The ecological role of a proteolytic psychrophile, \textit{Flavobacterium limicola} belonging to the \textit{Cytophaga-Flavobacteria} cluster, in the decomposition of particulate organic nitrogen in freshwater sediment

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Microbes of the \textit{Cytophaga-Flavobacterium} (CF) group, within the phylum \textit{Bacteroidetes}, have been considered contributors to the early decomposition of particulate organic matter (POM) in sediments because of their abundance in the ecosystem and their great ability to degrade macromolecules on artificial media. However, there is no report on the ability of members of this group to decompose POM in sediment. We investigated the POM-decomposition capabilities of members of the CF group, \textit{Flavobacterium limicola} strains ST-82$^T$, ST-10 and ST-92 isolated from freshwater sediment as proteolytic psychrophiles, in sterilized sediment slurries. All strains of \textit{F. limicola} grew and survived at over $10^8$ cfu per ml for more than 30 days in the sediment slurries at 5°C. Protease activity levels in the slurries inoculated with the strains were 3–5 times higher than the control level for over 30 days of incubation at 5°C. Concentrations of total dissolved nitrogen (TDN) released by the hydrolysis of particulate organic nitrogen (PON), significantly increased only in the slurries inoculated with \textit{F. limicola} strains. Approximately 70–80% of the TDN released was converted to NH$_4^+$-N in these inoculated slurries. The results clearly demonstrate that \textit{F. limicola} strains ST-82$^T$, ST-10 and ST-92 are able to secrete an extracellular protease and hydrolyze PON to TDN and thereby mineralize TDN to NH$_4^+$-N in freshwater sediment. This is the first report on the decomposition and mineralization of PON by members of the CF group in benthic environments.

Key words: \textit{Cytophaga-Flavobacterium} group, decomposition, particulate organic nitrogen (PON), freshwater sediment

The mineralization of organic matter in sediments is an important component of carbon, nitrogen and nutrient cycles in aquatic environments. The process is important for the self-purification of these environments and causes the benthic regeneration of key nutrients for primary production through the diffusion of inorganic compounds such as nitrogen and phosphorus to the overlying water column, particularly in shallow eutrophic lakes and marines\cite{19,27,28}. Thus, the decomposition of organic compounds in sediments has a great influence on aquatic ecosystems.

The conversion of particulate organic matter (POM) to dissolved organic matter (DOM) is generally considered the first and rate-limiting stage in the mineralization process\cite{12,21}. This process is catalyzed by extracellular macromolecule-hydrolyzing enzymes. Hence, a number of studies on enzymatic decomposition of POM in sediments have been reported to date\cite{6,12,16,17,20}. However, functionally important microbes, the providers of those enzymes, have not been identified and characterized in detail.

Members of the \textit{Cytophaga-Flavobacterium} (CF) group within the phylum \textit{Bacteroidetes} are now considered sig-
nificant contributors to organic matter decomposition in natural environments for the following reasons: (i) most previously cultivated species within the CF group have had the capability to hydrolyze various macromolecules such as polysaccharides and polypeptides, and (ii) they are abundantly found in a wide variety of ecosystems, based on molecular ecological techniques. Recent studies revealed that members of the CF group were also found in significant numbers in benthic environments and became dominant in response to an input of organic substrates in the ecosystems. These findings imply that they play a key role in the primary decomposition of organic matter in sediments. However, there is no report showing directly their ability to degrade POM in sediment.

We previously isolated novel organic polymer-degrading bacteria, Flavobacterium limicola strains ST-82, ST-10 and ST-92, belonging to the CF group from freshwater sediment. These strains are psychrophilic and freshwater bacteria that can particularly hydrolyze proteinous compounds. In addition, they utilize amino acids as the best substrate for their growth. Therefore, we estimate that they may be of importance as degraders of particulate organic nitrogen (PON) in freshwater sediments during the low temperature seasons. In this study, we aimed to clearly demonstrate their ecological role, to degrade and mineralize PON, in freshwater sediment.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains used in this study were Flavobacterium limicola strains ST-82, ST-10 and ST-92 isolated from freshwater sediment. Cultivation media for these strains were PM medium, a tenth-strength NB (nutrient broth, Difco Laboratories, Detroit, USA) medium, and 1% and 0.1% skim milk media.

The strains were routinely cultured on PM agar or 0.1% skim milk agar. Liquid cultivation was performed in 300-ml Erlenmeyer flasks containing 100 ml of a medium under rotary agitation (200 rpm) at the desired temperatures. Growth was monitored by measuring optical density at 600 nm with a spectrophotometer.

Determination of protease activity

Protease activity was assayed by using the dye-labeled particular protease substrate, HPA (Hyde Powder Azure, Sigma, St. Louis, MO). The reaction was performed at 40°C for 30 min in a 2.0 ml tube containing 11.2 mg of HPA, 0.5 ml of enzyme solution and 1.0 ml of 20 mM Tris-HCl buffer (pH 8.0). After centrifugation (6,000×g for 10 min at 4°C), the absorbance of the soluble stained hydrolyzate was determined at 600 nm.

Characterization of extracellular protease activity

For enzyme secretion, bacterial strains were cultured in 0.1% skim milk medium at 15°C for 5 days. After centrifugation (8,000×g for 15 min at 4°C), the culture supernatants were filtrated with 0.2 μm membrane filters.

The extracellular protease activity in the enzyme solution was assayed at different pHs ranging from 3 to 12. The buffers used were the following: for pH 3.0 to 6.0, 20 mM sodium citrate-NaOH buffer; for pH 6.0 to 8.0, 20 mM sodium phosphate buffer; for pH 7.5 to 8.5, 20 mM Tris-HCl buffer; for pH 8.5 to 12.0, glycine-NaOH buffer; and for pH 11.0 to 12.0, sodium phosphate-NaOH buffer.

To test the effect of temperature on protease activity, the assays were performed at 20, 30, 40, 50, 60, 70 and 80°C in 20 mM Tris-HCl buffer (pH 8.0).

For inhibition studies, protease inhibitors used in this study were 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 1 mM pepstatin (Sigma), 1 mM leupeptin (Sigma) and 1 mM EDTA (Wako, Osaka, Japan). The mixture of each protease inhibitor and enzyme solution was incubated in 20 mM Tris-HCl (pH 8.0) at 40°C for 15 min. Protease activity was measured as described above.

PON-decomposition test in sterilized sediment slurry

a) Sterilized sediment slurry

Sediment samples were collected from a shallow eutrophic lake, Kasumigaura, Japan (November 1998). Sterilization of the sediment was performed by irradiation with 5.0 Mrad from a 60Co source after freeze-drying treatment. A sterilized 500-ml Erlenmeyer flask, which contained 10 g of the sterilized sediment and 300 ml of filter-sterilized lake water collected at the time of sediment sampling, was inoculated with 20 ml of bacterial suspension in the lake water. Bacterial strains were freshly precultured in the 0.1% skim milk medium. Incubation of the sediment slurries was carried out on a rotary shaker (100 rpm) at 5°C.

b) Monitoring of growth and protease production

Growth of the inoculated strains in the sediment slurry was monitored by the plate counting method using 1% skim milk agar plates incubated at 5°C.

Protease production by the inoculated strains in the sediment slurry was monitored with the HPA assay. Enzyme solution was extracted from 5 ml of the sediment slurry using 5 ml of extraction buffer (2% Triton X-100 containing...
2 g/l polyvinylpyrrolidone in 10 mM Tris-HCl) at 30°C for 1h. After centrifugation (6,000×g for 15 min at 30°C), 1.6 ml of the extract was incubated with 11.2 mg of HPA for 20 h. The incubation temperature was adjusted to 30°C for sensitive detection of the protease secreted by the strains. After centrifugation (6,000×g for 10 min at 4°C), the absorbance of the supernatant was determined at 600 nm.

c) Chemical analysis

The ability of our isolates to degrade PON was evaluated from the amount of dissolved nitrogen compounds released as a result of the decomposition of PON in sediment. After centrifugation (3,000×g for 15 min at 4°C) of the slurry sampled from the experimental systems, a water sample was prepared with a 0.2 μm membrane filter. Concentrations of dissolved ammonium ion (NH₄⁺), and nitrite and nitrate ions (NOₓ⁻) in the water sample were analyzed by ion chromatography (TRAACS-II, Bran Luebbe, Hamburg, Germany). The total dissolved nitrogen (TDN) concentration was measured by ion chromatography after alkaline potassium peroxodisulfate digestion.

The bioavailable nitrogen in sediment was determined as enzymatically hydrolyzable nitrogen (EHN) by direct digestion with the proteolytic enzyme. The analytical method for enzymatically hydrolyzable amino acids (EHAA) was previously established by Mayer et al. to estimate bioavailable nitrogen, humic-like substances contained in sediment samples. We slightly modified their method in order to measure the bioavailable nitrogen in sediment more easily and rapidly. The procedure of direct digestion with proteinase-K was almost the same, but our method differs from their method in measuring enzymatically hydrolyzable nitrogen as the released TDN concentration instead of amino acids. A 2.0-ml tube containing 0.10 g of freeze-dried sediment and 1.35 ml of sodium phosphate buffer (pH 8.0) was incubated with 0.15 ml of proteinase-K (1 mg/ml) for 6 h at 40°C. After centrifugation (6,000×g for 10 min at 5°C) and filtration with a 0.2 μm membrane filter, the released TDN in the water sample was determined as described above.

Results and Discussion

Effect of culture conditions on extracellular protease production by F. limicola

The effects of the composition of the media on the extracellular protease production by F. limicola strains ST-82T, ST-10 and ST-92 were investigated using PM medium, 1/10 NB medium and 1% and 0.1% skim milk media. The 0.1% skim milk medium gave the highest level of protease activity in culture broth per cell density (OD₆00) (data not shown). The strains were therefore cultivated using the 0.1% skim milk medium for enzyme secretion.

The effect of three different incubation temperatures (5, 15 and 23°C) on protease production was evaluated. Interestingly, although the optimum growth temperature of each strain was in the range of 15 to 20°C, maximum protease production (protease activity per cell density) was exhibited at the lowest temperature, 5°C (Fig. 1). In addition, protease production tended to decrease as the temperature increased to 15 and 23°C (Fig. 1). Feller et al. and Russell implied in their reviews that such a regulation of protease secretion dependent on growth temperature is a common feature of

![Fig. 1. Effect of growth temperature on protease production by F. limicola, strains [a] ST-82T, [b] ST-10 and [c] ST-92.](image-url)
psychrophilic microorganisms. Our results support this speculation.

**Enzyme properties**

Extracellular proteases from *F. limicola*, strains ST-82, ST-10 and ST-92, had similar biochemical traits (the results for strain ST-82 are shown as representative data in Fig. 2). The optimum temperature for extracellular protease activity was around 50°C (Fig. 2 [a]). The optimum pH was approximately 8.0 to 9.0 (Fig. 2 [b]). The enzyme activities were detected at pH 6.2 to 11.6. The enzyme inhibitors were tested for the ability to block the hydrolysis of HPA. The protease activity was remarkably inhibited (less than 10% activity) by the chelating agent EDTA (Fig. 2 [c]). Other inhibitors did not significantly affect the activity. This result indicates that the extracellular protease produced by *F. limicola* can be classified as a metalloprotease.

**Growth of and protease production by F. limicola in sediment slurry**

The growth and protease levels of *F. limicola* strains ST-82, ST-10 and ST-92 were monitored in sediment slurries at 5°C. All strains grew in 7 days, and their CFU counts reached $2.51 \times 10^8$, $2.20 \times 10^8$ and $2.04 \times 10^8$ cfu per ml, respectively (Fig. 3 [a]). They survived for another 20 days at over $10^8$ cfu per ml after they reached the stationary phase. Protease activity levels in sediment slurries inoculated with ST-82, ST-10 or ST-92 were 3–5 times higher than the control level after 7 days of incubation (Fig. 3 [b]). These higher levels were maintained for 30 days. The findings
indicate that *F. limicola* strains ST-82, ST-10 and ST-92 are able to produce extracellular protease in freshwater sediment at significantly low temperature (5°C), and to grow and survive by utilizing substrates hydrolyzed with the enzyme.

**PON-decomposition ability of *F. limicola* in sediment slurry**

The abilities of *F. limicola* strains ST-82, ST-10 and ST-92 to degrade and mineralize PON contained in sediment were evaluated by monitoring the concentrations of TDN, NH$_4^+$-N and NO$_x$-N released as a result of the decomposition of PON. TDN concentrations in the sediment slurries inoculated with ST-82, ST-10 or ST-92 notably increased for 10 days (Fig. 4 [a]). The amount of TDN released from PON in the slurries inoculated with ST-82, ST-10 and ST-92 reached 7.05, 7.29 and 6.24 mgN per liter after 30 days of incubation. No significant accumulation of TDN was found in the control. Likewise, the release of NH$_4^+$-N was also observed in the sediment slurries inoculated with ST-82, ST-10 and ST-92, and the amount of NH$_4^+$-N reached 5.27, 5.25 and 5.13 mgN per liter after 30 days (Fig. 4 [b]). No increase in the NH$_4^+$-N concentration was found in the control. The time course of NO$_x$-N release was almost identical in all experimental systems, and the amount released was only 0.4–0.5 mgN per liter after 30 days (Fig. 4 [c]).

These findings clearly suggest that *F. limicola* strains ST-82, ST-10 and ST-92 have the capability to degrade PON in sediment at low temperatures. In fact, the release of TDN corresponded to the production of protease in the sediment slurries inoculated with *F. limicola*. In addition, approximately 70–80% of the TDN released was in the form of NH$_4^+$-N after 30 days of incubation. Such a release of dissolved nitrogen compounds was not found in the sediment slurries inoculated with *F. limicola* strains at 25°C (data not shown). Therefore, the strains ST-82, ST-10 and ST-92 are able not only to hydrolyze PON to TDN with their extracellular protease but also to mineralize TDN to NH$_4^+$-N in freshwater sediment particularly at low temperatures.

To evaluate how these strains degrade and mineralize PON in sediment, the concentration of enzymatically hydro-

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**Table 1. Percentages of the amount of TDN or NH$_4^+$-N released by *F. limicola* strains ST-82, ST-10 and ST-92, as enzymatically hydrolyzable nitrogen (EHN) in sediment after 30 days of incubation at 5°C.**

<table>
<thead>
<tr>
<th>Inoculated strain</th>
<th>TDN [mgN/g-dgw]</th>
<th>TDN/EHN [%]</th>
<th>NH$_4^+$-N/EHN [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST-10</td>
<td>0.233</td>
<td>58.3</td>
<td>42.0</td>
</tr>
<tr>
<td>ST-82$^T$</td>
<td>0.227</td>
<td>56.4</td>
<td>42.3</td>
</tr>
<tr>
<td>ST-92</td>
<td>0.130</td>
<td>49.9</td>
<td>41.0</td>
</tr>
</tbody>
</table>
lyzable nitrogen (EHN) in the sediment used was determined. The release of TDN by enzyme digestion was evident in 6 h (data not shown), and reached a maximum value of 0.40 mgN per g-dry weight of sediment. This value accounted for 7.5% of all nitrogen in the sediment, and was not significantly different from the previously reported result of approximately 10%\(^{18}\). The amount of PON decomposed and mineralized by \textit{F. limicola} was evaluated as a percentage of TDN or NH\(_4\)-N to EHN, as summarized in Table 1. These results suggest that the strains ST-82\(^{1}\), ST-10 and ST-92 are able to hydrolyze approximately 50–60% of PON in sediment to TDN, and to mineralize about 40% of PON in sediment to NH\(_4\)-N.

**Conclusion**

In this study, we clearly demonstrated that psychrophilic, organic polymer-degrading bacteria, \textit{F. limicola} strains ST-82\(^{1}\), ST-10 and ST-92, had the ability to secrete an extracellular protease and hydrolyze PON to TDN, and then to mineralize TDN to NH\(_4\)-N in freshwater sediment slurries incubated aerobically at low temperatures. The function of proteolytic psychrophiles such as \textit{F. limicola} to aerobically decompose and mineralize PON play an ecologically important role in the sediment of shallow eutrophic lakes such as Lake Kasumigaura for the following reasons: (i) temperatures in freshwater sediments are 4–18°C, which are appropriate for the metabolic activity of psychrophiles, during the autumn-winter-spring seasons, three fourths of the year\(^{2,27}\); (ii) a few centimeters of the sediment is kept relatively oxic during these seasons\(^{13,15,16,18}\); (iii) the POM content of sediments frequently increases in early autumn due to sedimentation of POM derived from a number of blue-green-algae that grow during the summer\(^{11}\); (iv) the most common form of PON found in sediment is proteinaceous compounds such as polymerized amino acids or peptides\(^{8,11,16-18}\).

Recent molecular ecological studies have revealed that members of the \textit{Cytophaga-Flavobacterium} group are the main constituents of microbial communities in a wide variety of ecosystems including benthic environments\(^ {13-15,20,24}\). Researchers had speculated that microbes of the CF group play an important role in the early decomposition of POM in ecosystems because of their great ability to degrade macromolecules on artificial media\(^{5,11,22}\). However, to date, there had been no study confirming their ability to degrade POM in sediments. This is the first report to reveal the PON-decomposing and -mineralizing abilities of members of the CF group in freshwater sediment. Some members within this group may have great affinity for complex organic compounds in freshwater sediment. In fact, we found that the cultivation of sediment agar plates inoculated with the freshwater sediment sample brought about preferential growth of members closely related to \textit{F. limicola} (over 70% of colonies selected) (data not shown). This finding supports the speculation described above. More investigations are needed to evaluate their function in situ, that is the decomposition or mineralization of POM, in benthic environments through a molecular ecological approach.

**References**