAA (μmol L^{-1})																				
L-amino acids	1.42 ± 1.23	0.66 ± 0.39	1.30 ± 0.79	1.45 ± 1.24	1.12 ± 0.73	8.64	2.53	8.82	3.78	3.28	1.23	2.29	1.27	2.87	0.64	2.84	0.32	6.29	3.52	1.36
D-amino acids	0.03 ± 0.02	0.08 ± 0.07	0.15 ± 0.04	0.08 ± 0.01	0.05 ± 0.00	1.81	0.09	0.24	0.18	0.12	0.05	0.12	0.14	0.64	0.06	0.15	0.02	0.80	0.24	0.09
D- and L-amino acids	1.46 ± 1.26	0.74 ± 0.45	1.45 ± 0.84	1.53 ± 1.25	1.17 ± 0.74	10.45	2.62	9.06	3.96	3.39	1.27	2.41	1.41	3.51	0.70	2.99	0.34	7.10	3.76	1.46
Total %D	13%	11%	13%	23%	24%	22%	8%	5%	9%	13%	8%	12%	21%	34%	20%	7%	12%	23%	15%	18%
DFAA (μmol L^{-1})																				
L-amino acids	0.07	0.36	0.12	0.02	0.02	5.93	0.18	1.50	0.49	0.18	0.02	0.09	0.04	0.00	0.00	2.10	0.42	0.03	0.67	0.03
D-amino acids	0.01	0.03	0.03	0.00	0.00	0.75	0.02	0.08	0.08	0.04	0.00	0.01	0.01	0.00	0.00	0.17	0.04	0.04	0.00	0.00
D- and L-amino acids	0.08	0.39	0.15	0.02	0.02	6.68	0.21	1.58	0.57	0.21	0.02	0.11	0.04	0.00	0.00	2.26	0.45	0.07	0.67	0.03
DCAA (μmol L^{-1})																				
L-amino acids	1.36	0.30	1.18	1.43	1.10	2.71	2.35	7.32	3.28	3.10	1.21	2.20	1.23	2.87	0.64	0.75	–	6.26	2.85	1.33
D-amino acids	0.02	0.05	0.12	0.08	0.05	1.06	0.07	0.16	0.10	0.08	0.04	0.10	0.13	0.64	0.06	–	–	0.76	0.24	0.09
D- and L-amino acids	1.38	0.35	1.30	1.51	1.15	3.77	2.42	7.48	3.38	3.18	1.25	2.30	1.37	3.51	0.70	0.73	–	7.03	3.09	1.43
PAA (μmol L^{-1})																				
L-amino acids	55.86	78.95	38.11	9.56	11.84	439.96 ± 5.24	293.80 ± 17.90	56.98	39.53	17.63										
D-amino acids	1.24	1.31	0.84	0.32	0.44	7.68 ± 0.39	5.28 ± 0.53	0.94	0.76	0.38										
D- and L-amino acids	57.10	80.27	38.95	9.88	12.27	447.64 ± 5.64	299.08 ± 17.37	57.92	40.29	18.01										
Total %D	5%	4%	5%	7%	8%	4%	5%	4%	4%	5%										
% live bacteria	0.3%	0.7%	0.2%	0.4%	0.1%	0.1%	0.3%	0.3%	0.3%	0.3%										

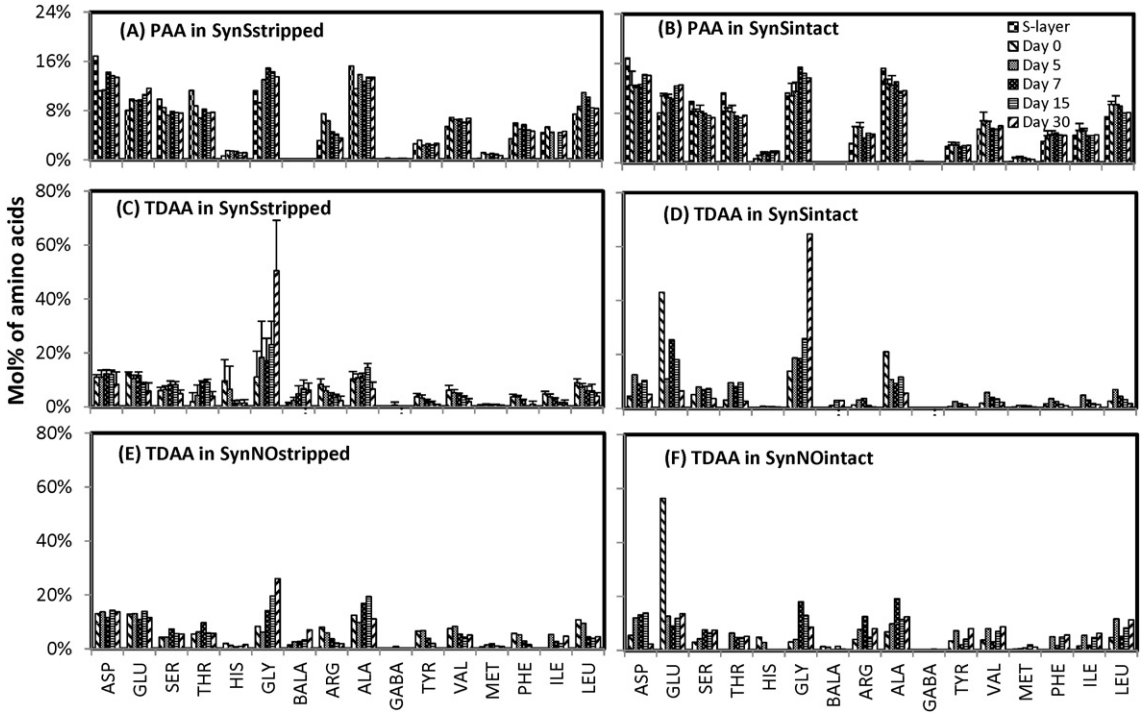

Fig. 2. Relative abundance of amino acid composition in S-layer protein, particles of SynSstripped (A) and SynSintact (B); relative abundance of TDAA in seawater from SynSstripped (C) and SynSintact (D); relative abundance of TDAA in seawater from SynNOstripped (E) and SynNOintact (F) incubations. The error bars are standard deviations from replicate analysis. The analytical errors of individual PAA from external standards range from 2% to 30% with an average of 8% (n = 3), and those of individual TDAA range from 2% to 36% with an average of 12% (n = 10).

over the next 30 d to a concentration of 18 μmol L^{-1} (Table 2). In contrast, much lower initial PAA concentration (57 μmol L^{-1}) was observed in the stripped SynS cell incubation. After an initial elevation to 80 μmol L^{-1} at day 2, PAA concentration in SynSstripped incubations also decreased rapidly to a concentration of 12 μmol L^{-1} after 30 days. The initial difference in PAA at the beginning of the incubation was probably due to the loss during the stripping treatment and subsequent rinses. As PAA concentrations in both stripped and intact SynS cell incubations decreased over time, PAA composition also exhibited noticeable changes (Fig. 2A and B). For example, mol% ASP and ALA decreased gradually in contrast to increased mol% GLU and GLY with incubation time.

Degradation states of samples taken over the incubation period were investigated using the principal component analysis technique of Dauwe et al. (1999) (Fig. 3), who quantitatively evaluated the change in amino acid composition during degradation of hydrolyzed amino acids in marine sediments. This method has been applied to marine particles as well (Keil et al., 2000; Sheridan et al., 2002; Xue et al., 2011). Here, the degradation index (DI) ranged from −0.5 to 0.6 in both

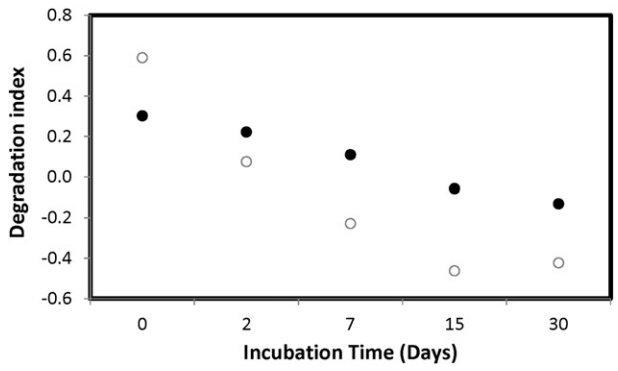

Fig. 3. Change of Dauwe degradation index of particulate amino acids during degradation of SynSstripped (open circles) and SynSintact (solid circles) cells;

stripped and intact SynS, and decreased over time. A higher DI was observed on Day 0 in stripped compared to intact cells, suggesting that the stripping procedure caused a change in PAA composition. In spite of this initial difference, DI of both SynSstripped and SynSintact cells gradually decreased during the 30-d incubation, although that of SynSstripped decreased faster and to a lower DI. The faster decrease of DI in SynSstripped than SynSintact on the same time scale suggests that the amino acid composition of stripped cells was subject to greater change due to heterotrophic microbial activity during the incubation.

Both dissolved free and combined amino acids were measured in the seawater medium during all incubations. Dissolved free amino acids (DFAA) were initially high in intact cell incubations (mostly due to glutamic acid as mentioned below) but rapidly declined to low values after only 2 d; in stripped cell incubations DFAA were low throughout the entire incubation (Table 2). The early decline of DFAA in the intact cell incubation suggests that labile organic matter was released initially and rapidly decomposed, while stripped cells did not release DFAA, or the stripping process had removed them. A slight peak in DCAA concentrations was observed in all four incubations, usually after 7–15 days (Table 2), which may result from the release of DCAA by heterotrophic degradation as observed in previous studies (Ogawa et al., 2001). As with DFAA, DCAA in intact SynS cells were generally higher than stripped ones initially, while this difference in DCAA concentration was not observed in SynNO incubations.

Total dissolved amino acids in the initial seawater medium used here exhibited a molecular composition generally similar to that of other seawater (Dittmar et al., 2001; Kuznetsova et al., 2004; Kaiser and Benner, 2009). However, this composition changed during the degradation of both stripped and intact cyanobacterial cells (Fig. 2C, D, E and F). In TDAA of SynS and SynNO cell incubations, mol% GLU was initially very high, 40% and 56%, respectively. Much (37 and 61%) of the initial TDAA was free rather than combined glutamic acid, and was lost quickly in the first two days. GLU was not enriched in stripped cell incubations. Mol% glycine (GLY) in TDAA progressively increased over the 30 days in all incubations except that of intact SynNO (without S-

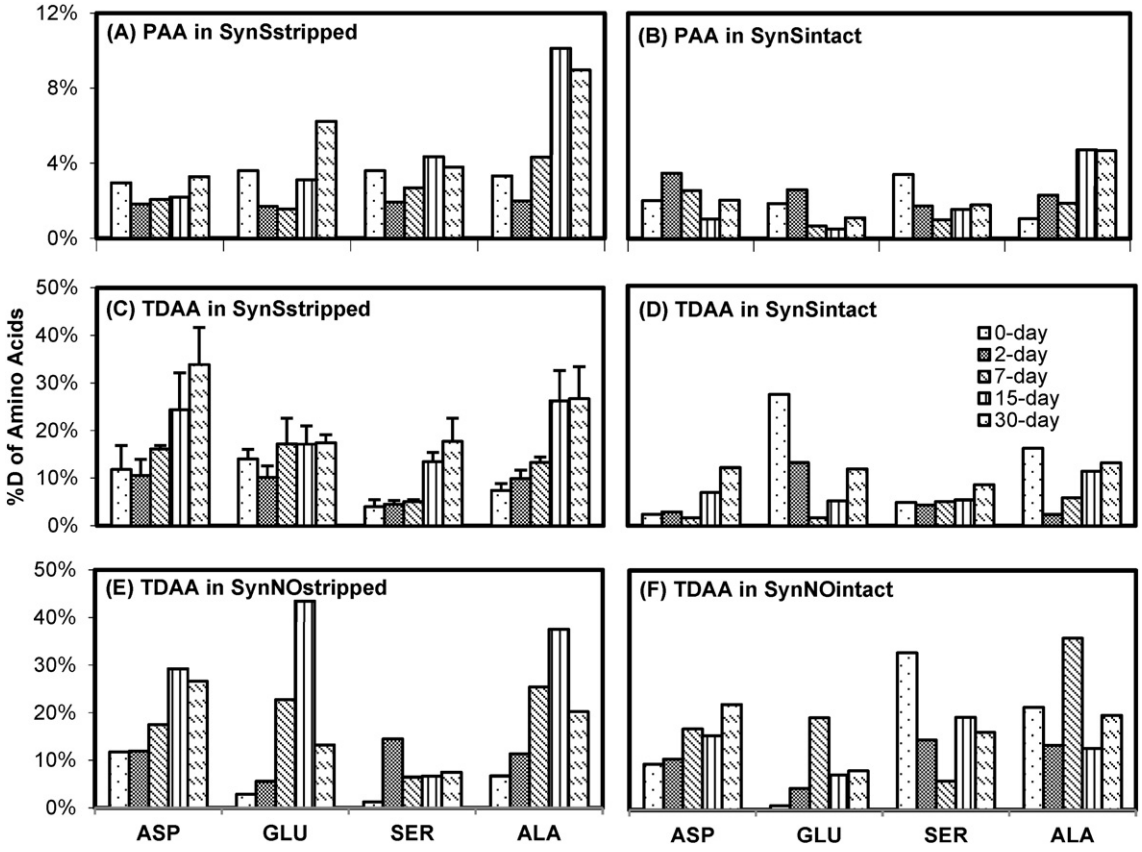

Fig. 4. Change in the %D of ASP, GLU, SER, and ALA during the incubations of stripped and intact cells. Particulate amino acids during the incubations of SynSstripped (A) and SynSintact (B). Dissolved hydrolyzable amino acids during the incubations of SynSstripped (C) and SynSintact (D). Dissolved hydrolyzable amino acids during the incubations of SynNOstripped (E) and SynNOintact (F). The error bars are standard deviations from replicate analysis. The analytical errors of %D (relative standard deviations of external standards) are 7% and 1% in average in TDAA and PAA (n = 10 and 3).

layer), and increased more in SynS than SynNO cultures. The non-protein amino acid, BALA, preferentially accumulated during all incubations except that of intact SynNO. Another microbial derived non-protein amino acid, GABA, was below the detection limit for most of the samples. Accumulation of BALA is found in marine environments, most likely as a decarboxylation product of ASP (Lee and Cronin, 1982; Cowie and Hedges, 1994), which is consistent with progressive degradation occurring during our incubations.

3.3. Changes in D-amino acids in particles and surrounding seawater over time

Four D-amino acids, D-ASP, D-GLU, D-SER and D-ALA, were measured in particles and surrounding seawater during incubations. In particles, a general decrease in the sum of these four D-amino acid concentrations was observed over time, ranging from 1.24 to 0.44 μmol L^{-1} in SynSstripped and from 7.68 to 0.38 μmol L^{-1} in SynSintact (Table 2).

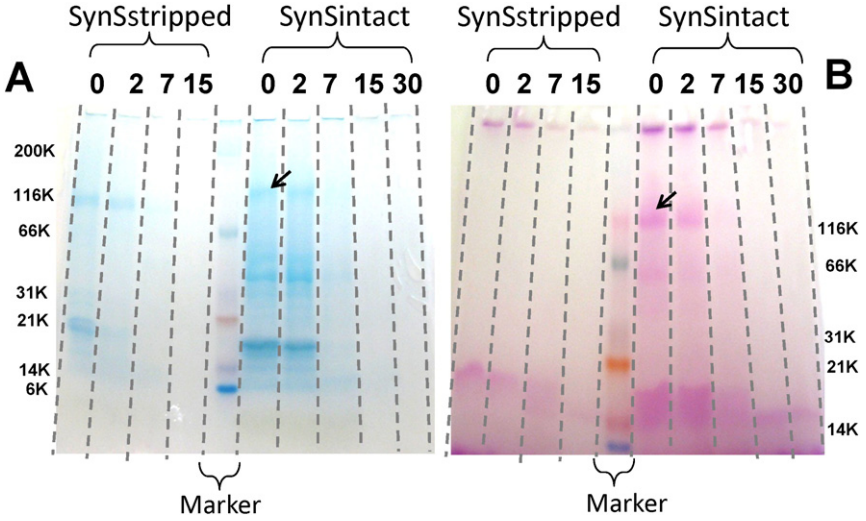

Fig. 5. SDS polyacrylamide gels of CBB-stained (A) and PAS stained (B) materials. Degradation of SynSstripped over time was showed in the left parts after stained by CBB (A) and PAS (B), respectively; Degradation of SynSintact over time was showed in the right parts after stained by CBB and PAS, respectively. Arrowheads show the protein band with molecular weight of about 130 kDa. The molecular weight of marker proteins was listed next to the gels.

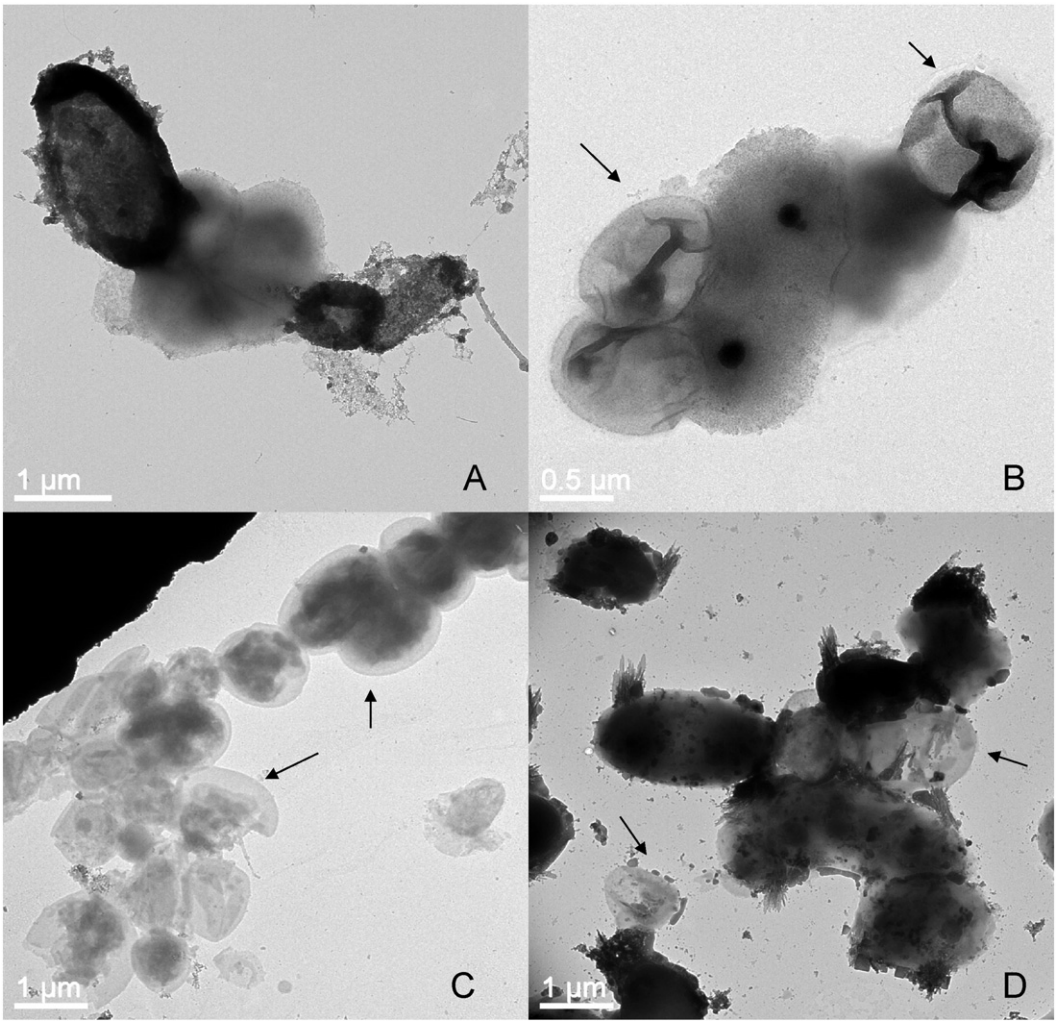

Fig. 6. TEM of SynSstripped (A) and SynSintact (B) after 2-days incubation; TEM of SynNOstripped (C) and SynNOintact (D) after 2-days of incubation. Arrows show fragments of cell membrane with smooth surface.

This trend is consistent with the decreases observed in total PAA concentrations. Total %D in SynSintact particulate samples was relatively low and did not change much over time except for a small increase in D-ALA (Table 2 and Fig. 4B). Total %D values in stripped cell incubations were on average relatively higher than in intact cells, and slightly increased over time (Table 2). By the end of the incubations, D-ALA accounted for a major portion of D-amino acids in both stripped and intact cell incubations (Fig. 4A and B), which is consistent with previous findings that D-ALA is the major D-amino acid constituent in marine particles (Dittmar et al., 2001).

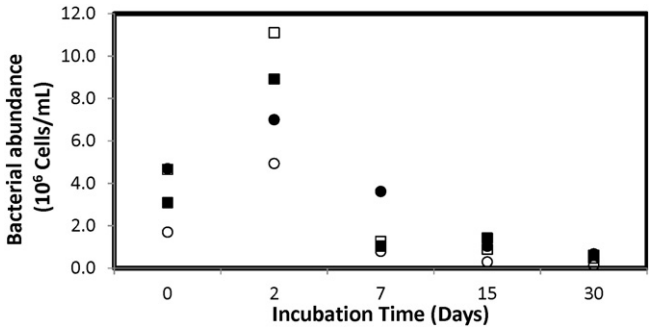

Fig. 7. Change of bacterial abundance during the degradation of SynSstripped (open circles), SynSintact (solid circles), SynNOstripped (open squares) and SynNOintact (solid squares);

Unlike in particles, concentrations of total dissolved hydrolyzable D-amino acids were generally lower at the beginning of incubations and gradually increased to a peak, 0.15–0.80 μmol L^{-1}, before declining at the end of the incubations (Table 2). Exceptions were the higher D-amino acid at the beginning of intact cell incubations, which rapidly decreased by the second day. This pattern of higher D-amino acids in the middle of the incubation was similar in both stripped and intact SynS, although concentrations were somewhat higher in intact cell incubations. Peak concentrations in SynNO incubations were somewhat higher than those of SynS. Although error is high in the DFAA and DCAA calculations due to their low concentrations, DCAA appeared to contribute most to the peak in D-amino acids. The %D in both total and individual amino acids in TDAA was usually higher than in PAA (Table 2 and Fig. 4). This is consistent with previous observations of relatively higher %D in seawater than in particles (Dittmar et al., 2001). Total %D was also generally higher in stripped than intact cell incubations, while this difference was not observed in SynNO incubations (Table 2). The %D of individual amino acids in stripped cells increased during the incubations (Fig. 4C and E), whereas intact cells showed no clear trend with time. In intact but not stripped cell incubations, the %D in GLU, SER and ALA was high initially, but quickly decreased in the first two days before increasing again (Fig. 4C–F). The progressive increase in %D was much less clear in incubations of intact SynNO without an S-layer, where only D-

ASP clearly increased throughout the incubation. It is unclear why %D in SynNOstripped and SynNOintact shows different distribution from that in SynS incubations during the 30-d degradation. Moreover, why the higher %D-SER and %D-ALA exist in SynNOintact relative to SynNOstripped also remains unexplained.

3.4. Changes in CBB-stainable and PAS-stainable materials over time

CBB selectively stains molecules with basic amino acids like arginine (ARG), histidine (HIS) and lysine (LYS), while PAS targets molecules with diol functional groups, like sugars. Therefore, proteinaceous materials can be separated and identified using gel electrophoresis and CBB staining, while glycoproteins with both peptide and sugar chains can be stained by both CBB and PAS. Changes in concentrations of proteinaceous materials during the seawater incubation experiments with stripped and intact SynS were traced with both CBB and PAS staining (Fig. 5A and B). Initially, intact SynS cells added to seawater (SynSintact at day 0, Fig. 5A) had a protein distribution identical to intact cells, including the 130 kDa S-layer protein (Fig. 1A). In seawater with stripped SynS cells added (SynSstripped at day 0, Fig. 5A), however, only two major proteins were identified, implying that a large amount of proteins was lost when the cells were resuspended in seawater. In the incubations of both stripped and intact SynS cells, all proteins were decomposed within 15 days. Glycoproteins, including S-layer, disappeared at the same time as other proteins (Fig. 5B). No evidence for preferential preservation of glycoproteins, including S-layer, was observed during the 30-d incubations.

3.5. Changes in cyanobacteria morphology and bacterial abundance over time

Synechococcus cell morphology was visualized after 2 days of incubation using transmission electron microscopy (TEM). Intact cells had a characteristic elliptical shape and spread evenly on the TEM grid (Fig. 1B); in comparison, the degraded cells tended to aggregate (Fig. 6). In incubations of both stripped and intact cells of both *Synechococcus* species, the cellular material lost integrity and was no longer distinct after 2 d, which indicates a change in cell morphology for all four cases. Cell membranes were still visible in all cases (see arrows in Fig. 6B–D), except in cells of SynSstripped with S-layer stripped off. This difference in appearance between Fig. 6A and B–D is consistent with the stripping treatment having removed the outer membrane from SynS, but not from the SynNO cells, which had no S-layer. Cell morphology changed dramatically at the beginning of cell degradation, consistent with the idea that cell lysis is the first step of cell degradation.

Bacterial abundance, as measured by epifluorescence microscopy after DAPI staining, varied from 1.4×10^5 to 1.1×10^7 cells mL^{-1} with the highest cell numbers on Day 2 in all four incubations (Fig. 7). SynNOstripped incubations had the highest cell abundance on Day 2, followed by SynNOintact, SynSintact and SynSstripped. The bacterial abundance of SynSintact was always higher than that of SynSstripped, while SynNOstripped was higher than SynNOintact on Day 2, after which the bacterial abundances were similar for the rest of the incubations.

Contribution of live heterotrophic bacteria to POC was evaluated based on the bacterial abundance and an estimated cellular carbon content of heterotrophic bacteria (6.3 fg C $cell^{-1}$, Kawasaki et al., 2011) as well as POC from the PAA (PAA/POC = 0.54, Kawasaki et al., 2011). A live bacterial contribution of 0.1% to 0.3% was observed in intact cell degradation, while the bacterial contribution in stripped cell degradations ranged from 0.1% to 0.7%. The result suggests that live heterotrophic bacteria cell content accounts for a very small portion of particulate organic carbon throughout the 30-day incubations compared with cyanobacterial cells and any coastal organic matter introduced initially.

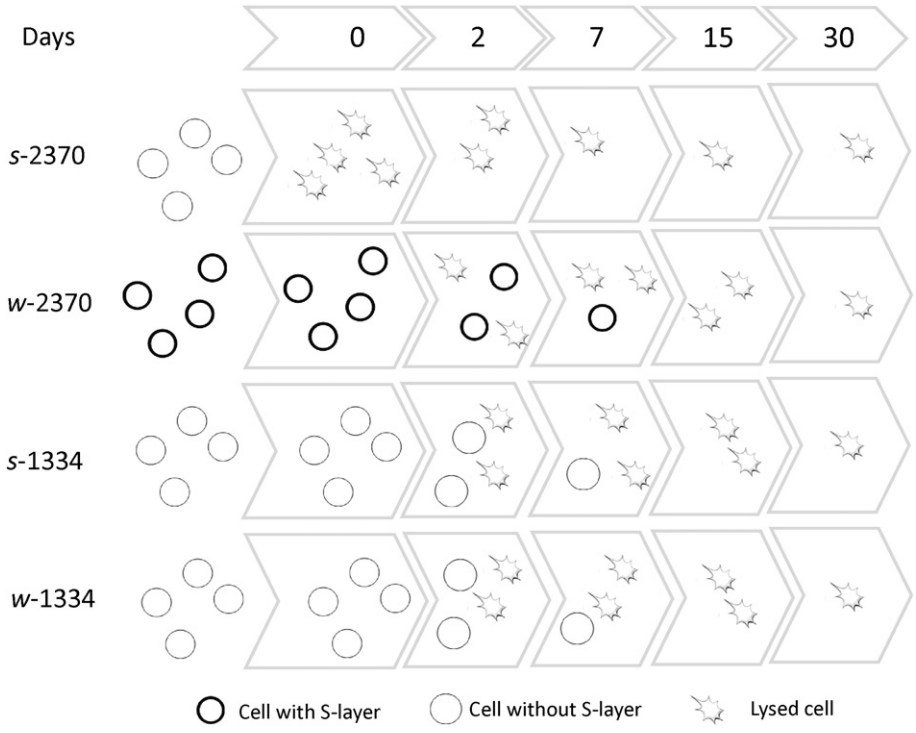

Fig. 8. Mechanism of early degradation of intact and stripped cells of *Synechococcus* CCMP2370 and CCMP1334 in the coastal seawater. At the beginning of incubation, only SynSintact has S-layer structures in the cell membrane (showed as black open circles), because S-layer in SynSstripped has been chemically removed, and SynNOstripped and SynNOintact were inherently absent in S-layer (showed as gray open circles). SynSstripped cells were lysed directly after rinsed with seawater (showed as asterisks), while SynSintact, SynNOstripped and SynNOintact still maintained cell shapes at 0-day, though they were subject to slower cell lysis in the following incubations from 2-days to 15-days. Lysed cells from all the four cases can be rapidly degraded so that cells in SynSstripped degraded faster than the other three incubations.

4. Discussion

4.1. Removal of S-layer by stripping treatment

McCarren et al. (2005) showed that S-layer proteins assemble to form a regularly arranged layer, and together with a polysaccharide sheath, cover the outer cell membrane of *Synechococcus*, effectively isolating the cell from ambient seawater. S-layer structure as a defensive barrier in live cells has been widely discussed (Sleytr et al., 1999; Smarda et al., 2002), and specifically emphasized for many Archaea, in which S-layer is a universal cell wall structure (Baumeister and Lembcke, 1992; Konig, 1988). Hence, intracellular materials are inaccessible to extracellular enzymes unless the S-layer is punctured or removed. In our experiments, the S-layer was removed from SynS cells with stripping buffer. The effectiveness of this removal is apparent from both TEM observation and SDS-PAGE gel analysis (Fig. 1). EDTA in the stripping buffer we used can chelate ions like Mg^{2+} or Ca^{2+} that are located at the center of individual subunits of the S-layer and stabilize the S-layer ultrastructure (Engelhardt and Peters, 1998). Removal of these ions from the S-layer structures by chelation is a likely cause of the disruption of S-layer structure, since previous studies suggest that subunits of S-layer are held together (and held to the underlying cell membrane structure) by weak non-covalent hydrophobic bonds and ionic interactions (Koval and Murray, 1984). However, EDTA apparently lacks the capacity to damage the deeper lipid bilayer and peptidoglycan membrane structures, since electron microscopy demonstrated that the stripping buffer effectively removed the outer membrane of SynS, but caused no visible damage to the membrane of SynNO, which has no S-layer. A comparison of stripped and intact cells provides us an opportunity to assess the importance to cell decomposition of the S-layer, particularly with regard to its conformation and its interaction with adjacent cellular components. This information is usually absent from studies of the purified S-layer protein itself.

4.2. Massive cell lysis caused by removal of S-layer

Given our understanding of the nature of the *Synechococcus* S-layer, we expected that the only difference between SynSstripped and SynSintact incubations at day 0 should be the absence of the S-layer protein in SynSstripped. However, molecular evidence shows that particulate amino acid concentrations per cell at day 0 were much lower in the stripped than the intact incubation, suggesting a loss of large amounts of cellular materials from individual stripped cells. This was also supported by the gel electrophoresis data, in which not only S-layer, but also many other proteins were missing at the beginning of stripped cell incubations (Fig. 5). Moreover, the PAA lost from stripped cells were not found in the TDAA, indicating that this portion of cellular material had been removed before resuspension of stripped cells in coastal seawater. Since only S-layer was removed after the stripping treatment, the cellular materials lost at the beginning of the SynSstripped incubation must have been removed when the stripped cells were rinsed with artificial seawater prior to resuspension in coastal seawater.

Thus the question: why did stripped cells maintain their intact shapes in the stripping buffer, but be lysed in artificial seawater? One plausible explanation is that the cell membrane was weakened by S-layer removal; the thinner membrane beneath the S-layer may not have been strong enough to maintain the cell structure under the osmotic difference between seawater and intracellular cytoplasm, in which the ionic strength is close to stripping buffer (Fig. 8). As a result, stripped cells may have been lysed, and cellular proteins removed by the artificial seawater before resuspension in the coastal seawater (containing its natural bacterial community), while intact cells protected by the S-layer structure were robust, and remained intact until released into the coastal seawater. A second possibility is that many membrane proteins are associated with the S-layer, so that removal of the S-layer structure caused a rapid change in or loss of these membrane proteins. For example, alkaline phosphatase has been found to detach from the cell membrane during proteomic extraction of *Synechococcus* (Saito et al., 2011). This was thought due to the change of S-layer structure during the extraction procedure. The different behaviors in stripped and intact cells are consistent with the idea that S-layer structure is similar to peptidoglycan in preventing cell lysis and in having other membrane proteins located on its unique ultrastructure.

4.3. More intensive degradation of stripped cells

Besides the initial differences between stripped and intact cells, the degradation behavior of cells with and without stripping treatment is also different. First, the degradation index (DI) shows that stripped cells experienced more intensive changes in PAA composition (Fig. 3) during the incubation, indicating that stripped cells were subject to more intensive degradation. Although the initial indices of SynSstripped and SynSintact were dissimilar, the decomposition processes in both incubations should be controlled by the same mechanism: the alteration of organic composition primarily by heterotrophic degradation by the same microbial community. Thus the change of DI during the incubations is a valid comparative indicator of degradation state in our experimental setting. Second, higher %D was observed later in stripped cell incubations; in particular, the relative abundance of D-ALA was found to increase progressively during the degradation (Table 2 and Fig. 4A, B). This phenomenon was much less obvious in incubations of intact cells, suggesting that D-amino acids were either produced or preferentially preserved in the particles of stripped cells. D-amino acids are found in both photosynthetic and heterotrophic bacterial cells due to their presence in peptidoglycan, teichoic acids, lipopeptides and some siderophores (Schleifer and Kandler, 1972; Kaiser and Benner, 2008 and references therein). The growth of heterotrophic bacteria may not be the major reason for the larger amount of D-amino acids in stripped cells considering that the contribution of bacteria to the total amino acids was <1% (Table 2), and that the pattern of bacterial numbers during the incubation (Fig. 7) did not mirror the increase in D-amino acids (Table 2). Thus, the greater increase in D-amino acids in stripped cell than in intact cell incubations most likely results from preferential accumulation. Some D-amino acid containing structures that are present in stripped cell incubations, e.g., peptidoglycan, add rigidity to the cell membrane and are selectively accumulated during decomposition of bacterially derived organic matter (Nagata et al., 2003).

Higher total %D in TDAA can be observed in the later samples of SynSstripped incubation relative to SynSintact (Table 2). Individual D-amino acids generally agree with this trend but with exceptions, e.g., the high %D-GLU and D-ALA at the beginning of SynSintact incubations (Fig. 4C and D). However, this difference between stripped and intact samples is less obvious between SynNOstripped and SynNOintact incubations, and the %D of individual amino acids shows somewhat different patterns (Fig. 4E and F). The D-amino acids in seawater are thought to originate from peptidoglycan remnants, or as the building blocks in the biosynthesis of peptidoglycan (McCarthy et al., 1998; Kawasaki and Benner, 2006), so that the progressive increase in D-amino acids during the incubations may result from either degradation of cyanobacterial cell walls or biosynthesis of heterotrophic bacterial walls along with the cell degradation. Therefore, the relative increase of D-amino acids in stripped vs. intact cell incubations is consistent with the speculation that stripped cells are more intensively decomposed. However, the inconsistent results observed in SynNOstripped and SynNOintact incubations implies that the behavior of D-amino acid during the cell degradation is more complicated, and a more detailed survey is required to link dissolved D-amino acid behavior to degradation.

4.4. Possible mechanisms for preservation of cyanobacterial cells by S-layer structures

4.4.1. S-layer as a glycosylated protein

Post-translational modifications like glycosylation have been discussed as important processes modifying the rigidity of proteins, particularly those associated with cell membrane structures (Upreti et al., 2003). The geochemical importance of glycosylation has also been investigated (Keil and Kirchman, 1993; Tsukasaki and Tanoue, 2010), which suggests that individual glycoproteins of great stability must exist, or that there are other processes involved in the accumulation of these glycopeptides in marine environments (Philben and Benner, 2013). Using synthetic peptides, Liu et al. (2010) found no difference in resistance to degradation between glycosylated and non-glycosylated tetrapeptides in a coastal environment, implying that mechanisms involved in the glycoprotein preservation in marine environments are complex.

Though S-layer protein is one of the most abundant glycoproteins in *Synechococcus* cells, we found no clear evidence that S-layer was better preserved than other major proteins based on PAS staining results and amino acid analysis of degrading cells (Fig. 5B). Indeed, based on the protein distribution from gel electrophoresis, the difference between S-layer protein and other major proteins during the incubation was much less than the difference between that of stripped and intact cells. The difference between stripped and intact cells resulted mostly from rapid loss of cellular materials without the protection of S-layer structure. The similarity between changes in glycoproteins and other proteins during degradation suggests that glycoprotein and other proteins were decomposed in a similar fashion. Thus, our results imply that selective preservation of glycoprotein is not as important as physical protection by S-layer in regulating the degradation of cellular materials of cyanobacteria. Previous studies have shown that glycans, the more hydrophilic part of S-layer protein, tend to be located at the outside of the S-layer structure and to face the ambient aquatic environment, while the underlying peptide part is more hydrophobic; this helps the S-layer better associate with other membrane structures (Schäffer and Messner, 2001). These S-layer glycans play a role in preventing the enzymatic hydrolysis of underlying peptide conjugates (Sleytr et al., 2014). Therefore, the rapid decomposition of S-layer together with other cellular materials may result from the intensive cell lysis that occurs during initial degradation, and which damages the integrity of S-layer structure so that S-layer glycan can no longer defend the cell from enzymatic attack. In this sense, our study of S-layer emphasizes the importance of the integrity of membrane glycoprotein ultrastructure in regulating degradation at the early stage of diagenesis.

4.4.2. Comparison of S-layer to other prokaryotic membrane structures

Bacterial cell membranes are thought to preserve bacterial organic matter as a result of the recalcitrant nature of some membrane components, e.g., lipid bilayer, membrane protein and peptidoglycan, or of their ability to protect other molecules from decomposition. Evidence of the refractory nature of peptidoglycan can be seen in its slower degradation rate in seawater incubation experiments (Nagata et al., 2003) and in the presence of peptidoglycan fragments in marine organic matter from various marine environments (Lee and Bada, 1977; McCarthy et al., 1998; Kaiser and Benner, 2008). The lipid bilayer is characterized by its high hydrophobicity, which repels water and protects associated components from enzymatic attack (Nagata et al., 1998). Membrane proteins like porin and FTR1, either imbedded inside or anchored on the bilayer, are relatively more hydrophobic, and may be less accessible to hydrolytic enzymes (Tanoue et al., 1995).

However, S-layer has a very different nature from the membrane proteins associated closely with either lipid bilayer or peptidoglycan. The S-layer overlays most other membrane structures except the polysaccharide sheath at the outermost surface. Therefore, S-layer is relatively independent from other membrane structures (McCarren et al., 2005). This may explain why removal of S-layer does not apparently damage other membrane structures, nor can S-layer be protected by other membrane structures. Besides the contribution of its glycosylated structure, the rigidity of S-layer is largely a result of its unique 3-D structure, i.e., how S-layer proteins are assembled together and cover the outermost surface of cells (Engelhardt and Peters, 1998). On the other hand, our work suggests that S-layer structure can slow the degradation of other cellular materials. It has been found that peptidoglycan is often thinner in gram-positive bacteria with S-layer (Sleytr and Glauert, 1976), suggesting that S-layer has a similar function to peptidoglycan in maintaining cell structure and adding membrane rigidity. This unique assemblage pattern allows S-layer, a relatively labile cellular component, to function in protecting cellular materials from enzymatic attack (Sleytr et al., 2014). Additionally, fine-grain gypsum ($CaSO_4$ $2H_2O$) and calcite ($CaCO_3$) can deposit on the S-layer structure of freshwater cyanobacteria, another way S-layer structure regulates prokaryotic biogeochemical processes in aquatic environments (Schultze-Lam et al., 1992).

4.4.3. Importance of S-layer as a defensive barrier during early diagenesis

Although S-layer protein itself shows no higher resistance to decomposition than the majority of proteinaceous materials from *Synechococcus*, several different lines of evidence support the idea that stripping off S-layer proteins caused more intensive degradation of cellular materials. Considering the fact that stripping off S-layer structure caused massive cell lysis and removal of cytoplasm as depicted schematically in Fig. 8, we speculate that cell lysis exposed the intracellular materials to the ambient environment, so that they were more available to hydrolytic enzymes, and thus subject to more intensive degradation. On the other hand, the intact cells were better protected by the intact membrane structure. One limitation of this study is that this work was conducted once in the lab, whereas decomposition of prokaryotic cells can be impacted greatly by the highly dynamic and variable geochemical processes in natural environments. Moreover, the mechanism of CCMP1334 (SynNO) degradation was unclear. Thus, degradation incubations with sufficient coverage and replication should be used in future work.

Overall, our study on the S-layer in cyanobacteria suggests a novel protective mechanism of prokaryotic cellular structure that has seldom been explored from a geochemical perspective. Even though S-layer proteins are easily decomposed, and post-translational glycosylation apparently has limited impact on protecting S-layer proteins from degradation, the unique planar ultrastructure of the S-layer and its location overlying most other membrane structures appears to slow the degradation of prokaryotic cellular materials, most likely by preventing cell lysis and limiting the access of enzymes to cellular materials inside the cell. In contrast, it appears that the rigidity of individual biomolecules plays a less important role in controlling the early degradation of prokaryotic cells. This finding tempers our traditional view of proteins as a major component of the most labile organic matter during early diagenesis in the ocean. With proper organization or assemblage, labile organic matter like proteins can also build up a robust defensive barrier not only to maintain the regular cellular physiology but also to protect the cells from decomposition after cell death. This feature of membrane glycoproteins like S-layer in regulating the degradation of prokaryotes by physical protection may thus help preserve cyanobacterial cellular materials so that they can be delivered to the deep ocean (e.g., Dong et al., 2010; Lomas and Moran, 2011).

A better understanding of the S-layer is particularly critical for those prokaryotes like Archaea, in which S-layer is a universal cell wall component instead of peptidoglycan (Baumeister and Lembcke, 1992; Konig, 1988), the function of which has been poorly understood in the oceans (Ingalls et al., 2006; Karner et al., 2001).

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