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Discovery of a novel alginate lyase from *Nitratiruptor* sp. SB155-2 thriving at deep-sea hydrothermal vents and identification of the residues responsible for its heat stability

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Running title: *NitAly, a novel alginate lyase from a deep-sea bacterium*

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ABSTRACT

Extremophiles are expected to represent a source of enzymes having unique functional properties. The hypothetical protein NIS_0185, termed NitAly in this study, was identified as an alginate lyase-homolog protein in the genomic database of ε-Proteobacteria *Nitratiruptor* sp. SB155-2, which was isolated from deep-sea hydrothermal vents at a water depth of 1,000 m. Among the characterized alginate lyases in the polysaccharide lyase family 7 (PL-7), the amino acid sequence of NitAly showed the highest identity (39%) with that of red alga *Pyropia yezoensis* alginate lyase PyAly. Recombinant NitAly (rNitAly) was successfully expressed in *Escherichia coli*. Purified rNitAly degraded alginate in an endolytic manner. Among alginate block types, PolyM was preferable to polyG and polyMG as a substrate, and its end degradation products were mainly tri-, tetra-, and penta-saccharides. The optimum temperature and pH were 70ºC and around 6, respectively. A high concentration of NaCl (0.8–1.4 M) was required for maximum activity. In addition, a 50% loss of activity was observed after incubation at 67ºC for 30 min. Heat stability was decreased in the presence of 5 mM DTT, and Cys80 and Cys232 were identified as the residues responsible for heat stability, but not lyase activity. Introducing 2 cysteines into PyAly based on homology modeling using *Pseudomonas aeruginosa* alginate lyase PA1167 as the template enhanced its heat stability. Thus, NitAly is a functional alginate lyase, with its unique optimum conditions adapted to its environment. These insights into the heat stability of NitAly could be applied to improve that of other PL-7 alginate lyases.

Alginate consists of 2 uronic acids, β-ᴅ-mannuronic acid (M) and its C5 epimer α-ʟ-guluronic acid (G) (1, 2). These units are linearly linked by a 1-4 glycosidic bond, and are arranged in an M-consecutive sequence (M-block), G-consecutive sequence (G-block), and M/G random sequence (MG-block). Only a limited number of organisms produce alginate in nature. As a typical example, brown algae biosynthesize alginate as a cell wall component (3), while some types of mucoid bacteria, such as *Pseudomonas* spp. and *Azotobacter vinelandii*, produce alginate as an exopolysaccharide (4).

Alginate lyase enzymes catalyze the cleavage of the 1-4 glycosidic bond of alginate via a β-elimination mechanism (5). These enzymes are found in organisms that have either alginate-metabolizing systems or alginate-biosynthesis systems. In alginate metabolization, endo-type alginate lyase(s) attack alginates and degrade them to oligosaccharides. Then, exo-type alginate lyase(s) release monosaccharides, and 4-deoxy-ʟ-erythro-5-hexoseulose uronic acid (DEH) is generated by non-enzymatic conversion. DEH is reduced to 2-keto-3-deoxygluconate (KDG) by a DEH-specific reductase (6-8), with KDG possibly being metabolized by the Entner–Doudoroff (ED) pathway (9, 10). The ability to degrade alginate is required to synthesize the alginate polymer because *Pseudomonas aeruginosa* does not produce alginate when inactive alginate lyase is expressed (11). In addition, alginate-producing bacteria use this ability to escape from biofilms containing alginate (12). Although a similar alginate biosynthesis process may be present in brown algae, candidate genes for alginate lyases have not been found, even following the completion of genomic analysis (13).

To date, dozens of alginate lyases have been isolated and characterized. According to the CAZy database (http://www.cazy.org/) (14), alginate lyases are structurally classified into 7 polysaccharide lyase families, PL-5, -6, -7, -14, -15, -17, and -18. At present, PL-7 alginate lyases are the best characterized of all classified alginate lyases, and are well documented. In alginate-producing bacteria, the alginate lyases PA1167 from *P. aeruginosa* (15) and AlyA1, AlyA2, and AlyA3 from *A. vinelandii* (16) have been characterized. Many PL-7 alginate lyases have also been identified in alginate-producing bacteria, i.e., *Flavobacterium* sp. (17, 18), *Sphingomonas* sp. (19-21), *Vibrio* sp. (22-26), *Streptomyces* sp. (27-29), *Corynebacterium* sp. (30), *Agarivorans* sp. (31, 32), *Pseudoalteromonas* sp. (33), *Photobacterium* sp. (34), *Klebsiella pneumonia* (35), *Zobellia galactanivorans* (36), *Saccharophagus degradans* (37), and an uncultured bacterium (38). Within the last year, a novel alginate lyase, PyAly belonging to the PL-7 family, was identified from the red alga *Pyropia yezoensis* (39). This discovery was unexpected because *P. yezoensis* is not considered to have any alginate utilization systems. While its physiological role remains obscure, PyAly showed alginate degradation activity in an endolytic manner and its gene was confirmed to be derived from eukaryotic cells, not from contaminating prokaryotic bacteria. Moreover, we noticed that the amino acid sequence of PyAly showed a significant degree of identity with that of the hypothetical protein NIS_0185 (GenBank accession no. YP_001355656) from *Nitratiruptor* sp. SB155-2, a bacterium of the ε-Proteobacteria phylum that was isolated from deep-sea hydrothermal vents at a water depth of 1,000 m (40, 41). In such extreme environments, alginate producers, such as brown algae, are not present and alginate-utilizing organisms have yet to be found. This finding of amino acid sequence similarity led us to question whether the product of NIS_0185 could have alginate-degradation activity.

In this study, the enzymatic properties of the *Nitratiruptor* sp. SB155-2 hypothetical protein NIS_0185, which was termed NitAly, were investigated using recombinant NitAly protein (rNitAly). Our results are expected to reveal the residues responsible for the heat stability of NitAly, which could be used to improve the heat tolerance of PyAly.

RESULTS

Cloning and sequencing of NitAly—NitAly was identified by the analysis of the genomic sequence of *Nitratiruptor* sp. SB155-2, as a protein homolog of the red alga alginate lyase PyAly. This gene was annotated as a hypothetical protein, NIS_0185, in the genomic sequence of *Nitratiruptor* sp. SB155-2 from the NCBI Genome database. The gene was amplified with a set of specific primers (Table 1) using DNA isolated from *Nitratiruptor* sp. SB155-2 as the template. The amplified product was estimated to have a length of about 700 bp, based on agarose gel electrophoresis results, and was sequenced. The nucleotide sequence was completely identical to that of NIS_0185, with 243 amino acids being deduced. Twenty-two residues at the N-terminal were predicted to comprise the secretion signal using the SignalP 3.0 software program (42), and the mature protein was considered to consist of 221 residues with a total molecular weight of 26,692 Da.

A BLAST search showed that the deduced amino acid sequence of NitAly shared a significant degree of identity with *Caminibacter mediatlanticus* hypothetical protein (55%), *Hippea jasoniae* hypothetical protein (46%), and *Nautilia profundicola* predicted alginate lyase (44%) (Fig. 1). Interestingly, these bacteria were also isolated at deep-sea hydrothermal vents (43-45). Among the characterized PL-7 alginate lyases, the amino acid sequence of NitAly showed the highest identity with that of PyAly (39%) (Fig. 1). Lower identities were detected for alginate lyases from deep-sea sediments, i.e., *Agarivorans* sp. JAM-A1m alginate lyase A1m (16%) (31) and *Vibrio* sp. alginate lyase A9mT (20%) (25). Other characterized PL-7 alginate lyases were found to have a relatively low degree of identity with NitAly, e.g., 17% for FlAlyA from *Flavobacterium* sp. UMI-01 and 13% for PA1167 from *P. aeruginosa*. Among the listed sequences in Fig. 1, 13 residues were fully conserved; namely, Arg85, Glu87, Leu88, Arg89, Gln123, His125, Trp214, Tyr220, Phe221, Lys222, Gly224, Tyr226, and Gln228 in NitAly. Of these, Arg85, Gln123, His125, and Tyr226 corresponded to the catalytic residues proposed to exist in some PL-7 alginate lyases, due to their location at an active cleft based on the resolved crystal structures of PL-7 alginate lyases (15, 21).

Alginate degradation activity of NitAly—rNitAly was successfully expressed in *E. coli* using the pCold expression system and purified (Figs. 2A and B). The yield was about 1.2 mg protein per 1 L culture. The molecular weight of purified rNitAly was estimated at approximately 29 kDa under reducing conditions using SDS-PAGE. This value corresponded reasonably well with the calculated mass of rNitAly (29,636 Da) (Fig. 2B, left lane). Under non-reducing conditions, rNitAly appeared at a lower position, at approximately 27 kDa (Fig. 2B, right lane). This result indicated that it has a compact conformation, due to an internal disulfide bond forming via the 2 Cys residues (Cys80 and Cys232) in rNitAly. Therefore, we used PEG-Mal to evaluate the formation of the disulfide bond. PEG-Mal showed that rNitAly migrated less after pretreatment with DTT compared with when no pretreatment was used (Fig. 2C). The number of free thiol groups in rNitAly detected by the 4-PDS method was 0.01 ± 0.01 and 1.4 ± 0.2/rNitAly molecules under non-reducing and reducing conditions, respectively. Thus, Cys80 and Cys 232 primarily form an internal disulfide bond in rNitAly.

We subsequently investigated 9 commercially available substrates for polysaccharide lyases that might be degraded by rNitAly; namely, alginate, pectin, xanthan gum, polygalacturonic acid, heparin, hyaluronic acid, chondroitin sulfate A, chondroitin sulfate B, and chondroitin sulfate C. The substrates were incubated in a solution containing 10 mM sodium phosphate (pH 6.0), 1 M NaCl, 0.1 mg/mL BSA, 0.1 mg/mL rNitAly, and 0.25% (w/v) polysaccharide for 24 h at 50ºC. Then, each mixture was analyzed by TLC, with only alginate producing degradation products (data not shown). Therefore, we used alginate as the substrate for rNitAly. Next, the alginate degradation activity of rNitAly was evaluated based on the rate of decrease in the viscosity of an alginate solution produced by the enzyme reaction. As shown in Fig. 3, the relative viscosity rapidly decreased within 1 h, and declined gradually thereafter. Along with the decrease in viscosity, the absorbance of the reaction mixture at 235 nm increased linearly. Thus, rNitAly might degrade alginate endolytically, with the lyase reaction introducing the double bonds to the degradation products.

The substrate preference of rNitAly was assayed using polyM, polyG, polyMG, and sodium alginate as the substrates (Fig. 4A). polyM was the most preferable substrate for rNitAly, whereas polyG was the least preferable. The activity level of rNitAly against polyMG and alginate was about one-third of that against polyM. Thus, rNitAly may preferably attack consecutive M residues of alginate.

The degradation products of polyM produced by digestion using rNitAly were analyzed by TLC (Fig. 4B). After 8 h, oligosaccharides with a degree of polymerization of 3 and above were generated; however, most substrates remained undigested at this time point. The original substrate spot disappeared, and the main degradation products that were detected included tri-, tetra-, and penta-saccharides after 24 h and later. Disaccharides were not detected, even after 48 h; therefore, the smallest end products of alginate digestion by rNitAly were identified as trisaccharides, whereas the major end products were tetra- and penta-saccharides.

Enzymatic properties of NitAly—To determine the optimum conditions for rNitAly, its alginate lyase activity was assayed using polyM as the substrate under different conditions. rNitAly activity gradually increased as the temperature rose from 10 to 70ºC (Fig. 5A). Maximum activity (1,620 U/mg) was observed at 70ºC.

The effect of pH on rNitAly activity was also assayed. Optimum pH was around 6, and relative activities of 80% or above were observed between pH 5.0 and 6.5 (Fig. 5B). The activity of rNitAly noticeably decreased at pH 7 and above. Thus, rNitAly preferred acid conditions to neutral or alkaline conditions for alginate degradation.

Next, the effect of NaCl concentration on rNitAly activity was investigated (Fig. 5C). Alginate degradation activity was fully activated in the presence of 0.8–1.4 M NaCl; however, its activity was extremely low in the presence of 0.15 M NaCl. The effects of other monovalent cations were investigated. All tested cations (K^+, Li^+, and Cs^+) showed similar activation with Na^+ (Table 2). These finding showed that rNitAly requires a high concentration of monovalent cations for maximum activity.

The effects of divalent cations, trivalent cations, and chelating reagents were examined (Table 3). Among the tested reagents, Sn^{2+}, Pb^{2+}, Fe^{2+}, and Fe^{3+} inhibited rNitAly activity by 84.6%, 81.6%, 68.8%, and 86.1%, respectively. Ni^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+}, Hg^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, Co^{2+}, Al^{3+}, EDTA, and EGTA had no significant effect on rNitAly activity. Although Hg^{2+} is a blocking agent for cysteine thiol groups, it did not inhibit rNitAly activity. Thus, the disulfide bond in rNitAly (Figs. 1 and 2) is not responsible for alginate degradation through the lyase reaction.

Effects of temperature and pH on NitAly stability—The heat stability of rNitAly was investigated by measuring the residual alginate lyase activity of rNitAly after incubation at different temperatures for 30 min (Fig. 6A). Heat treatment did not affect rNitAly activity between 0 and 30ºC. The loss of activity was observed after incubation at 40ºC and above, and reached 50% after incubation at 67ºC for 30 min. Interestingly, rNitAly showed residual alginate lyase activity of about 10% even after treatment at 100ºC for 30 min.

Extending the duration of heat treatment of rNitAly at 20 and 30ºC to 16 h slightly decreased its residual activity (Fig. 6B). After incubation for 16 h under these conditions, rNitAly still showed over 90% of its original activity, while its residual activity was reduced to about 70 and 20% by incubation at 40 and 50ºC, respectively, for 16 h.

The pH stability of rNitAly was investigated by measuring its residual activity after incubation under different pH conditions for 8 h at 4ºC (Fig. 6C). rNitAly exhibited the highest stability at pH 5. The residual activity of rNitAly significantly decreased at pH 4 and 7–10.

Effects of DTT on the optimum temperature and heat stability of NitAly—While measuring the alginate lyase activity of NitAly at 30ºC, we noticed that the absorbance of the reaction solution at 235 nm did not increase linearly. The slope of the increase in absorbance over time decreased after 1.5 h, unlike that shown in Fig. 2, when DTT was added to the reaction mixture to reduce the protein disulfide bonds. In comparison, incubation at 30ºC in the absence of DTT did not significantly affect rNitAly activity (Fig. 6B). Thus, we investigated how DTT affected the optimum temperature and heat stability of rNitAly. Figure 7A shows the temperature-dependency of rNitAly activity in the presence of 5 mM DTT. Under this condition, the activity of rNitAly between 10 and 50ºC was slightly lower in comparison with its activity in the absence of DTT (Fig. 5A). Yet, the optimum temperature for the activity of rNitAly in the presence of DTT remained at 70ºC, corresponding to that observed without DTT (Fig. 5A). In addition, the specific activity of rNitAly at 70ºC was not significantly different in the absence (1,620 U/mg) and presence (1,590 U/mg) of DTT. Yet, the presence of DTT markedly decreased the heat stability of rNitAly. rNitAly activity fell to 78% of its original activity after incubation at 30ºC for 30 min in the presence of DTT (Fig. 7B). In comparison, incubation at 20ºC or below had no effect on rNitAly activity. Compared with the decrease in rNitAly activity in the absence of DDT, it showed a marked further decline in the presence of DTT at incubation temperatures of 40–70ºC. rNitAly activity declined to 50% after incubation at 50ºC, which was 17ºC lower than the temperature that produced the same decrease in activity in the absence of DTT (Fig. 6A). Thus, DTT tended to have no effect on the optimum temperature and specific activity of rNitAly. In contrast, DTT had a major effect on the heat stability of rNitAly, probably due to the reduction of disulfide bond(s).

Identification of the residues responsible for the heat stability of rNitAly—In the amino acid sequence of NitAly, 3 cysteines were found at the positions of 17, 80, and 232 (Fig. 1). Since Cys17 was located in the predicted signal sequence region, rNitAly did not contain this amino acid. Hence, we focused on the other 2 cysteines that we suggested form an internal disulfide bond (Fig. 2). Homology modeling of NitAly also supported that Cys80 and Cys232 are physically located close to one another in the folded protein (Fig. 8A). Therefore, Cys80 and/or Cys232 of NitAly were replaced with Ala by point mutation to produce the mutant proteins rNitAlyC80A, rNitAlyC232A, and rNitAlyC80A/C232A. Then, the optimum temperature and heat stability of each mutant were investigated in the absence of DTT. All of these mutants showed maximum activity at 70ºC, with specific activities of 1,520 U/mg, 1,630 U/mg, and 1,480 U/mg for rNitAlyC80A, rNitAlyC232A, and rNitAlyC80A/C232A, respectively (Figs. 9A, C, and E). In addition, the effect of temperature on the activity of all mutants was similar to that of wild-type NitAly. Thus, the replacement of Cys80 and/or Cys232 with Ala had no significant effect on the activity and optimum temperature of rNitAly. Further experiments revealed that alterations to the residual activity of each mutant after incubation at different temperatures in the presence of DTT were indistinguishable to that of the wild-type rNitAly protein (Figs. 9B, D, and E). A loss of 18–24% activity was observed in all mutants after incubation at 30ºC in the absence of DTT, whereas the wild-type protein showed no loss of activity under the same conditions. Incubation temperatures of 48, 50, and 46ºC caused a 50% loss of activity for rNitAlyC80A, rNitAlyC232A, and rNitAlyC80A/C232A, respectively. Overall, the replacement of Cys80 and/or Cys232 caused the heat stability of NitAly to decline. Thus, the disulfide bond that forms between Cys80 and Cys232 is responsible for the heat stability of NitAly.

Improvement to the heat stability of rPyAly—NitAly showed the highest amino acid sequence identity with that of the red alga alginate lyase PyAly among the characterized family PL-7 alginate lyases (Fig. 1). The heat stability of PyAly appeared to be inferior to that of NitAly. For instance, PyAly showed residual activity of 50% after incubation at 32.5ºC for 30 min, and all activity ceased at incubation temperatures above 50ºC (39). Alignment of the amino acid sequences of PyAly and NitAly (Fig. 1) suggested that 2 residues in PyAly, Gly79 and Asp230, correspond to the Cys80 and Cys232 residues, respectively, in NitAly. These 2 residues of PyAly may be positioned close to each other based on the molecular structure obtained by homology modeling (Fig. 8B). In addition, Cys residues were not found in the whole sequence of PyAly. Thus, Gly79 and/or Asp230 in PyAly were replaced with Cys (single Cys mutant: rPyAlyG79C or rPyAlyD230C, double Cys mutant: rPyAlyG79D230C). These mutant proteins and the wild-type protein (rPyAlyWT) were expressed and purified. First, the effect of temperature on the alginate lyase activity of these mutant proteins was assayed. Maximum activity was observed at 35ºC for PyAlyG79C and PyAlyD230C, and at 40ºC for PyAlyD230C. Specific activity was 1,820 U/mg for rPyAlyWT, 1,740 U/mg for PyAlyG79C, 1,710 U/mg for PyAlyD230C, and 1,780 U/mg for PyAlyG79C/D230C (Fig. 10). Therefore, Cys mutation(s) at Gly79 and/or Asp230 in PyAly had no significant effect on the optimum temperature and activity of NitAly. Interestingly, rPyAlyG79C/D230C activity significantly declined to 61% of its original activity after incubation at 50ºC for 30 min. In comparison, the relative activity of the other mutant and wild-type

PyAly enzymes showed declined to 20–28%. Thus, the dual replacement of Gly79 and Asp230 residues in PyAly with Cys residues improved the heat stability of the enzyme at 50ºC.

The heat stability of the mutant and wild-type PyAly enzymes was then evaluated. The heat stability of both PyAlyG79C and PyAlyD230C was mostly identical to that of rPyAlyWT (Fig. 11). A 50% loss and a complete loss of activity occurred at about 33ºC and above 50ºC, respectively, in both mutants. However, the introduction of the 2 Cys residues to PyAly enhanced heat stability (Fig. 11D). A temperature of 45ºC caused a 50% loss of activity in PyAlyG79C/D230C, which was more than 10ºC higher than the temperatures causing similar losses of activity in rPyAlyWT, PyAlyG79C, and PyAlyD230C. Furthermore, PyAlyG79C/D230C showed residual activity of 25% after incubation at 50ºC, whereas rPyAlyWT, PyAlyG79C, and PyAlyD230C showed no residual activity. Moreover, the enhanced heat stability of PyAlyG79C/D230C was removed in the presence of DTT (Fig. 11D). Thus, the disulfide bond that formed by introducing the 2 Cys residues to PyAly improved the heat stability of the enzyme without decreasing its alginate lyase activity.

Location of the NitAly gene in the alginate biosynthetic cluster of Nitratiruptor sp. SB155-2 genome—BLAST searches of a series of predicted proteins from *Nitratiruptor sp.* SB155-2 against *P. aeruginosa* proteins located within the alginate biosynthesis operon revealed that 7 genes encoding the *Nitratiruptor sp.* SB155-2 homologs were adjacent to one another in the *Nitratiruptor sp.* SB155-2 genome; namely, the *P. aeruginosa* proteins AlgD (GDP-mannose dehydrogenase), Alg8 (glycosyltransferase), Alg44 (c-di-GMP binding), AlgE (outer-membrane porin), AlgG (mannuroic acid C5-epimerase), AlgL (alginate lyase), and AlgA (phosphomannose isomerase/GDP-mannose pyrophosphatase). Thus, these genes may constitute an operon system in *Nitratiruptor* sp. SB155-2, supporting that NitAly performs alginate biosynthesis in its natural habitat. Yet, candidate genes for AlgK and AlgX, which are involved in alginate polymerization, were not found. These proteins are needed to form the alginate biosynthesis machinery complex (46), whereby AlgK interacts with AlgE, Alg44, and AlgX. Furthermore, AlgX interacts with Alg44 and the specifically interacting serine protease MucD (47). In addition, AlgI, AlgJ, and AlgF were not found, despite being involved in the *O*-acetylation of alginate. Thus, proteins with these functions may have low homology with known proteins, and may be the currently unidentified proteins encoded by genes such as *nis_0180–nis_0184* and *nis_0186* located near the *nitaly* (*nis_0185*) gene.

DISCUSSION

This study identified NitAly as a functional alginate lyase belonging to the PL-7 family. Compared with other characterized PL-7 alginate lyases, NitAly activity was maximum at 1,620 U/mg, 70ºC and pH 6. This activity was comparable to the maximum activity of PyAly at 35ºC and pH 8 (39), and was approximately 2.5% that of FlAlyA at 30ºC and pH 8 (48). When comparing the maximum activity of rNitAly with other PL enzymes, abalone alginate lyase HdAly belonging to PL-14 has about 1.3-fold higher activity at 35ºC and pH 8 (49).

One important finding of this study was that a disulfide bond between Cys80 and Cys232 is critical for the high heat stability of NitAly (Figs. 7–9). The breaking of this bond, by a reducing reagent or point mutation, caused heat stability to noticeably decline. These Cys residues are fully conserved among alginate lyases, showing significant homology to NitAly that are produced by bacteria isolated from deep-sea hydrothermal vents, but are not fully conserved among alginate lyases from terrestrial bacteria, marine bacteria isolated from deep-sea sediments, and red algae (Fig. 1). *C. mediatlanticus* (43), *H. jasoniae* (44), and *N. profundicola* (45) are thermophilic bacteria isolated from deep-sea hydrothermal vent sites. These species produce uncharacterized proteins that show homology with NitAly (Fig. 1). If these uncharacterized proteins have alginate lyase activity, we could confirm that the 2 conserved Cys residues are important for their high heat stability.

Interestingly, rNitAly did not completely lose its activity, regardless of the presence of DTT, even after incubation at 100ºC for 30 min (Fig. 7B). This result was observed in the wild-type, as well as the C80A, C232A, and C80A/C232A mutants of rNitAly (Fig. 9). Thus, other heat tolerant structure(s) may exist in NitAly, separate to the disulfide bond between Cys80 and Cys232. Although there is no firm explanation for this additional heat tolerance yet, regions with a high conservation of residues between NitAly and its homologous proteins from thermophilic bacteria (including proteins other than PL-7 alginate lyases) may be involved. For example, the 49Asp–59Phe, 152His–165Ser, and 198Lys–202Asn regions of NitAly represent possible candidates. Thus, further studies are required to understand the relationship between the structure of NitAly and several of its properties, including its heat stability. At present, we are attempting to resolve the crystal structure of NitAly.

Although the high optimum temperature and heat stability of NitAly were expected, considering the habitat occupied by *Nitratiruptor sp.* SB155-2, other unexpected, unique properties were also found. NitAly optimally functions under acidic conditions, rather than neutral and alkaline conditions. To date, the characterized PL-7 alginate lyases have shown maximum activity at pH 7–9. In contrast, NitAly showed a markedly decreased activity at this higher pH range, with its optimum pH being around 6 (Fig. 5B). This mild acidophilicity may reflect the natural habitat of *Nitratiruptor* sp. SB155-2, where deep-sea hydrothermal vent fluids (typical pH ~4) mix with sea water (typical pH ~8). It is not possible to determine the exact pH in such an environment due to local and steep fluctuations in pH. However, in general, the environment should be more acidic than that at the sea surface. Consequently, NitAly is adapted to acidic conditions. Thus, NitAly may be secreted to function outside of the cell, rather than in the periplasmic space.

Our results imply that bacterial communities inhabiting deep-sea hydrothermal vents use alginate. Previous reports on the existence or utilization of alginate in this type of environment are not available. One of the best characterized alginate-producing bacteria, *P. aeruginosa*, is a member of microbiota that reside in the soil, water, plants, and animals. The exocellular matrix produced by *P. aeruginosa* contains alginate as a component (4). This matrix functions as a biofilm to protect the bacterium from attack and to physically attach it to target sites (50). If *Nitratiruptor* sp. SB155-2 produces alginate, which is a component of exopolysaccharides matrices in nature, it might have the capacity to adhere to biotic and abiotic surfaces, as well as the cells of other *Nitratiruptor* sp. SB155-2. The adherent capacity of alginate stems from its ability to bind divalent metals (such as Ca^{2+}, Sr^{2+}, and Ba^{2+}) to form a cross-linked sticky gel, in accordance with the egg-box molecular model (51-54). Alginate gels show high heat stability compared with gels consisting of other polysaccharides (e.g., agar or carrageenan) and are stable at 0–100ºC (55). The heat resistance property of alginate gels means that they could be used around deep-sea hydrothermal vents where cold seawater associates with hydrothermal vent fluids emerging at temperatures of 300–400ºC (56). In addition, *Nitratiruptor* sp. SB155-2 thrives at a water depth of 1,000 m, where the pressure is 101 atmospheres (= 10.1×10^5 Pa). Under such high pressure, alginate gels are stable. A previous study reported no significant change in the strength of an alginate gel after treatment at a pressure of 10.5×10^7 Pa (57).

In conclusion, we showed that *Nitratiruptor* sp. SB155-2 has a gene encoding a functional alginate lyase enzyme, NitAly, that is mildly acidophilic and heat stable. Because the *nitaly* gene appears to be a member of an alginate biosynthetic operon (Fig. 12), this bacterium may be able to produce alginate. If this is the case, NitAly might be involved in alginate biosynthesis to form and decompose an exocellular matrix (such as a biofilm).

EXPERIMENTAL PROCEDURES

Host strain and vectors—*Escherichia coli* DH5α (Nippon Gene, Tokyo, Japan) and pTac-1 vector plasmid (BioDynamics, Tokyo, Japan) were used for DNA cloning. *E. coli* BL21(DE3) (Nippon Gene) and pCold I plasmid vector (Takara Bio, Shiga, Japan) were used for recombinant protein expression.

Reagents—Sodium alginate with a viscosity of 80–120 cP was purchased from Wako Pure Chemical Industries (Osaka, Japan). PolyM, polyG, and polyMG blocks were prepared by the method of Gacesa and Wusteman (58). Restriction enzymes and DNA modifying enzymes were purchased from Nippon Gene. All other chemical reagents were purchased from Wako Pure Chemical Industries, unless otherwise stated.

DNA cloning and sequencing of NitAly—Genomic DNA was isolated from *Nitratiruptor* sp. SB155-2, as described previously (41). A primer set of Nit-F and Nit-R (Table 1) was used in combination with Platinum® *Taq* DNA Polymerase (Thermo Fisher Scientific, Waltham, MA) to amplify the full-length gene encoding the hypothetical protein NIS_0185 (GenBank accession no. YP_001355656). Polymerase chain reaction (PCR) was conducted using temperature settings of 95ºC for 5 min, followed by 30 cycles of 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 90 s. The final step for extension was performed at 72ºC for 7 min. Amplified DNA was ligated into the pTac-1 vector, and was sequenced using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA).

Construction of expression plasmid for rNitAly—Restriction sites of BamHI and XbaI were introduced into the 5′- and 3′-termini of the NitAly gene using the Nit-BamF and Nit-XbaR primer sets (Table 1), respectively, by PCR. The reaction conditions were the same as those described in the previous section. Amplified DNA was digested with BamHI and XbaI after subcloning in the pTac-1 vector. DNA fragments were isolated and recovered from agarose gel following agarose gel electrophoresis, and were ligated in the pCold I vector digested with BamHI and XbaI. After DNA sequencing, BL21(DE3) cells were transformed with the recombinant pCold I construct.

Expression and purification of rNitAly—Transformed cells were cultured in lysogeny broth medium supplemented with 50 μg/mL ampicillin at 37ºC for 12 h. Then, the medium was cooled at 15ºC for 1 h and isopropyl β-D-1-thiogalactopyranoside was added at a final concentration of 0.1 mM. After incubation at 15ºC for 12 h, cells were harvested by centrifugation at 5,000 × *g* for 15 min. Pellets were suspended and sonicated in a buffer containing 10 mM imidazole-HCl (pH 7.4), 0.5 M NaCl, 1% (v/v) Triton™ X-100, and 0.05 mg/ml lysozyme. After centrifugation at 10,000 × *g* for 15 min, the supernatant was mixed with 250 μL Ni-NTA agarose in a conical tube, and the tube was rotated at 4ºC for 30 min. Resins were washed with a 20-fold volume of 30 mM imidazole (pH 7.4) and 0.5 M NaCl. Protein elution was conducted with a solution containing 250 mM imidazole (pH 7.4) and 0.5 M NaCl. Fractions containing the targeted proteins were combined and dialyzed against 20 mM sodium acetate (pH 5.0) and 0.1 M NaCl at 4ºC for 8 h. Purified proteins were stored on ice and were assayed within 3 days. Protein concentration was determined by the method of Bradford (59), using bovine serum albumin (BSA) fraction V as the protein standard.

Site-directed mutagenesis of alginate lyase proteins—The listed primers (Table 1) were designed for the site-directed mutation of rNitAly and rPyAly. Mutagenesis was conducted using recombinant plasmid rNitAly-pCold I (in this study) or rPyAly-pCold I (39) as a template with a Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA). The mutant proteins of NitAly (rNitAlyC80A, rNitAlyC232A, and rNitAlyC80A/C232A) were purified using the same method as that described for rNitAly in the previous section. Wild-type rPyAly and its mutant proteins (rPyAlyG79C, rPyAlyD230C, and rPyAlyG79C/D230C) were purified using the same method as that used for rPyAly, as reported previously (39).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)—SDS-PAGE was conducted on a 10 or 12% (v/v) polyacrylamide gel using the method of Porzio and Pearson (60). After electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol/10% (v/v) acetic acid, and the background of the gel was destained with 5% (v/v) methanol/7% (v/v) acetic acid. Protein Molecular Weight Marker (Broad) (Takara Bio) was used as the molecular weight marker.

Labeling of Cys residues in rNitAly—Purified rNitAly (0.1 mg) was incubated in 20 mM Tris-HCl (pH 7.5) and 2.5% SDS in the presence and absence of 20 mM DTT at 25ºC for 5 h. Then, the proteins were precipitated by 10% trichloroacetic acid, and were dissolved in 20 mM Tris-HCl (pH 7.5), 3% SDS, and 10 mM methoxypolyethylene glycol maleimide (PEG-Mal) (PEG average Mn 5,000, Sigma-Aldrich). After incubation at 25ºC for 30 min, the samples were analyzed by SDS-PAGE.

Determination of the number of free thiol *groups in rNitAly*—Purified rNitAly (0.1 mg) was incubated in 10 mM Tris-HCl (pH 7.5) and 6 M guanidinium chloride in the presence and absence of 5 mM DTT at 25ºC for 2 h. Then, the number of free thiol groups was determined using 4,4'-dithiodipyridine (4-PDS) by measuring absorbance at 324 nm using the method of Le and Means (61).

Assay for alginate lyase activity—The decrease in the viscosity of alginate solutions produced by the alginate lyase enzyme reactions was measured at 30ºC using an Ostwald-type viscometer. The alginate lyase activities of rNitAly and its mutants were assayed in a solution containing 10 mM sodium phosphate (pH 6.0), 1.0 M NaCl, 0.25% (w/v) sodium alginate, 0.1 mg/mL BSA, and 0.005 mg/mL enzyme, unless otherwise stated. For PyAly and its mutants, the reaction mixture contained 10 mM sodium phosphate (pH 8.0), 0.1 M NaCl, 0.25% (w/v) sodium alginate, 0.1 mg/mL BSA, and 0.005 mg/mL enzyme. Alginate lyase activity was measured by an increase in absorbance at 235 nm, due to the formation of a double bond between C-4 and C-5 at the non-reducing end by the cleavage of alginate. Enzyme reactions were monitored using an U-3010 spectrophotometer (Hitachi, Tokyo, Japan) equipped with a SP-12R thermal control unit (TAITEC, Koshigaya, Japan). One unit (U) of alginate lyase was defined as the amount of enzyme that increased absorbance at 235 nm by 0.01 in 1 min.

Alginate lyase activity of rNitAly in the presence of divalent cations, trivalent cations, or chelator reagent was measured by the 2-thiobarbituric acid (TBA) method (62). Assay conditions were the same as those described in the previous section. One unit of activity was defined as the amount of enzyme required to liberate 1 μmol of β-formyl-pyruvic acid per min at 50ºC. Because one unit in this assay was experimentally determined to be equivalent to an increase of 0.62 in the absorbance at 235 nm in 1 min, the calculated activity was converted to activity comparable to that measured by the absorbance at 235 nm.

Thin-layer chromatography (TLC)—After each enzyme reaction, ethanol was added to the mixture at a final concentration of 80% (v/v), and the resulting solution was stored at -20ºC for 2 h. Pellets were produced by centrifugation at 12,000 × *g* for 15 min, and were washed with ethanol 3 times. Dried pellets were dissolved with 10 mM sodium phosphate (pH 8.0), and were analyzed by TLC using a silica gel 60 plate (Merck, Darmstadt, Germany). The resulting solvent contained ethyl acetate, acetic acid, and water (2:2:1, v:v:v). Sugars developed on the TLC plate were detected by spraying 10% (v/v) sulfuric acid in ethanol followed by heating at 130ºC for 10 min.

Computational analysis of proteins—The presence of a signal peptide was predicted using the program SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (42). Homology modeling of protein structures was carried out using the PHYRE2 protein fold recognition server (http://www.sbg.bio.ic.ac.uk/phyre2/) (63). The predicted protein structures were visualized using the program CCP4mg (64).

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Author contributions: AI designed the study and wrote the paper. AI, MA, and TO carried out DNA cloning, the construction of the protein expression system, and enzymatic characterization. SN performed the culture of *Nitratiruptor* sp. SB155-2 and the extraction of genomic DNA.

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FIGURE LEGENDS

FIGURE 1. Comparison among the amino acid sequences of NitAly, its homologous proteins, and several characterized family PL-7 alginate lyases. *NitAly*, *Nitratiruptor* sp. SB155-2 hypothetical protein NIS_0185 (GenBank accession no. WP_012081562); *Caminibacter*, *Caminibacter mediatlanticus* hypothetical protein (GenBank accession no. WP_007473671); *Hippea*, *Hippea jasoniae* hypothetical protein (GenBank accession no. WP_051904545); *Nautilia*, *Nautilia profundicola* predicted alginate lyase (GenBank accession no. WP_015901805); *PyAly*, *Pyropia yezoensis* alginate lyase PyAly (GenBank accession no. BAI66416) (39); *PA1167*, *Pseudomonas aeruginosa* PAO1 alginate lyase PA1167 (GenBank accession no. AAG04556) (15); *A1m*, *Agarivorans* sp. JAM-A1m alginate lyase A1m (GenBank accession no. AB426616) (31); *A9mT*, *Vibrio* sp. alginate lyase A9mT (GenBank accession no. AB473598) (25); *FlAlyA*, *Flavobacterium* sp. UMI-01 alginate lyase FlAlyA (18). Residues invariant among all listed proteins are indicated with blue boxes. Residues identical with those of NitAly are indicated with yellow boxes. Cys80 and Cys232 in NitAly and their corresponding conserved Cys residues in other proteins are highlighted with red. An arrowhead shows the cleavage site of the signal peptide in NitAly predicted by the SignalP 3.0 software program. Catalytic residues proposed in PL-7 alginate lyases (15, 21, 65) are shown by solid circles. The level of identity of each other protein with NitAly is shown at the end of each sequence, respectively.

FIGURE 2. Bacterial expression and purification of rNitAly. *A*, schematic drawing of rNitAly. *B*, SDS-PAGE of purified rNitAly. "Reduced" and "non-reduced" indicate that the samples were prepared in the presence and absence of 20 mM DTT, respectively. *C*, SDS-PAGE of PEG-Mal labeled rNitAly pretreated in the presence and absence of 20 mM DTT.

FIGURE 3. Evaluation of an alginate lyase activity of rNitAly. Relative viscosity (closed circles) and absorbance at 235 nm (open circles) were measured in a mixture of 10 mM sodium phosphate (pH 6.0), 1 M NaCl, 0.1 mg/mL BSA, 1% (w/v) sodium alginate, and 0.005 mg/mL rNitAly at 30ºC at the indicated time points.

FIGURE 4. Substrate preferences of rNitAly and degradation products. *A*, substrate preferences of rNitAly. Enzyme reactions were conducted in a solution containing 10 mM sodium phosphate buffer (pH 6.0), 1 M NaCl, 0.1 mg/mL BSA, 0.005 mg/mL rNitAly, and 0.5% (w/v) substrate (polyM, polyG,

polyMG, or sodium alginate) at 50ºC for 15 min. A relative activity of 100% was equivalent to 1,290 U/mg. Assays were done 3 times and the data were shown as mean ± SD. *B*, thin-layer chromatography analysis of the degradation products of polyM following incubation with rNitAly. The enzyme reaction was conducted using polyM as the substrate under the same conditions described for Fig. 4A, and was stopped by the addition of 4 volumes of ice cold ethanol at the indicated time point.

FIGURE 5. Effects of temperature, pH, NaCl, and divalent metals on rNitAly activity. *A*, temperature dependence of alginate lyase activity of rNitAly. Enzyme reactions were conducted in a solution containing 10 mM sodium phosphate (pH 6.0), 1 M NaCl, 0.1 mg/mL BSA, 0.005 mg/mL rNitAly, and 0.5% (w/v) polyM for 15 min at the indicated temperatures. Relative activity of 100% was equivalent to 1,620 U/mg. *B*, pH dependence of the alginate lyase activity of rNitAly. Assays were performed as described for Fig. 5A, except that reaction mixtures were incubated at 50 °C for 15 min in 10 mM sodium acetate for pH 4.0–5.1 (open circles), 10 mM sodium phosphate for pH 5.7–7.4 (closed circles), 10 mM Tris-HCl for pH 8.1–8.5 (closed triangles), and 10 mM glycine-NaOH for pH 9.2–10.4 (open triangles). Relative activity at 100% was equivalent to 1,230 U/mg. *C*, NaCl dependence of the alginate lyase activity of rNitAly. Assays were performed as described for Fig. 5A at 50 °C, except that different concentrations of NaCl were used, as indicated. Relative activity at 100% was equivalent to 1,430 U/mg. *D*, the effects of divalent metals on the alginate lyase activity of rNitAly. Assays were performed as described for Fig. 5A at 50 °C (Control). Each indicated metal ion or EDTA was added at a final concentration of 1 or 5 mM, respectively. Relative activity at 100% was equivalent to 1,410 U/mg. All assays were repeated 3 times and the data are shown as mean ± SD.

FIGURE 6. Effects of temperature and pH on rNitAly stability. *A*, heat stability of rNitAly. For this assay, 0.2 mg/mL rNitAly in 10 mM sodium phosphate (pH 6.0) and 1 M NaCl were incubated for 30 min at the indicated temperatures and placed on ice. Enzyme activity was then assayed as described for Fig. 5A at 50 °C. Relative activity at 100% was equivalent to 1,230 U/mg. *B*, Effects of time extension of heat treatment on the heat stability of rNitAly. For this assay, 0.2 mg/mL rNitAly in 10 mM sodium phosphate (pH 6.0) and 1 M NaCl were incubated for the indicated time at 20 °C (open circles), 30 °C (closed circles), 40 °C (open triangles), and 50 °C (closed triangles) and placed on ice. Assays were performed as described for Fig. 6A. Relative activity at 100% was equivalent to 1,210 U/mg. *C*, pH stability of rNitAly. For this assay, 0.2 mg/mL rNitAly in 10 mM sodium phosphate (pH 6.0) and 1 M NaCl was dialyzed against 1,000 volumes of 1 M NaCl and 10 mM sodium acetate (pH

4.0 or 5.0) (open circles), 10 mM sodium phosphate (pH 6.0 or 7.0) (closed circles), 10 mM Tris-HCl (pH 8.0) (open triangle), and 10 mM glycine-KOH (pH 9.0) (closed triangle) at 4°C for 8 h. Assays were performed as described for Fig. 6A. Relative activity at 100% was equivalent to 1,410 U/mg. All assays were repeated 3 times and the data are shown as mean ± SD.

FIGURE 7. Effects of DTT on the temperature dependence and heat stability of NitAly. *A*, Temperature dependence of alginate lyase activity of rNitAly in the presence of DTT. Assays were performed as described for Fig. 5A, except that DTT was added to the reaction mixture at a final concentration of 5 mM. Relative activity at 100% was equivalent to 1,590 U/mg. *B*, Heat stability of rNitAly in the presence of DTT. Samples were preincubated as described for Fig. 6A, except that DTT was added at a final concentration of 5 mM. Assays were performed as described in Fig. 7A at 50 °C. Relative activity at 100% was equivalent to 1,080 U/mg. All assays were repeated 3 times and the data are shown as mean ± SD.

FIGURE 8. Homology modeling of NitAly and PyAly. The structures of NitAly (*A*, residues 34–242) and PyAly (*B*, residues 13-216) were predicted using the PHYRE2 program (63) and *P. aeruginosa* alginate lyase PA1167 (PDB entry 1vav) as the template (15) with 100% confidence, respectively. *A,* Cys80 and Cys232 are shown by red globules. The residues Arg85, Gln123, His125, and Ty226, which correspond to the suggested catalytic residues in PA1167 (15, 21), are shown by pink sticks. *B*, Gly79 and Asp230 are shown by red globules.

FIGURE 9. Effects of cysteine residue mutations in rNitAly on temperature dependence and heat stability. *A*, *C*, and *E*, Temperature dependence of alginate lyase activity of rNitAlyC80A (*A*), rNitAlyC232A (*C*), and rNitAlyC80A/C232A (*E*). Assays were performed as described for Fig. 5A. Relative activities at 100% were equivalent to 1,520 U/mg (*A*), 1,630 U/mg (*C*), and 1,480 U/mg (*E*). *B*, *D*, and *F*, Heat stability of rNitAlyC80A (*B*), rNitAlyC232A (*D*), and rNitAlyC80A/C232A (*F*). Assays were performed as described for Fig. 6A. Relative activity at 100% was equivalent to 970 U/mg (*B*), 1,060 U/mg (*D*), and 960 U/mg (*F*). All assays were repeated 3 times and the data are shown as mean ± SD.

FIGURE 10. Effects of replacing the residues of rPyAly with cysteine on its temperature dependence. Enzyme reactions were conducted in a solution containing 10 mM sodium phosphate (pH 8.0), 0.1 M NaCl, 0.1 mg/mL BSA, 0.25% (w/v) polyM, and 0.005 mg/mL rPyAly (*A*), rPyAlyG79C (*B*),

rPyAlyD230C (*C*), or rPyAlyG79C/D230C (*D*) for 15 min at the indicated temperatures. Relative activity at 100% was equivalent to 1,820 U/mg (*A*), 1,740 U/mg (*B*), 1,710 U/mg (*C*), and 1,780 U/mg (*D*). All assays were repeated 3 times and the data are shown as mean ± SD.

FIGURE 11. Effects of replacing residue(s) of rPyAly with cysteine on its heat stability. Enzyme solution containing 10 mM sodium phosphate (pH 8.0), 0.1 M NaCl, and 0.15 mg/mL rPyAly (*A*), rPyAlyG79C (*B*), rPyAlyD230C (*C*), or rPyAlyG79C/D230C (*D, -DTT*) was incubated for 30 min at the indicated temperatures and placed on ice. For rPyAlyG79C/D230C, 5 mM DTT was also added during incubation (*D, +DTT*). Enzyme activity was then assayed as described for Fig. 10 at 30 °C. Relative activity at 100% was equivalent to 1,730 U/mg (*A*), 1,620 U/mg (*B*), 1,540 U/mg (*C*), 1,600 U/mg (*D, -DTT*), and 1,420 U/mg (*D, +DTT*). All assays were repeated 3 times and the data are shown as mean ± SD.

FIGURE 12. Assignment of genes in the alginate biosynthesis operon of *P. aeruginosa* and their homologs in *Nitratiruptor* sp. SB155-2. Genes encoding proteins with homology between *P. aeruginosa* and *Nitratiruptor* sp. SB155-2 are connected by dotted lines. Each pentagon is classified by a different color based on known function in *P. aeruginosa*; blue, precursor biosynthesis; green, alginate polymerization; brown, alginate export; violet, epimerization of mannuroic acid to guluronic acid; red, alginate degradation; gray, *O*-acetylation. Yellow-painted pentagons show genes with unidentified functions in *Nitratiruptor* sp. SB155-2.

Table 1. Primers used in this study.

Primer	Sequence
For NitAly-DNA cloning	
Nit-F	5′-ATGCACCAACTAAAAGTTTTG-3′
Nit-R	5′-CTATTTCATTGTTAATGAATC-3′
For rNitAly expression	
Nit-BamF	5′-GCGGATCCCACGATGCGCCCTACGCTAT-3′
Nit-XbaR	5′-CGTCTAGACTATTTCATTGTTAATGAAT-3′
For rNitAlyC80A, rNitAlyC232A, or rNitAlyC80A/C232A expression	
Nit-C80A-F	5′-ACCTTTTTCATGGCCGGGAAAAAACAT-3′
Nit-C80A-R	5′-ATGTTTTTTCCCGGCCATGAAAAAGGT-3′
Nit-C232A-F	5′-CAAGGAGACGGAGCCGCAAAAGTTTT-3′
Nit-C232A-R	5′-AAAACTTTTGCGGCTCCGTCTCCTTG-3′
For rPyAlyG79C, rPyAlyD230C, or rPyAlyG79C/D230C expression	
Py-G79C-F	5′-GTGTTTGTCATGTGTGGCGACTCAC-3′
Py-G79C-R	5′-GTGAGTCGCCACACATGACAAACAC-3′
Py-D230C-F	5′-GAGGGCAGCCCCTGCGCGAGGGTAGTG-3′
Py-D230C-R	5′- CACTACCCTCGCGCAGGGGCTGCCCTC-3′

Introduced restriction sites are underlined. Mutated sequences are double underlined.

Table 2. Effects of monovalent cations on the alginate lyase activity of NitAly.

	Relative activity[a] (%)		
Concentration	0.1 M	0.5 M	1.0 M
NaCl	9.2 ± 0.5	74.2 ± 2.1	100 ± 4.3[b]
KCl	8.3 ± 0.4	71.6 ± 1.8	113 ± 6.1
LiCl	10.3 ± 0.4	77.3 ± 3.2	101 ± 3.0
CsCl	8.7 ± 0.3	72.4 ± 1.2	114 ± 7.4

[a] Enzyme reactions were conducted in a solution containing 10 mM sodium phosphate (pH 6.0), 0.1 mg/mL BSA, 0.005 mg/mL rNitAly, 0.5% (w/v) polyM, and the indicated concentration of monovalent cation at 50ºC for 15 min.

[b] Relative activity at 100% was equivalent to 1,350 U/mg.

Table 3. Effects of divalent cations, trivalent cations, and chelate reagents on the alginate lyase activity of NitAly.

		Relative activity [a] (%)
	Control[b]	100 ± 2.7[c]
Divalent cation (1 mM)		
	$NiCl_2$	110 ± 6.3
	$ZnCl_2$	109 ± 4.5
	$MnCl_2$	109 ± 5.3
	$CdCl_2$	107 ± 3.2
	$HgCl_2$	107 ± 2.2
	$MgCl_2$	105 ± 6.2
	$CaCl_2$	104 ± 7.8
	$SrCl_2$	97.3 ± 4.3
	$CoCl_2$	95.1 ± 5.6
	$SnCl_2$	84.6 ± 3.2
	$PbCl_2$	81.6 ± 4.1
	$FeCl_2$	68.8 ± 3.2
Trivalent cation (1 mM)		
	$AlCl_3$	97.2 ± 4.3
	$FeCl_3$	86.1 ± 5.2
Chelate reagent (5 mM)		
	EDTA	95.8 ± 6.2
	EGTA	101 ± 3.8

[a] Enzyme activity in this experiment was measured by TBA method (62), due to the increment of absorbance at 235 nm in the absence of rNitAly through the addition of some metal ions, possibly as a result of gelation. Enzyme reactions were conducted in a solution containing 10 mM sodium phosphate (pH 6.0), 1 M NaCl, 0.1 mg/mL BSA, 0.005 mg/mL rNitAly, and 0.5% (w/v) polyM for the specified divalent cations, trivalent cations, or chelate reagents at 50ºC for 15 min.

[b] "Control" activity was measured in the absence of metal ions or chelate reagents.

[c] Relative activity at 100% was equivalent to 1,320 U/mg.

Fig. 1

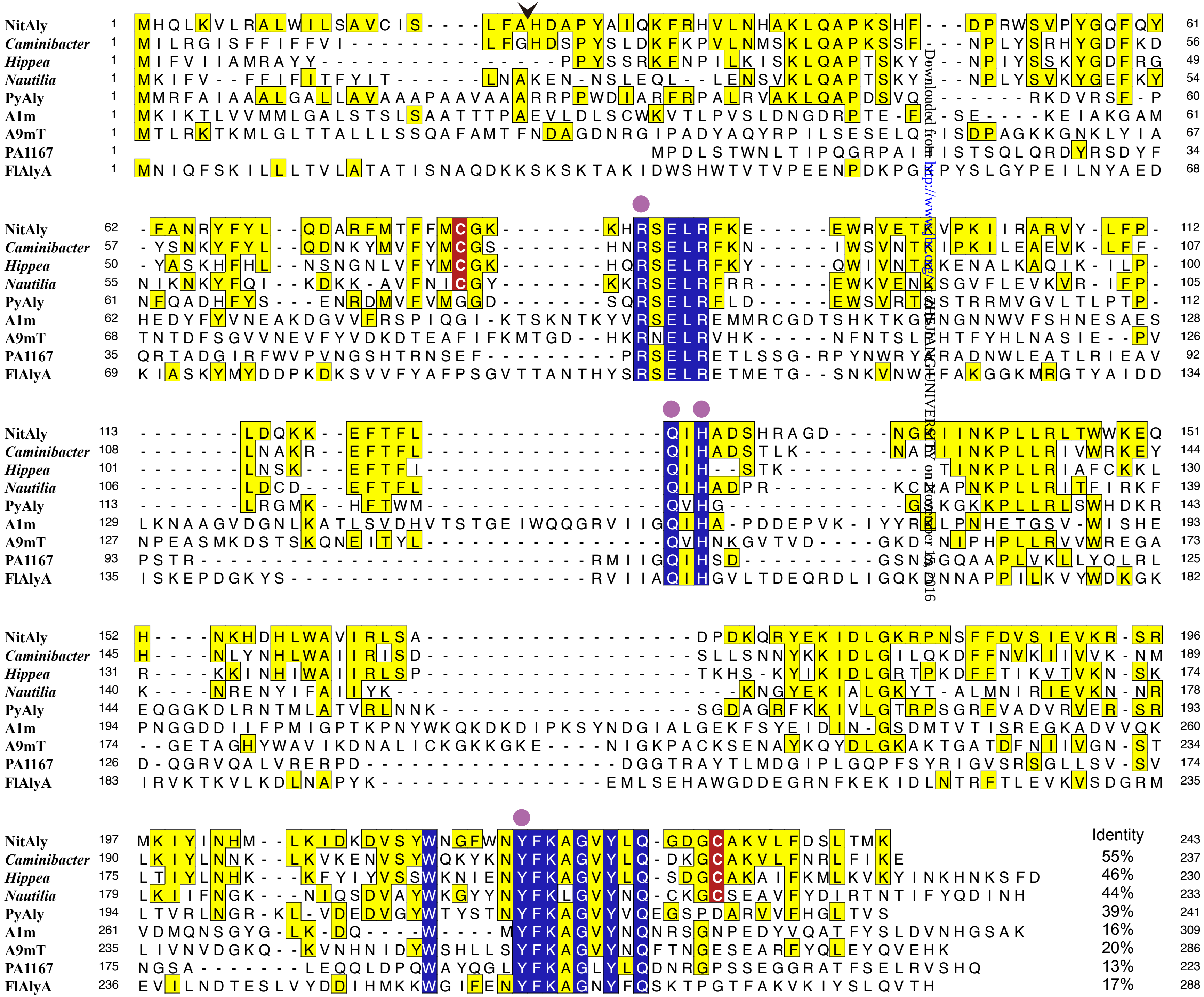

NitAly
Caminibacter
Hippea
Nautilia
PyAly
A1m
A9mT
PA1167
FlAlyA
Identity
55%
46%
44%
39%
16%
20%
13%
17%

Fig. 2

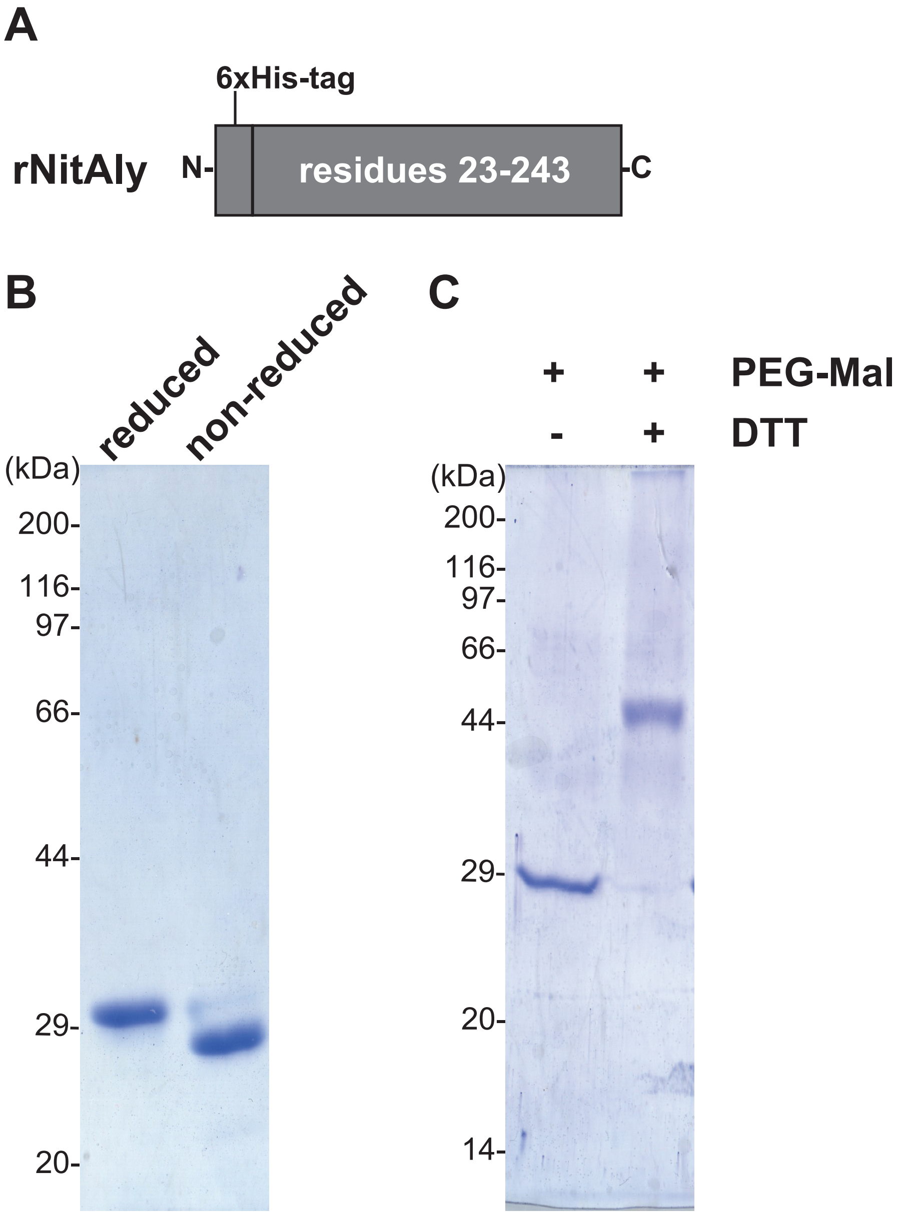

A
6xHis-tag
rNitAly
N-
residues 23-243
-C
B
reduced
non-reduced
(kDa)
200-
116-
97-
66-
44-
29-
20-
C
+ + PEG-Mal
- + DTT
(kDa)
200-
116-
97-
66-
44-
29-
20-
14-

Fig. 3

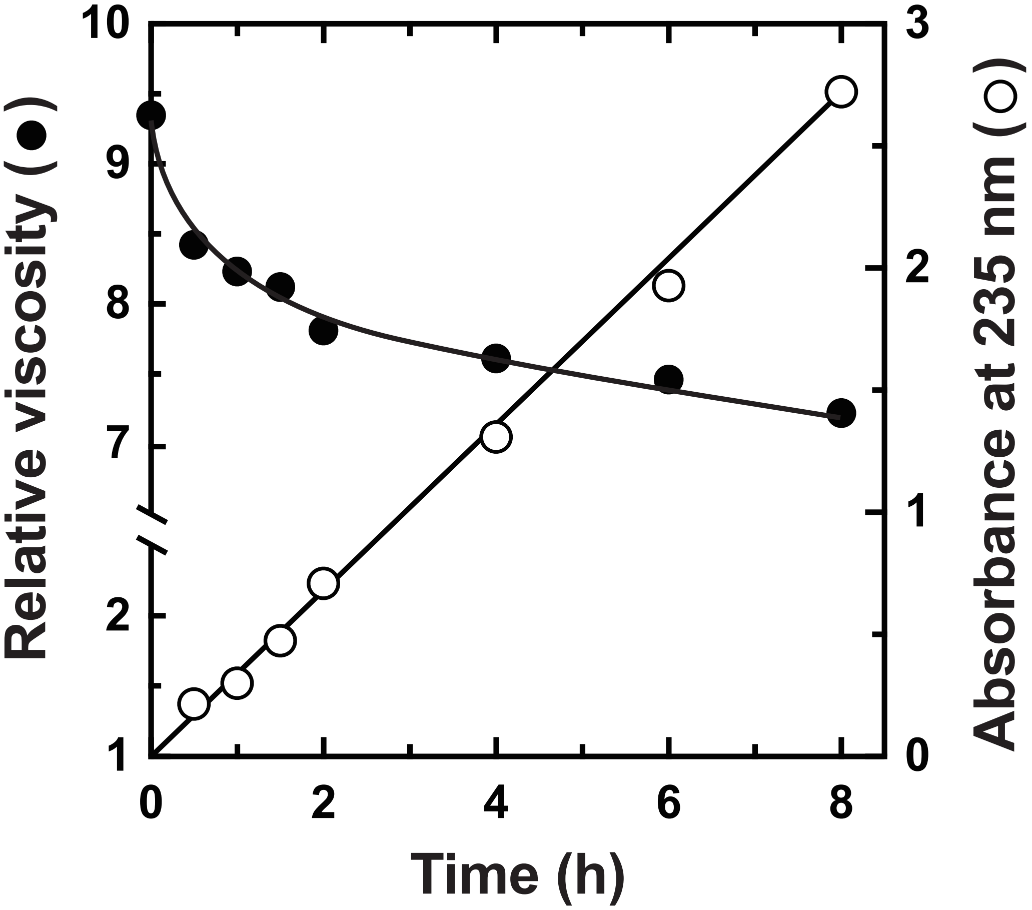

Relative viscosity (●)
Absorbance at 235 nm (○)
Time (h)
10
9
8
7
2
1
3
2
1
0
0
2
4
6
8

Fig. 4

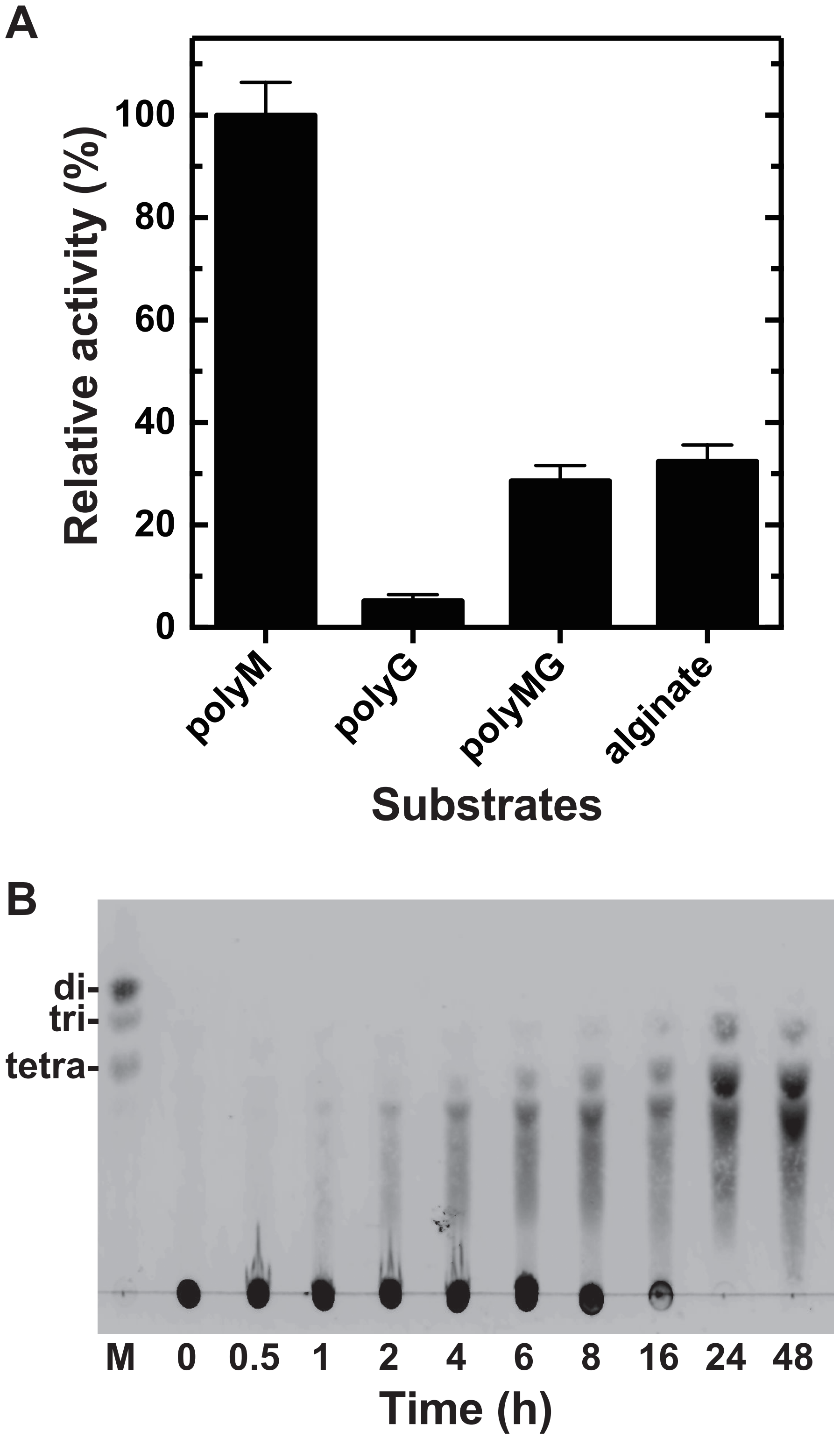

A
Relative activity (%)
0
20
40
60
80
100
polyM
polyG
polyMG
alginate
Substrates
B
di-
tri-
tetra-
M
0
0.5
1
2
4
6
8
16
24
48
Time (h)

Fig. 5

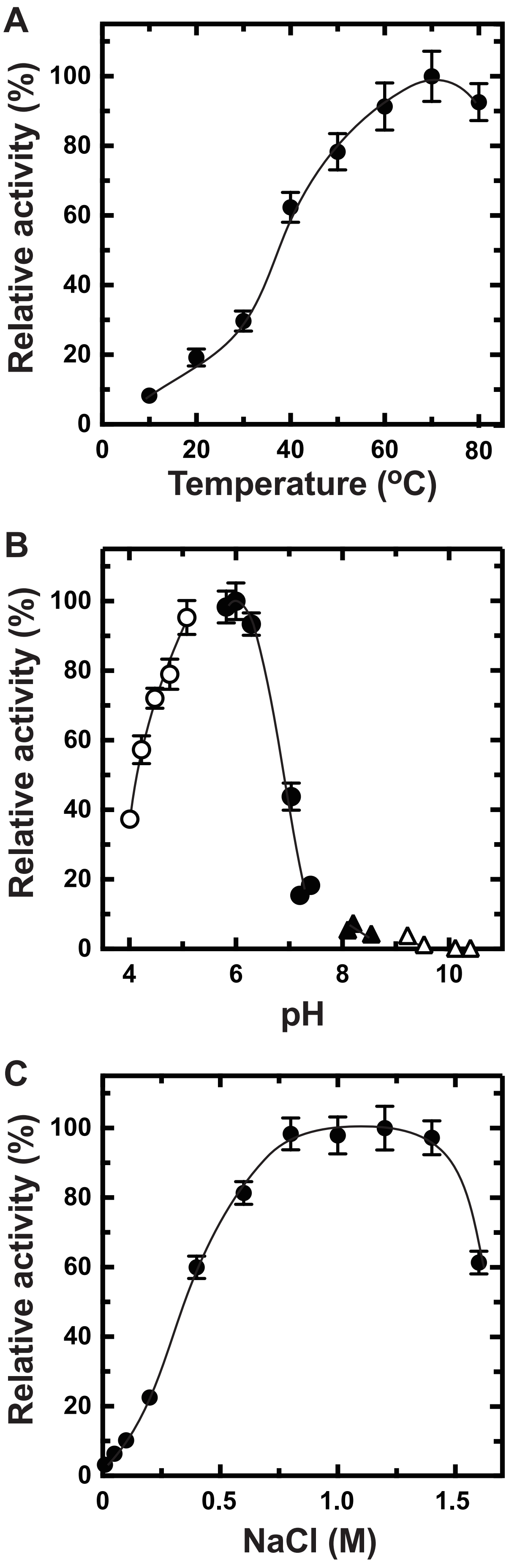

A
Relative activity (%)
0
20
40
60
80
100
0
20
40
60
80
Temperature (°C)
B
Relative activity (%)
0
20
40
60
80
100
4
6
8
10
pH
C
Relative activity (%)
0
20
40
60
80
100
0
0.5
1.0
1.5
NaCl (M)

Fig. 6

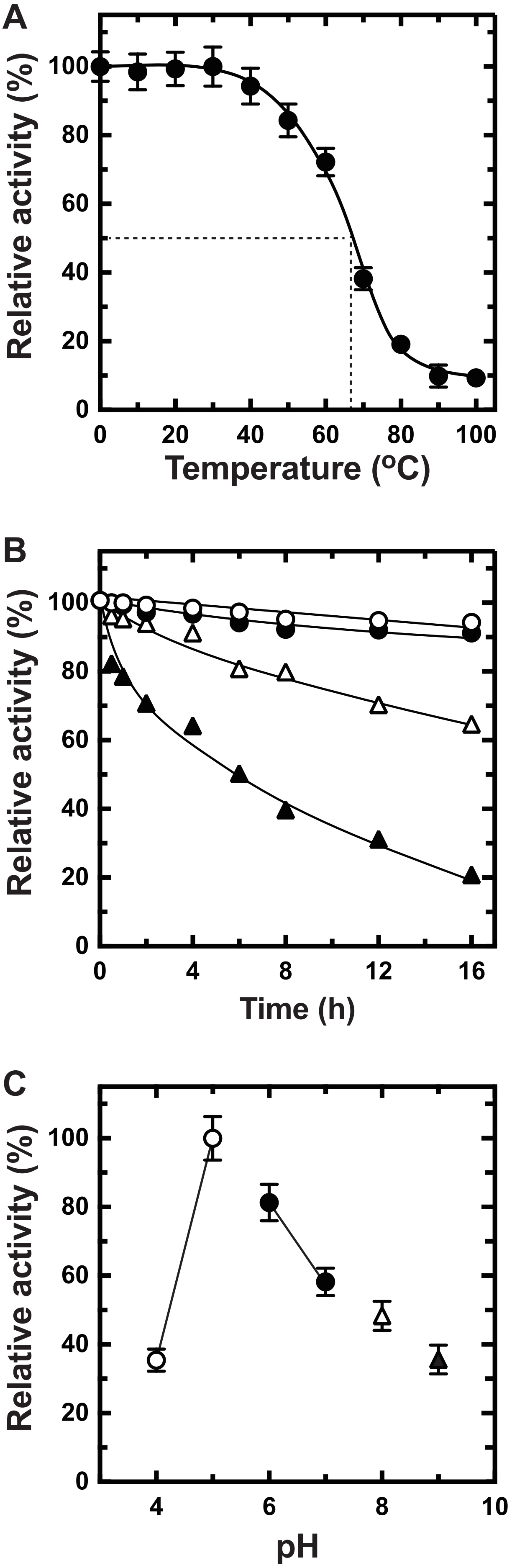

A
Relative activity (%)
0
20
40
60
80
100
0
20
40
60
80
100
Temperature (°C)
B
Relative activity (%)
0
20
40
60
80
100
0
4
8
12
16
Time (h)
C
Relative activity (%)
0
20
40
60
80
100
4
6
8
10
pH

Fig. 7

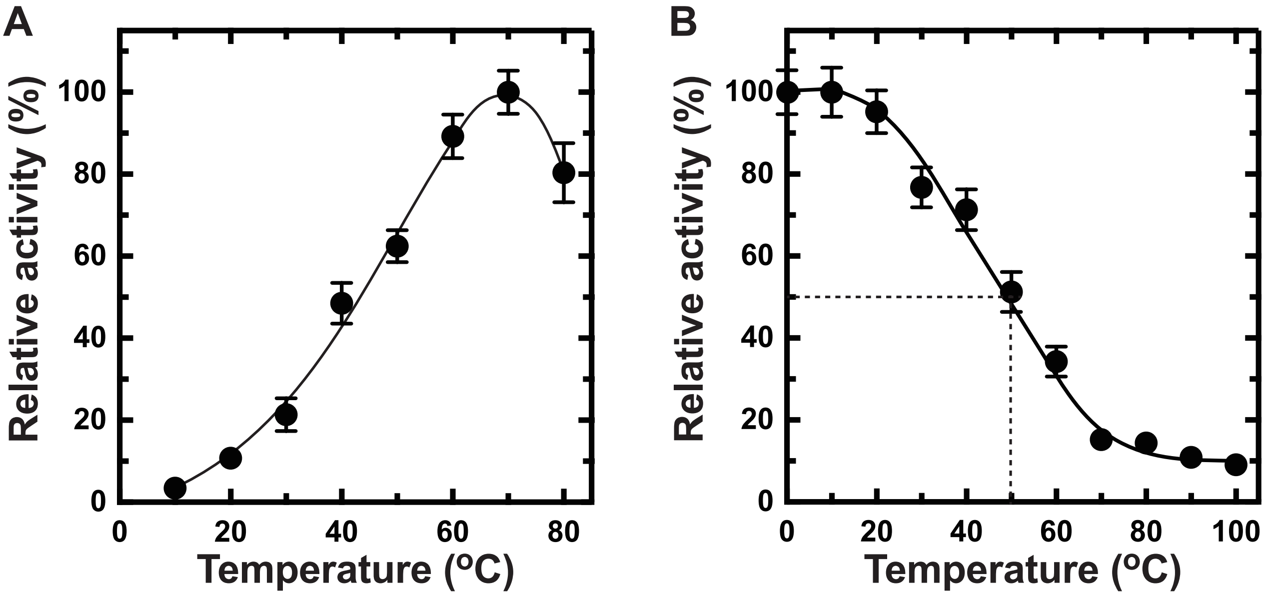

A
Relative activity (%)
0
20
40
60
80
100
Temperature (ºC)
0
20
40
60
80
B
Relative activity (%)
0
20
40
60
80
100
Temperature (ºC)
0
20
40
60
80
100

Fig. 8

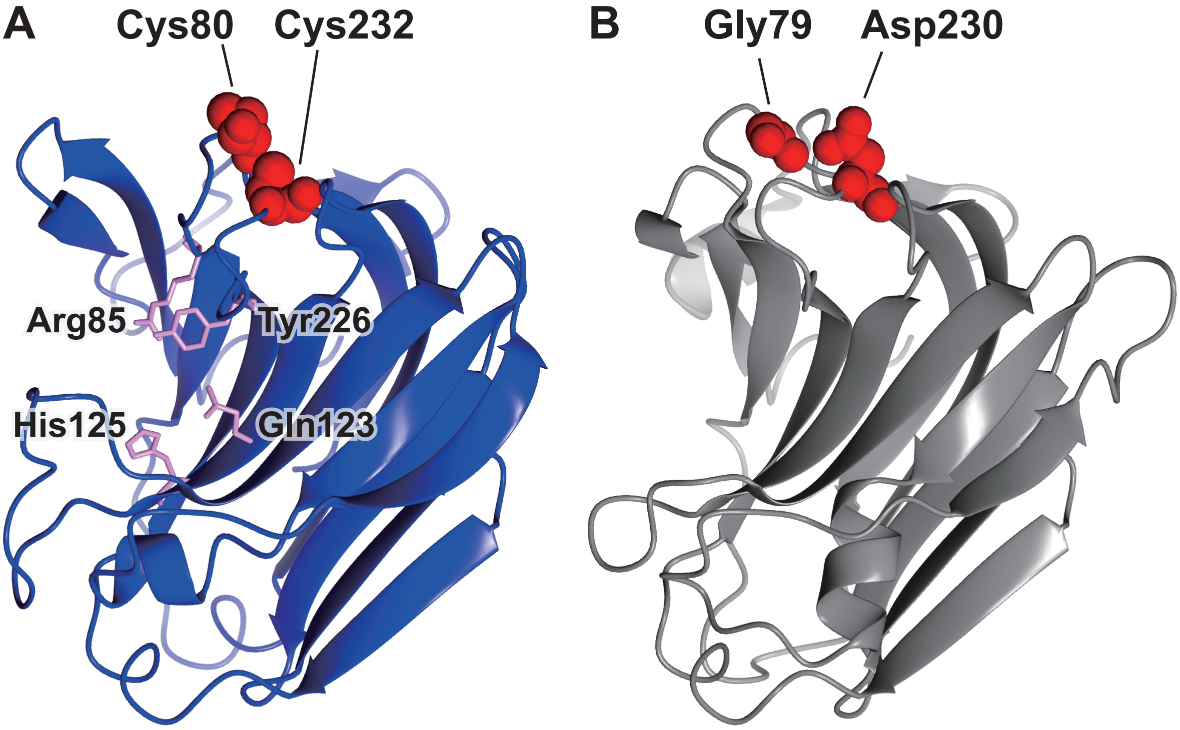


Fig. 9

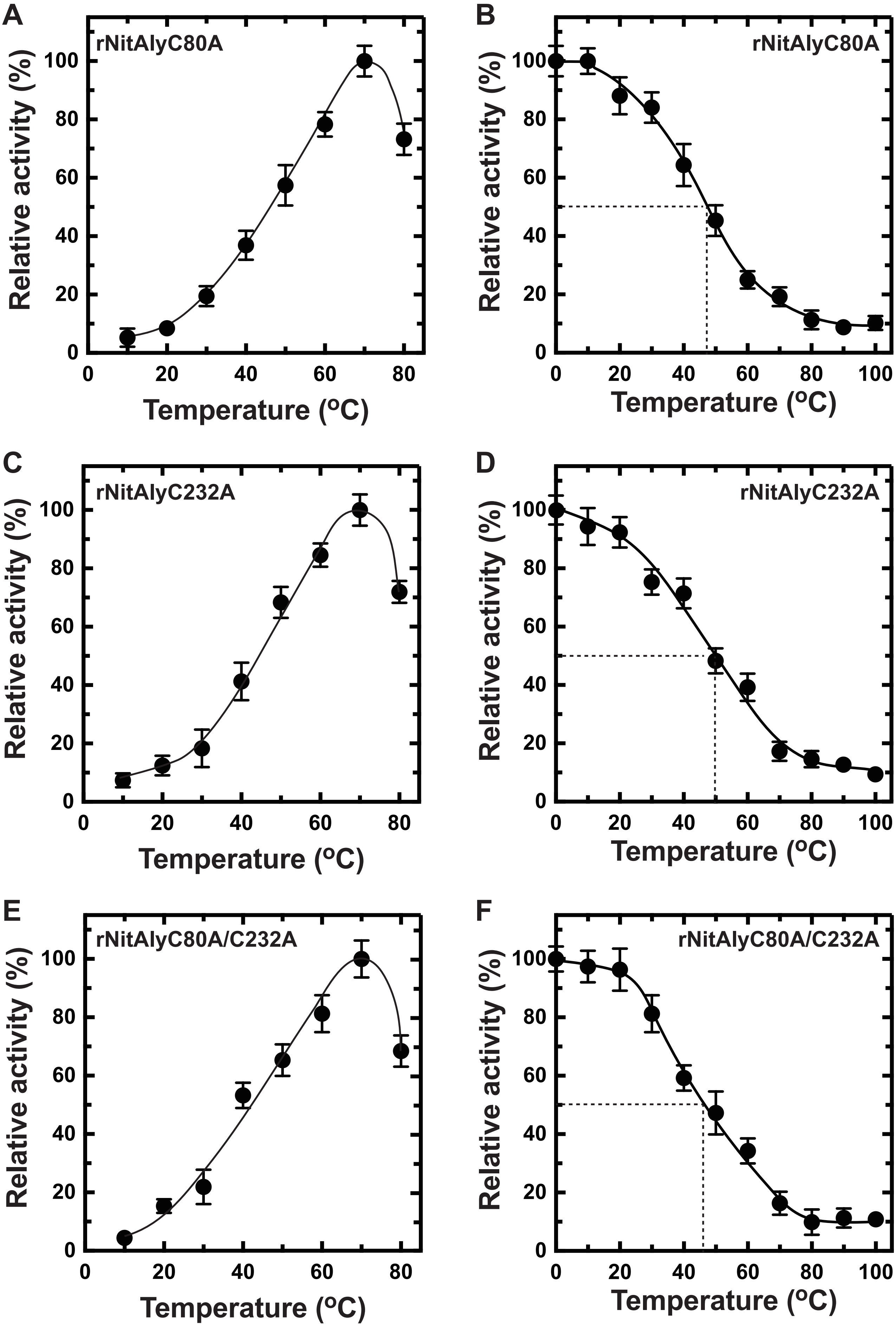

A
rNitAlyC80A
Relative activity (%)
Temperature (°C)
0
20
40
60
80
100
B
rNitAlyC80A
Relative activity (%)
Temperature (°C)
C
rNitAlyC232A
Relative activity (%)
Temperature (°C)
D
rNitAlyC232A
Relative activity (%)
Temperature (°C)
E
rNitAlyC80A/C232A
Relative activity (%)
Temperature (°C)
F
rNitAlyC80A/C232A
Relative activity (%)
Temperature (°C)

Fig. 10

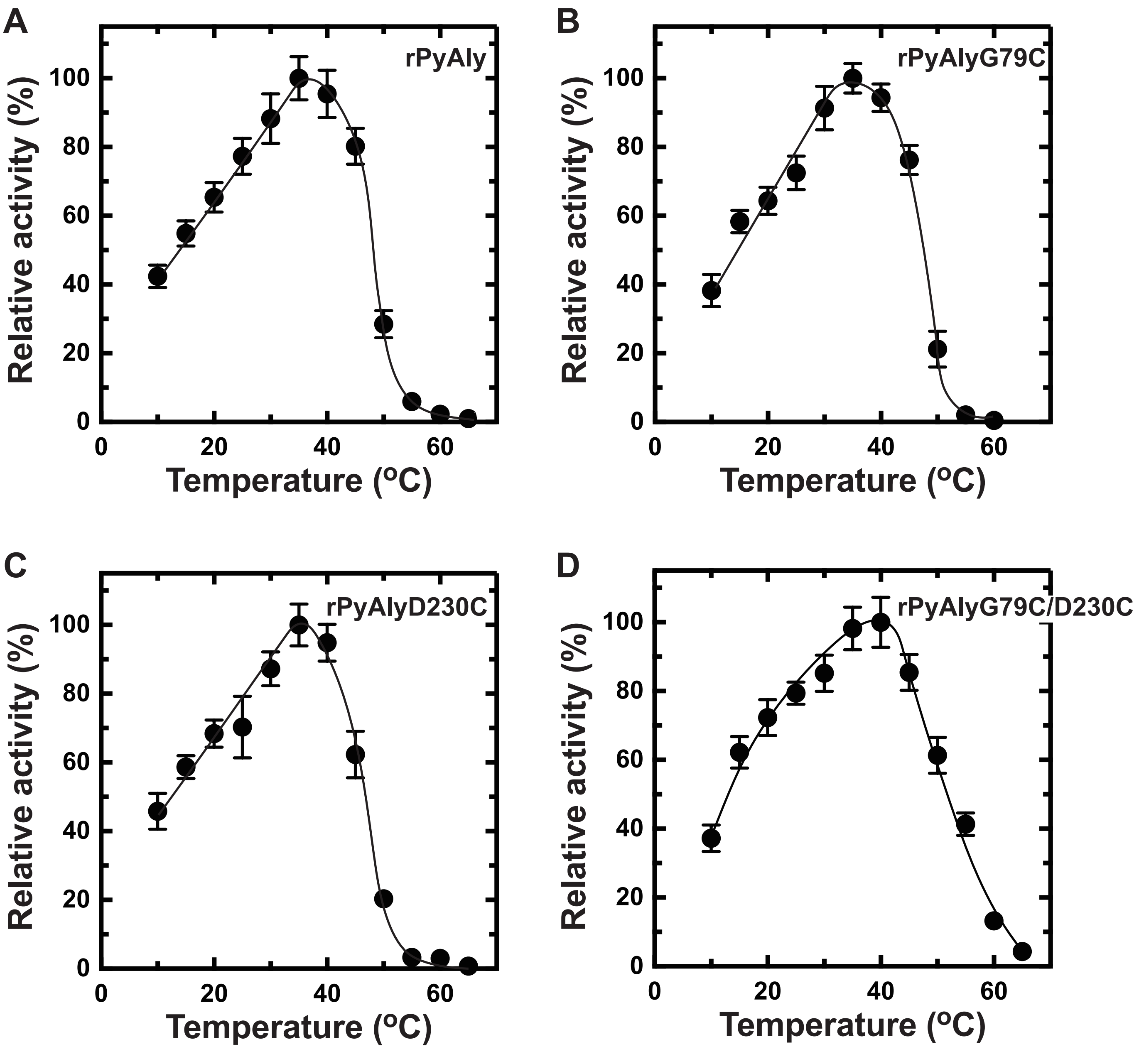

A
rPyAly
B
rPyAlyG79C
C
rPyAlyD230C
D
rPyAlyG79C/D230C
Relative activity (%)
0
20
40
60
80
100
Temperature (°C)
0
20
40
60

Fig. 11

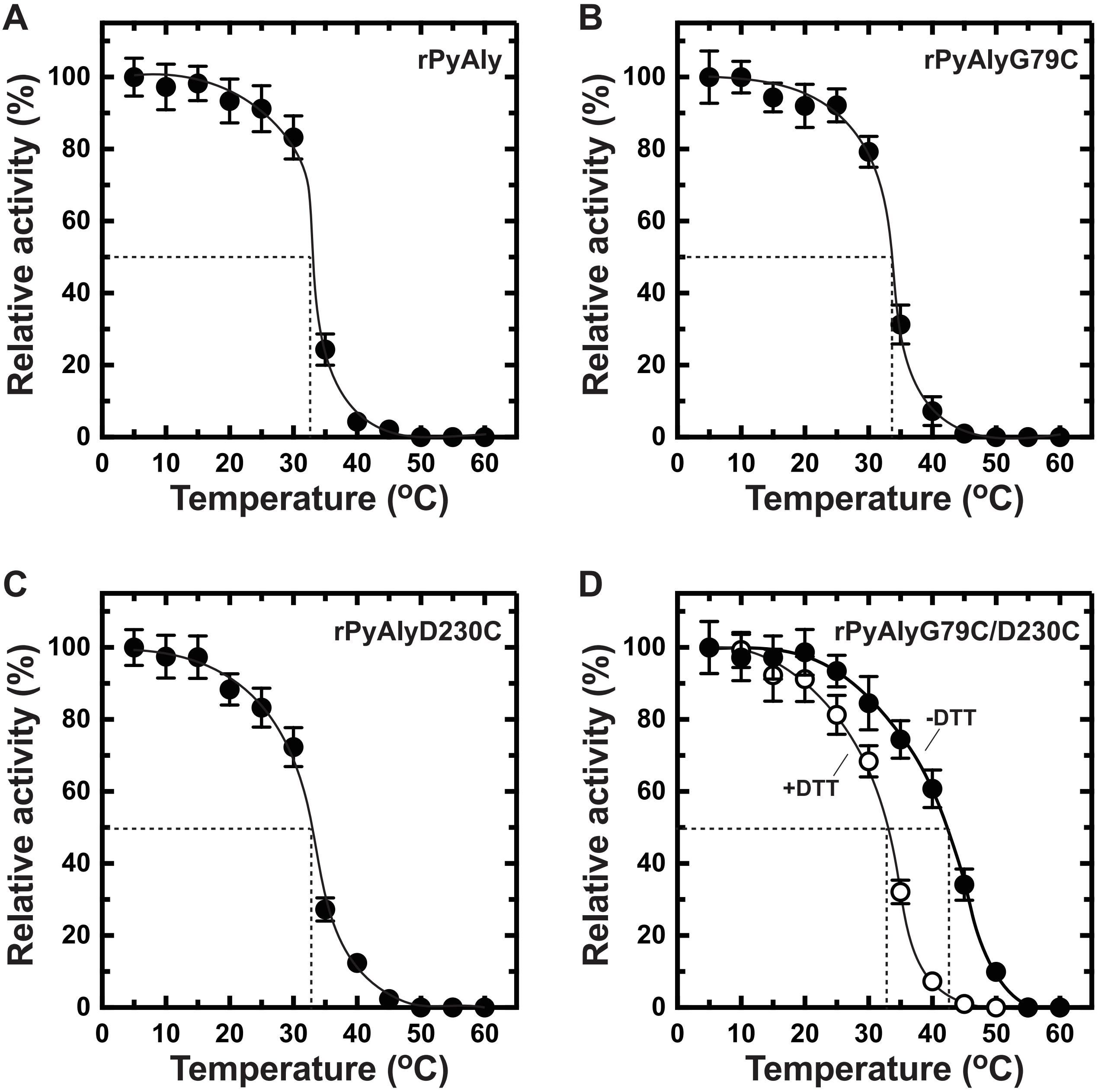
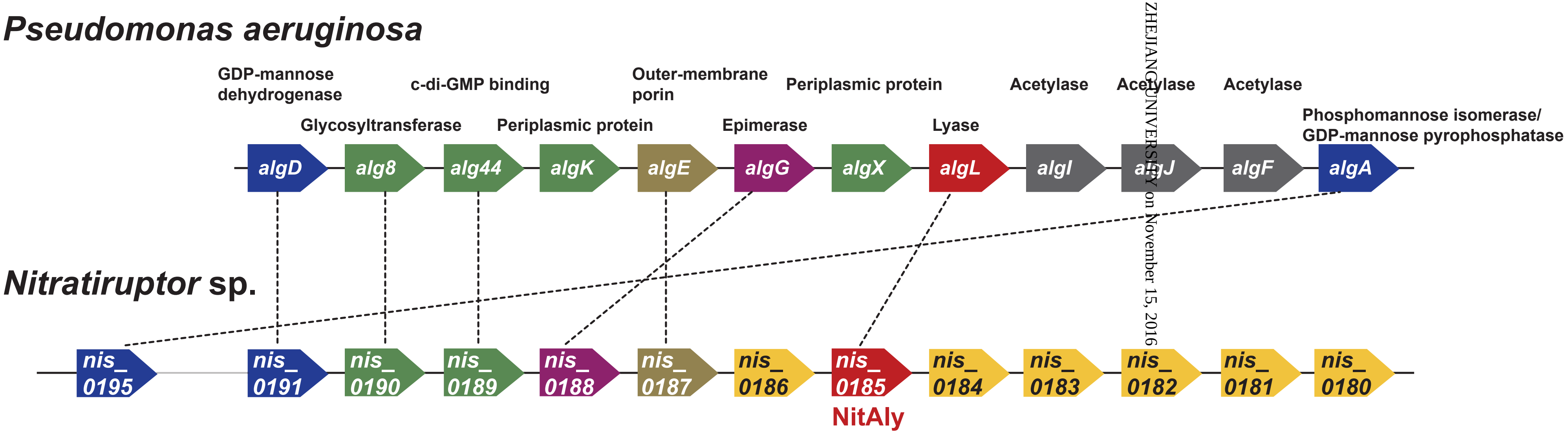

A
rPyAly
B
rPyAlyG79C
C
rPyAlyD230C
D
rPyAlyG79C/D230C
-DTT
+DTT
Relative activity (%)
0
20
40
60
80
100
Temperature (oC)
0
10
20
30
40
50
60

Fig. 12


Pseudomonas aeruginosa
GDP-mannose dehydrogenase
c-di-GMP binding
Outer-membrane porin
Periplasmic protein
Acetylase
Acetylase
Acetylase
Glycosyltransferase
Periplasmic protein
Epimerase
Lyase
Phosphomannose isomerase/ GDP-mannose pyrophosphatase
algD
alg8
alg44
algK
algE
algG
algX
algL
algI
algJ
algF
algA
Nitratiruptor sp.
nis_0195
nis_0191
nis_0190
nis_0189
nis_0188
nis_0187
nis_0186
nis_0185
nis_0184
nis_0183
nis_0182
nis_0181
nis_0180
NitAly

Discovery of a Novel Alginate Lyase from *Nitratiruptor* sp. SB155-2 Thriving at Deep-sea Hydrothermal Vents and Identification of the Residues Responsible for its Heat Stability

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