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Title: The role of calcium binding to the EF-hand-like motif in bacterial solute-binding protein for alginate import

Running Head: Bacterial alginate-binding protein

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Abstract (146/150 words)

Gram-negative Sphingomonas sp. A1 incorporates acidic polysaccharide alginate into the cytoplasm via a cell-surface alginate-binding protein (AlgQ2)-dependent ATP-binding cassette transporter (AlgM1M2SS). We investigated the function of calcium bound to the EF-hand-like motif in AlgQ2 by introducing mutations at the calcium-binding site. The X-ray crystallography of the AlgQ2 mutant (D179A/E180A) demonstrated the absence...
of calcium binding and significant disorder of the EF-hand-like motif. Distinct from the wild-type AlgQ2, the mutant was quite unstable at temperature of strain A1 growth, although unsaturated alginate oligosaccharides stabilized the mutant by formation of substrate/protein complex. In the assay of ATPase and alginate transport by AlgM1M2SS reconstructed in the liposome, the wild-type and mutant AlgQ2 induced AlgM1M2SS ATPase activity in the presence of unsaturated alginate tetrasaccharide. These results indicate that the calcium bound to EF-hand-like motif stabilizes the substrate-unbound AlgQ2 but is not required for the complexation of substrate-bound AlgQ2 and AlgM1M2SS.

Keywords
ABC transporter, calcium, EF-hand-like motif, solute-binding protein, X-ray crystallography

Introduction
ATP-binding cassette (ABC) transporters require the energy generated via ATP hydrolysis (ATPase) to translocate a variety of molecules across the cytoplasmic membrane (Davidson et al. 2008). ABC transporters can be either importers or exporters. ABC importers are mainly produced by prokaryotes, whereas ABC exporters are present in all species (Locher 2008). Bacterial ABC importers are classified into three types: type I, type II, and type III (Rice et al. 2014). Type I and II ABC importers require solute-binding protein (SBP) to transport substrate (Oldham et al. 2008). SBP consists of two large domains that are connected by hinge loops and adopts two conformations, namely, an open substrate-unbound conformation and a closed substrate-bound conformation.
ABC transporters are composed of two transmembrane domains (TMD) and two nucleotide-binding domains (NBD). ATPase activity by an NBD causes the TMD to change conformation from inward-facing to outward-facing. It is important for the substrate transport in order to alter the structural conformation (ter Beek et al. 2014).

Although SBPs were classified into six clusters based on structural features (Berntsson et al. 2010), a new seventh SBP cluster, characterized by an EF-hand-like calcium-binding motif has been proposed (Scheepers et al. 2016). Similarity in sequence and structure is observed among SBPs assigned to the seventh cluster, while there is little similarity between the seventh cluster and other clusters. The EF-hand-like helix–loop–strand motif in SBPs of the seventh cluster has a metal-binding loop that consists of nine amino acid residues, and is located near the SBP-TMD interaction surface. FusA, the SBP for fructooligosaccharide import in *Streptococcus pneumoniae*, is a member of the new SBP cluster identified by its EF-hand-like motif (Culurgioni et al. 2017). Mutations in the calcium-binding residues of the FusA EF-hand-like motif render *S. pneumoniae* unable to grow on nystose, a fructotetrasaccharide, as a sole carbon source. This result indicates that EF-hand-like motif-bound calcium is important for fructooligosaccharide import (Culurgioni et al. 2017).

Alginate is an acidic polysaccharide that consists of β-D-mannuronate (M) and α-L-guluronate (G) (Gacesa 1988). The gram-negative *Sphingomonas* sp. A1 (strain A1) directly incorporates the alginate polymer through the cell-surface pit (Hisano et al. 1996). The periplasmic SBP (AlgQ1 or AlgQ2) and inner-membrane ABC transporter (AlgM1M2SS) cooperatively transport alginate from periplasm to cytoplasm across the inner membrane (Momma et al. 2000). AlgM1 and AlgM2 correspond to the TMD, and
two AlgS molecules correspond to the NBD. AlgQ1 and AlgQ2 are mutually similar (sequence identity: 76%), and both bind alginate and pass the polymer to AlgM1M2SS at the same level (Nishitani et al. 2012). AlgQ1 or AlgQ2 binds alginate to form a closed conformation, and alginate-bound AlgQ1 or AlgQ2 interacts with AlgM1M2SS to enhance ATPase activity by AlgS (Kaneko et al. 2017). Both AlgQ1 and AlgQ2 have an EF-hand-like calcium-binding motif. In the complex structure of AlgQ2 and AlgM1M2SS, the EF-hand-like motif is located near the interface between AlgQ2 and AlgM2 (Figure 1a) (Maruyama et al. 2015), suggesting that calcium bound to the EF-hand-like motif plays a significant role in the interaction between AlgQ2 and AlgM1M2SS (Culurgioni et al. 2017).

The EF-hand motif has a helix–loop–helix conformation in which two helices lie in near-parallel arrangement, and the calcium ions are coordinated by residues within a loop. The representative EF-hand loop is composed of 12 amino acid residues and is called canonical EF-hand loop. There are variations in the length of the loop from 9 to 15 residues (Gifford et al. 2007, Delfina et al. 2015). Due to these variations, EF-hand-like motif is postulated. The EF-hand-like motif of SBPs in the seventh cluster has a metal-binding loop consisting of nine amino acid residues. Calcium in the calmodulin EF hand is known to function in signal transduction or protein interaction (Carafoli 2002; Ababou and Zaleska 2002). However, the role of calcium bound to the EF-hand-like motif in SBP interacting with ABC transporter remains unclear. This paper provides a structural and functional characterization of calcium-unbound SBP (AlgQ2) via X-ray crystallography and in vitro assay using ABC transporter (AlgM1M2SS) in liposomes.

Materials and methods
Preparation of alginate oligosaccharides

Sodium alginate (Nacalai Tesque, Extra Pure Reagent grade, product No. 31132-75) was treated with 0.1 mg/mL of endolytic poly(mannuronate) alginate lyase from Flavobacterium (Sigma-Aldrich) for 30 min at 25°C. The products were separated via anion-exchange chromatography [TOYOPEARL DEAE-650M (Tosoh)] and eluted with a gradient of ammonium bicarbonate 0 to 1 M. The oligosaccharide length was confirmed via thin-layer chromatography using standard markers. After lyophilization, the unsaturated alginate trisaccharide was dissolved in distilled water.

Unsaturated tetramannuronate Δ4M substrate was prepared from alginate as reported (Nishitani et al. 2012). To prepare PAΔ4M, Δ4M was labeled with 2-aminopyridine and purified via column chromatography as reported (Maruyama et al. 2015).

Site-directed mutagenesis of AlgQ2

To introduce alanine substitution in Asp179 and Glu180, inverse PCR was performed using a vector (pAQ2) used for Escherichia coli AlgQ2 expression (Momma et al. 2002) as a template, synthetic oligonucleotide DNA as a primer (Forward: GGCAACGGCAAGGCCGCTGCAATTCCGTTCATCAAC, Reverse: GTTGATGAACGGAATTGCAGCGGCCTTGCCGTTGCC; the mutation sites are underlined), and KOD FX Neo (TOYOBO) as a DNA polymerase. The PCR product was treated with DpnI and used to transform E. coli BL21(DE3)pLysS. E. coli BL21(DE3)pLysS cells harboring mutant pAQ2 were grown in LB medium containing 100 µg/mL of sodium ampicillin, collected via centrifugation at 4,000 g and 4°C for 20 min, and resuspended in 20-mM potassium phosphate buffer (pH 6.8). The cells were ultrasonically disrupted (201M Insonator, Kubota) at 0°C for 20 min, and cell extract was obtained via centrifugation at 15,000 g for 20 min. The cell extract was dialyzed against
20-mM potassium phosphate buffer (pH 6.8) and applied to a TOYOPEARL SP-650 M (Tosoh). The protein was eluted with a linear gradient of 0–0.5 M NaCl. The fractions containing the protein were dialyzed against 20-mM Tris-HCl buffer (pH 7.5). AlgQ2 wild-type (wtAlgQ2) was purified in the same procedure as mutant AlgQ2 (mtAlgQ2). All procedures were performed at 0°C–4°C.

Quantitative analysis of calcium with inductively coupled plasma (ICP)

Purified wtAlgQ2 and mtAlgQ2 were diluted with 20-mM Tris-HCl (pH 7.5) to a concentration of 10 µM determined based on the extinction coefficient calculated by the primary structure of AlgQ2 (the protein concentration was approximately determined to 1 mg/mL when the absorbance at 280 nm of the protein solution showed 2). Nitric acid (2 mL) was added to 2 mL of each sample and applied to microwave digestion with UltraWAVE (Milestone General). The samples were allowed to cool and mixed up with ultrapure water. These samples were applied to an ICP emission spectrometer (ICPS-8100, Shimadzu) to quantify calcium concentration.

X-ray crystallography of mtAlgQ2

mtAlgQ2 crystallization was performed in the presence of unsaturated alginate trisaccharide using the sitting-drop vapor diffusion method at 20°C for several weeks. mtAlgQ2 mixed with unsaturated alginate trisaccharide substrate was crystallized in 0.2 M sodium formate, 0.1 M 1,3-bis[tris(hydroxymethyl)methylamino]propane (pH 8.5), and 20% polyethylene glycol 3350. A crystal was picked up using a mounted nylon loop (Hampton Research) and placed directly into a cold nitrogen gas stream at −173°C. For cryoprotection, a protein crystal was soaked in the reservoir solution containing 20% glycerol. The diffraction data were collected at a wavelength of 1.0000 Å using a MAR225HE detector at BL38B1 beamline in SPring-8 (Hyogo, Japan) and processed.
with HKL2000 (Otwinowski and Minor 1997). The structure was solved by molecular
replacement using Molrep (Vagin and Teplyakov 1997) with the coordinates of wtAlgQ2
(PDB ID: 1J1N) as an initial search model and refined with Refmac5 (Murshudov et al.
1997). The winCoot program (Emsley and Cowtan 2004) was utilized for model
modification. To build the ligand, preinstalled coordinates and cif dictionaries were used
in winCoot. The final model was evaluated using PROCHECK (Laskowski et al. 1993).
The figures were prepared using Pymol (Schrodinger 2010). The atomic coordinates and
structure factors (code 6JHX for mtAlgQ2) were deposited in the Protein Data Bank,
Research Collaboratory for Structural Bioinformatics, Rutgers University, New
Brunswick, NJ (http://www.rcsb.org/).

**Differential scanning fluorimetry (DSF) analysis**

The thermal stability of wtAlgQ2 and mtAlgQ2 in the presence or absence of 20-μM
CaCl₂ and/or 50-μM Δ4M was investigated via DSF (Niesen et al. 2007). This experiment
was conducted using a real-time PCR instrument (MyiQ2, Bio-Rad). The reaction
mixture consisted of 1.7 μM of wtAlgQ2 or mtAlgQ2, 60-fold diluted SYPRO Orange
(Invitrogen), and 20-mM Tris-HCl (pH 7.5). The reaction mixtures were subjected to heat
treatment, and the temperature was increased from 25°C to 95°C by 0.5°C/cycle (10
s/cycle) for 141 cycles. Fluorescence emitted from SYPRO Orange bound to denatured
protein was measured by excitation at 492 nm and emission at 610 nm. Apparent melting
temperature (T_m) was calculated as the midpoint of increase in the fluorescence profile.
The fluorescence profile was analyzed with iQ5 (Bio-Rad) to determine the apparent T_m.

**Native PAGE**

Native PAGE was performed to examine the binding affinity of wtAlgQ2 or mtAlgQ2
with Δ4M. wtAlgQ2 or mtAlgQ2 (17 μM) was mixed with 0- to 22-μM Δ4M at 4°C to
avoid denaturation of mtAlgQ2. Electrophoresis was performed at 4°C and 10 mA for 2 h using 5% polyacrylamide gel and a running buffer consisting of 43-mM imidazole and 35-mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) (pH 7.4) (McLellan 1982) without heat pretreatment. The protein band densities on the gel were quantified using ImageJ (Schneider et al. 2012).

**ATPase activity of AlgM1M2SS**

Expression and purification of AlgM1M2SS [AlgM1(d0)M2(H10)SS(WT)], in which 10 histidine residues were added to the C terminus of AlgM2, were conducted as described (Maruyama et al. 2015). AlgM1M2SS was reconstructed in the liposome produced by L-α-phosphatidylcholine Type IV-S (Sigma-Aldrich) derived from soybean (Maruyama et al. 2015). The ATPase activity of the proteoliposome was measured in 200 µL of reaction mixtures containing 0.1-µM AlgM1M2SS reconstituted in the liposome, 5-mM MgCl₂, 5-mM ATP, 20-mM Tris-HCl (pH 8.0), 20-µM Δ4M or PAΔ4M, and 1-µM AlgQ2. The reaction mixtures were incubated at 37°C. The samples (30 µL) were collected from the reaction mixture at various times and added to 30 µL of 12% sodium dodecyl sulfate to stop the reaction. Inorganic phosphate was quantified via phosphomolybdic acid colorimetry (Chifflet et al. 1998). ATPase activity was measured as inorganic phosphate per min, and the same experiment was conducted in the absence of AlgM1M2SS.

**Alginate transport activity**

AlgM1M2SS was reconstructed in the liposome produced by L-α-phosphatidylcholine Type II-S (Sigma-Aldrich) derived from soybean as reported (Maruyama et al. 2015). To prepare ATP and MgCl₂ containing the proteoliposome, the mixture of proteoliposome, ATP, and MgCl₂ was freeze-thawed three times with liquid nitrogen and a water bath, followed by Nuclepore membrane (Whatman, pore size of 0.1 µm) filtration using a Mini-
Extruder (Avanti Polar Lipids). Alginate transport activity was measured in 200-µL reaction mixtures containing 0.1-µM AlgM1M2SS reconstituted in liposomes, 1-mM MgCl₂, 5-mM ATP, 20-mM Tris-HCl (pH 8.0), 20-µM PAΔ4M, and 1-µM AlgQ2. The reaction mixtures were incubated at 37°C for 1 h. After the reaction, the proteoliposome was collected via ultracentrifugation (4°C, 192,000 g, 30 min). The collected liposomes were diluted in pure water and disrupted by vortexing. The concentration of PAΔ4M was calculated from the fluorescence intensity (Ex, 310 nm; Em, 380 nm). Liposomes without AlgM1M2SS were reacted in 20-µM PAΔ4M and 1-µM wtAlgQ2 or mtAlgQ2 to subtract the values of the AlgM1M2SS transport activity under each condition. In the negative control [AlgQ2(−)/PAΔ4M(+) and AlgQ2(−)/PAΔ4M(−)], the mean value of the observed transport activity in liposomes without AlgM1M2SS in the presence of wtAlgQ2 and mtAlgQ2 was subtracted.

Results

Mutations at the EF-hand-like calcium-binding site

The crystal structure of AlgQ2 was described elsewhere (Momma et al. 2002). Five amino acid residues (Asn173, Asn175, Lys177, Asp179, and Glu180) coordinate calcium at the EF-hand-like motif of AlgQ2 (Figure 1b). mtAlgQ2 was constructed by replacing two (Asp179 and Glu180) of the five residues with alanine to disrupt the calcium-binding ability of the EF-hand-like motif. Recombinant wtAlgQ2 and mtAlgQ2 were expressed in E. coli and purified to homogeneity via column chromatography. The calcium concentration of each protein solution was quantified via ICP emission spectroscopy (Table 1). The amounts of calcium and protein were comparable in the wtAlgQ2 solution, whereas the calcium concentration was drastically reduced to 10% of the protein
concentration in the mtAlgQ2 solution. This result indicated that the mutations in calcium-coordinated amino acid residues dramatically reduced the affinity of AlgQ2 for calcium, as predicted.

**Crystal structure of calcium-unbound mtAlgQ2 in complex with substrate**

To obtain structural insights into the role of calcium at EF-hand-like motif, the mtAlgQ2 structure was determined via X-ray crystallography. The crystal of mtAlgQ2 was subjected to X-ray diffraction experiments. The statistics for data collection and structure refinement are summarized in Table 2.

The structure of mtAlgQ2 in complex with alginate trisaccharide was determined at 2.2 Å resolution (Figure 2a). No calcium was detected at the EF-hand-like motif in the crystal structure. Due to the unclear electron density map around the EF-hand-like motif loop, the residues (174–178) in the motif were omitted from the coordinates (Figure 2b). The results indicated that the mutations caused local loop destabilization. The structural identity between wtAlgQ2 and mtAlgQ2 was evaluated by superimposing the coordinates of two proteins (wtAlgQ2, PDB ID: 5H71) using the ASH program (Toh 1997; Standley et al. 2004) (Figure 2c). The global root-mean-square deviation (RMSD) was 0.47 Å for all Cα atoms, indicating that the mutations and/or the absence of calcium exerted a negligible effect on the overall structure, except for the local conformation of the EF-hand-like motif loop.

**Extreme destabilization of substrate-unbound mtAlgQ2**

The thermal stability of wtAlgQ2 and mtAlgQ2 was examined via DSF (Niesen et al. 2007). The fluorescence intensity derived from SYPRO Orange bound to denatured protein was measured via a melting curve analysis using a real-time PCR instrument. wtAlgQ2 in the absence of substrate, unsaturated tetramannuronate (Δ4M), demonstrated
an apparent $T_m$ of 45.6°C (Figure 3, red). mtAlgQ2 in the absence of Δ4M emitted intense fluorescence at 25°C (Figure 3, blue), indicating that mtAlgQ2 was denatured at 25°C or less. In the presence of Δ4M at 50 µM, the wtAlgQ2 apparent $T_m$ increased to 63.2°C (Figure 3 black) (Nishitani et al. 2012), and mtAlgQ2 was stabilized to an apparent $T_m$ of 55.7°C (Figure 3, green). These results indicated that both wtAlgQ2 and mtAlgQ2 were stabilized by forming a complex with alginate oligosaccharide and that mtAlgQ2 adopts an extremely unstable substrate-unbound form (open conformation) and stable substrate-bound form (closed conformation). Calcium bound to the EF-hand-like motif is, therefore, crucial for the stabilization of substrate-unbound form. Conversely, the additional calcium in the reaction mixture at 20 µM, about 10 times the concentration of protein (1.7 µM), had a negligible effect on the stability of both proteins in the presence or absence of Δ4M (Table 3).

**mtAlgQ2 and wtAlgQ2 exhibited a comparable substrate affinity at a low temperature**

To date, the affinity between AlgQ2 and alginate oligosaccharides was measured via UV absorption difference spectroscopy (Momma et al. 2005). However, the method was unsuitable for mtAlgQ2 being cloudy even at room temperature, probably due to protein aggregation. Alternatively, to investigate the molecular state depending on substrate binding, wtAlgQ2 or mtAlgQ2 mixed with 0 or 50 µM of Δ4M was subjected to native PAGE at 4°C (Figure 4a). mtAlgQ2 in the absence of Δ4M demonstrated smear bands, whereas wtAlgQ2 formed a single clear band with a larger migration due to more negative charge. mtAlgQ2 in the absence of the substrate was denatured by the effect of electric field (10 mA, 2 h), whereas wtAlgQ2 maintained its conformation under the same conditions. Because a part of mtAlgQ2 molecules was considered to be spread in a lane
or remain a well due to its instability during electrophoresis, mtAlgQ2 showed smear, unclear, and low intensity bands. Although both substrate-bound forms produced a clear protein band at the same position, the substrate-unbound forms appeared at different positions (Figure 4a). These data support the DSF analysis revealing that mtAlgQ2 is stabilized by forming a complex with Δ4M and X-ray crystallography, demonstrating that mtAlgQ2, similar to wtAlgQ2, adopts a closed conformation in the substrate-bound form.

wtAlgQ2 and mtAlgQ2 were mixed with various concentrations (0 to 22 µM) of Δ4M and subjected to native PAGE to investigate their alginate-binding ability (Figure 4b). The band intensity corresponding to Δ4M-bound AlgQ2 was quantified. The ratios of the substrate-bound complex formed at each substrate concentration were plotted (Figure 4c). The dissociation constant ($K_d$) was calculated by fitting isotherms to the Langmuir equation (Langmuir 1918). The $K_d$ of mtAlgQ2 (4.2 µM) was comparable with that of wtAlgQ2 (7.0 µM). This result was supported by the $K_d$ (2.8–15 µM) of wtAlgQ2 for alginate oligosaccharides previously determined via UV absorption difference spectroscopy (Momma et al. 2005). The local destabilization of the EF-hand-like motif in calcium-unbound AlgQ2 seems not to affect alginate binding.

**Induction of ATPase activity through the formation of a complex with mtAlgQ2 and AlgM1M2SS**

Alginate-bound AlgQ2 interacts with AlgM1M2SS to induce ATPase activity by AlgS (Momma et al. 2000; Kaneko et al. 2017). The reconstituted AlgM1M2SS ATPase activity in liposomes was measured in the presence or absence of AlgQ2 or substrate (Figure 5a). The AlgM1M2SS-unbound liposome yielded negligible ATPase activity in the presence of AlgQ2 (wtAlgQ2 or mtAlgQ2) and substrate Δ4M. The AlgM1M2SS ATPase activity in liposomes was enhanced by combining AlgQ2 (wtAlgQ2 or mtAlgQ2)
and Δ4M, whereas the proteoliposome exhibited a basal ATPase activity in the absence of AlgQ2 (wtAlgQ2 or mtAlgQ2) or Δ4M. The enhanced AlgM1M2SS ATPase activity with mtAlgQ2 and wtAlgQ2 in the presence of substrate indicated that Δ4M-bound mtAlgQ2 could form a complex with AlgM1M2SS and induce ATPase activity.

The alginate transport activity of AlgM1M2SS in liposomes was measured using pyridylamino-modified Δ4M (PAΔ4M) as a substrate (Figure 5b). In the absence of AlgQ2, no transport activity was observed in the presence or absence of PAΔ4M. The proteoliposome exhibited transport activity in the presence of wtAlgQ2 and PAΔ4M, whereas the alginate transport activity of AlgM1M2SS in the presence of mtAlgQ2 and PAΔ4M was nearly undetectable. This suggests that, distinct from wtAlgQ2, mtAlgQ2 cannot promote the conversion of AlgM1M2SS from outward-facing to inward-facing mode (Figure 6). Although, to date, there is no effect on pyridyl amination of the substrate on the assay of ATPase activity (Maruyama et al. 2015), the ATPase activity of the proteoliposome in the presence of mtAlgQ2 and PAΔ4M was measured (Figure 5c). mtAlgQ2 exhibited a slight enhancement of the ATPase activity of AlgM1M2SS even with PAΔ4M, whereas wtAlgQ2 significantly induced ATPase activity in the presence of PAΔ4M. ATPase activity in the presence of mtAlgQ2 and PAΔ4M corresponded to the basal level of the AlgQ2-free proteoliposome in the presence of PAΔ4M.

Native PAGE of AlgQ2 and PAΔ4M was also performed to examine the binding of wtAlgQ2 and mtAlgQ2 to PAΔ4M (Figure S1). In the case of mtAlgQ2, although the electrophoretic profile changed with the concentration of PAΔ4M, multiple protein bands were observed in a single lane. The total band intensity in each of lanes corresponding to PAΔ4M suggests that the more amounts of mtAlgQ2 were electrophoresed to a lane, but not accumulated in a well, owing to stabilization of mtAlgQ2 by forming a complex with
PAΔ4M. The complex formation of mtAlgQ2 and PAΔ4M was considered to be inhibited by exogenous PA, an artificially added label to the native substrate.

Discussion

ABC importers are categorized based on the structure as type I, II, or III. Important for all ABC importers to transport molecules across the cytoplasmic membrane, types I and II require SBP, whereas type III requires an energy-coupling factor (Rice et al. 2014; Oldham et al. 2008). AlgM1M2SS is a member of type I and requires SBP (AlgQ1 or AlgQ2) to transport alginate.

A large number (over 500) of SBP structures have been identified and classified into seven clusters (A to G) (Scheepers et al. 2016). A newly proposed cluster G consists of five structure-determined proteins: strain A1 AlgQ1 and AlgQ2, S. pneumoniae FusA (PDB ID: 5G60) (Culurgioni et al. 2017), Streptobacillus moniliformis Smon0123 for the import of glycosaminoglycans (PDB ID: 5GUB) (Oiki et al. 2017), and extracellular SBP Blon_2351 (PDB ID: 3OMB) from Bifidobacterium longum subsp. infantis (unpublished).

All SBPs in cluster G have an EF-hand-like motif to bind calcium or magnesium and a relatively large molecular size. Moreover, the EF-hand-like motif is conserved in the primary structures of numerous structure-undetermined SBPs, suggesting that there may be more SBPs belonging to cluster G.

The crystal structures of calcium-bound SBPs from Thermus thermophiles HB8 and Synechocystis PCC 6803 for the transport of lactic acid and bicarbonate have been solved, although both SBPs have no EF-hand-like motif. Calcium bound to T. thermophiles SBP is easily removed by ethylenediaminetetraacetate, and the calcium-unbound SBP remains stable (Akiyama et al. 2009). In these SBPs, calcium is coordinated by amino acid...
residues and a substrate. Therefore, calcium is incorporated with a substrate or a cofactor (Koropatkin et al. 2007). However, calcium bound to the EF-hand-like motif is independent of interactions between AlgQ2 and a substrate, as shown by X-ray crystallography (Figure 1c) and native PAGE (Figure 4a).

In the case of another member in cluster G, FusA, calcium bound to the EF-hand-like motif stabilizes local structure, and this EF-hand-like motif loop is suggested to directly interact with FusB (Culurgioni et al. 2017). FusB is the TMD of a fructooligosaccharide-importing ABC transporter based on our crystal structure of AlgM1M2SS in complex with AlgQ2. The stabilization of the EF-hand-like motif loop by calcium is likely responsible for the interaction between Glu221 of FusA and Arg59 of FusB (residue numbers) based on the sequences registered in the database (sp_1797 and sp_1798) and differ from the numbers used previously. Therefore, calcium at the EF-hand-like motif is considered important for substrate transport due to the interaction between the closed conformation of SBP and the inward-facing ABC transporter (Culurgioni et al. 2017). On the other hand, no significant interaction between residues of the EF-hand-like motif in closed AlgQ2 and inward-facing AlgM1M2 was observed (Figure 1a). In fact, substrate Δ4M-bound mtAlgQ2 interacted with AlgM1M2SS to induce ATPase activity (Figure 5a), indicating that calcium bound to the EF-hand-like motif is independent of interaction between closed AlgQ2 and inward-facing AlgM1M2SS.

*S. pneumoniae* producing FusA with a mutated EF-hand-like motif cannot grow on nystose minimal medium, although the mutant FusA maintains affinity with fructooligosaccharides at 25°C. In the case of strain A1, the apparent T_m of mtAlgQ2 was less than 25°C (Figure 3), suggesting that calcium-binding restriction causes global destabilization of substrate-unbound AlgQ2. Since strain A1 is a medium-temperature
bacterium showing optimum growth at around 35°C, this destabilization of substrate-unbound SBP is considered lethal for strain A1 in an alginate-minimal medium. The mutation caused local destabilization of the EF-hand-like motif loop in the closed conformation of both AlgQ2 and FusA (Figure 2b) (Culurgioni et al. 2017). In the open conformation, calcium bound to EF-hand-like motif in AlgQ2 plays a role in the stabilization of the overall structure, and in the closed conformation, it stabilizes the EF-hand-like motif loop.

The substrate transport mechanism of ABC importers has been well studied in MalFGK2, the maltose transporter in *E. coli* and a type I ABC importer (Oldham et al. 2007; Gould et al. 2009; Oldham et al. 2013; Bao et al. 2015). From the substrate transport scheme proposed in the MalFGK2 and ATPase induction mechanism of AlgM1M2SS (Kaneko et al. 2017), the transport of alginate by AlgQ2 and AlgM1M2SS is assumed to involve five (I to V) steps as follows (Figure 6). (I) AlgQ2 binds alginate to form a closed conformation. (II) Substrate-bound AlgQ2 interacts with inward-facing AlgM1M2SS to induce ATPase activity and AlgSs binding of ATP. (III) AlgQ2 and AlgM1M2SS change to outward-facing mode. (IV) AlgQ2 passes alginate to AlgM1M2SS. (V) AlgQ2 liberates from AlgM1M2SS, and AlgM1M2SS changes to inward-facing mode. The enhancement of ATPase activity by mtAlgQ2 in the presence of substrate Δ4M suggests that the conformational change occurs from inward- to outward-facing mode in the TMD (AlgM1M2) and from open to closed conformation in the NBD (AlgSS). With PAΔ4M substrate, no transport activity was detected because PAΔ4M is unsuitable for inducing ATPase activity by mtAlgQ2, suggesting that the transport procedure stops at step I.

In conclusion, calcium bound to the EF-hand-like motif is crucial for stabilizing
substrate-unbound AlgQ2 in the open conformation, especially at temperatures greater than 25°C, not for the direct interaction between substrate-bound AlgQ2 in the closed conformation and inward-facing AlgM1M2SS. Finally, this demonstrates that calcium bound to AlgQ2 is essential for alginate import at strain A1-growing temperature.

Supplementary material
Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability
The data underlying this article will be shared on reasonable request to the corresponding author.

Author contribution
K. O and Y. M. performed the experiments. K. O., Y. M., R. T., B. M, K. M., and W. H. analyzed the data. Y. M. and W. H. designed the study. K. O., Y. M., R. T., and W. H. wrote the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Ter Beek J, Guskov A, and Slotboom D J. Structural diversity of ABC transporters. *J Gen
Physiol 2014;143:419-35.


Table 1. Calcium content of purified AlgQ2

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Table 2. Data collection and refinement statistics

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Figure legends

Figure 1. X-ray crystal structures of ABC transporter and AlgQ2. (a) (Left) The complex structure of AlgQ2 and AlgM1M2SS (PDB ID: 4TQU). Magenta, AlgQ2; green, AlgM1; cyan, AlgM2; orange, AlgS. Calcium ion in AlgQ2 is indicated by a yellow ball. (Right) Magnified image at the interface between AlgQ2 and AlgM2. (b) EF-hand-like calcium-binding site of AlgQ2. (c) calcium (yellow ball) is far from the substrate (red/white balls)-binding site of AlgQ2.

Figure 2. X-ray crystal structure of mtAlgQ2. (a) Overall structure of mtAlgQ2. Gray and red-colored sticks indicate unsaturated alginate trisaccharide. (b) (Left) Structure of mutated EF-hand-like motif. Red, EF-hand-like motif helix; green, loop. (Right) The figure shows the $2F_o-F_c$ map contoured at 1.2 $\sigma$ around the EF-hand-like motif. (c) Superimposing of wtAlgQ2 and mtAlgQ2 for all C$\alpha$ atoms. Blue, mtAlgQ2; orange, wtAlgQ2. Calcium ion is indicated by a yellow ball.

Figure 3. Thermal shift assay by DSF. Top, fluorescent profile. Bottom, negative derivative curve plot of the fluorescent profile. Red, wtAlgQ2 without $\Delta$4M; blue, mtAlgQ2 without $\Delta$4M; black, wtAlgQ2 with $\Delta$4M; green, mtAlgQ2 with $\Delta$4M.

Figure 4. Native PAGE profile of AlgQ2. (a) wtAlgQ2 or mtAlgQ2 with or without 50-$\mu$M $\Delta$4M. (b) Binding ability of wtAlgQ2 (upper) and mtAlgQ2 (lower) to $\Delta$4M in proportion to increasing substrate concentration. (c) Profile of formation of AlgQ2 and $\Delta$4M complex. Circle; wtAlgQ2, square; mtAlgQ2.

Figure 5. ATPase and transport activity of AlgM1M2SS. (a) ATPase activity in the presence (+) or absence (−) of each component in the reaction mixture. ATPase activity was represented as phosphate (nmol) produced by 1-mg AlgM1M2SS per 1 min. (b) Transport activity. Transport activity used PA$\Delta$4M as a substrate in the presence (+) or
absence (−) of each component in the reaction mixture. Activity of AlgM1M2SS in the presence of wtAlgQ2 was taken as 100%. Negative values indicate no transport activity calculated by subtraction of the mean values of the observed transport activity in liposomes without AlgM1M2SS. (c) ATPase activity using PAΔ4M as a substrate in the presence (+) or absence (−) of each component in the reaction mixture. Assays were performed three times, and the error bars represent SE.

Figure 6. Mechanistic model of ABC transporter for alginate import. The dynamics of ABC transporter machinery model. Pink, wtAlgQ2; purple, mtAlgQ2; light yellow, calcium; gray, alginate; green, AlgM1; blue, AlgM2; dark yellow, AlgS.
Graphical abstract. The calcium bound to EF-hand-like motif stabilizes the substrate-unbound AlgQ2, while calcium-free AlgQ2 adopts a closed conformation in complex with substrate.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
**Figure S1.** PAΔ4M-binding ability of wtAlgQ2 and mtAlgQ2 with increasing PAΔ4M concentration. Top, wtAlgQ2; bottom, mtAlgQ2. The difference in the band profile from Figure 4b was thought to be due to positive-charge donated by PA.