A solute-binding protein in the closed conformation induces ATP hydrolysis in a bacterial ATP-binding cassette transporter involved in the import of alginate

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The Gram-negative bacterium Sphingomonas sp. A1 incorporates alginate into cells via the cell-surface pit without prior depolymerization by extracellular enzymes. Alginate import across cytoplasmic membranes thereby depends on the ATP-binding cassette transporter AlgM1M2SS (a heterotetramer of AlgM1, AlgM2, and AlgS), which cooperates with the periplasmic solute-binding protein AlgQ1 or AlgQ2; however, several details of AlgM1M2SS-mediated alginate import are not well-understood. Herein, we analyzed ATPase and transport activities of AlgM1M2SS after reconstitution into liposomes with AlgQ2 and alginate oligosaccharide substrates having different polymerization degrees (PDs). Longer alginate oligosaccharides (PD ≥ 5) stimulated the ATPase activity of AlgM1M2SS but were inert as substrates of AlgM1M2SS-mediated transport, indicating that AlgM1M2SS-mediated ATP hydrolysis can be stimulated independently of substrate transport. Using X-ray crystallography in the presence of AlgQ2 and long alginate oligosaccharides (PD 6–8) and with the hummid air and glue-coating method, we determined the crystal structure of AlgM1M2SS in complex with oligosaccharide-bound AlgQ2 at 3.6 Å resolution. The structure of the ATP-binding cassette transporter in complex with non-transport ligand-bound periplasmic solute-binding protein revealed that AlgM1M2SS and AlgQ2 adopt inward-facing and closed conformations, respectively. These in vitro assays and structural analyses indicated that interactions between AlgM1M2SS in the inward-facing conformation and periplasmic ligand-bound AlgQ2 in the closed conformation induce ATP hydrolysis by the ATP-binding protein AlgS. We conclude that substrate-bound AlgQ2 in the closed conformation initially interacts with AlgM1M2SS, the AlgM1M2SS–AlgQ2 complex then forms, and this formation is followed by ATP hydrolysis.

ATP-binding cassette (ABC) transporters are present in cytoplasmic membranes of all species, and the translocation of substrates by transporters is coupled with ATP hydrolysis (1). ABC transporters comprise two transmembrane domains and two nucleotide-binding domains. The transmembrane domains form the translocation pathway, and the nucleotide-binding domains hydrolyze ATP to generate energy for transport (2). ABC transporters are classified as exporters and importers (3), and whereas ABC exporters are ubiquitous from bacteria to humans, ABC importers are mainly found in prokaryotes (4) and require accessory solute-binding proteins (5).

Several ABC importers, such as MalFGK2 (6), the maltose transporter in Escherichia coli, and the E. coli vitamin B12 transporter BtuCD (7) have been well-characterized. When importing substrate, ABC importers change conformations from the inward-facing to outward-facing states (5). However, the mechanisms that regulate this conformational change and ATP hydrolysis of ABC importers are not fully understood.

Interactions between the ABC transporter MalFGK2 and the periplasmic maltose-binding protein MalE induce ATP hydrolysis (8). Moreover, a mutant MalE was shown to bind sucrose (non-transport ligand) and enhance the ATPase activity of MalFGK2, suggesting that ATP hydrolysis is induced by ligand-bound MalE but not by the translocated substrate (i.e., maltose).

Recently, two models of solute-binding protein MalE conformations in contact with MalFGK2 were described as closed (6) and open states (9). However, these findings were based on in vitro assays, and the structure of the ABC transporter in complex with the solute-binding protein with a non-transport ligand has not been directly characterized.

The abbreviations used are: ABC, ATP-binding cassette; M, β-1-manuronic acid; G, α-1-guluronic acid; FACE, fluorophore-assisted carbohydrate electrophoresis; PA, pyridylamino; HAG, humid air and glue-coating; ADA, N-(2-acetamido)iminodiacetic acid; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane sulfonate; PVA, polyvinyl alcohol; PD, polymerization degree; RMSD, root mean square deviation; strain A1, Sphingomonas sp. strain A1.

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Alginic is a linear acidic polysaccharide that comprises β-D-mannuronate (M) and its C-5-epimer α-L-guluronic (G). The Gram-negative and alginate-assimilating bacterium *Sphingomonas* sp. strain A1 incorporates polysaccharides into cells through cell-surface pits (10). Genes that are involved in alginate import and degradation form a cluster on the genome of strain A1 and encode the periplasmic alginate-binding proteins AlgQ1 and AlgQ2; the cytoplasmic membrane-bound ABC transporter (AlgM1M2SS); the cytoplasmic endotype alginate lyases A1-I, A1-II, and A1-III; the exotype alginate lyase (A1-IV); and the alginate-dependent transcription factor AlgO (11, 12). Alginate concentrated in the cell-surface pit is incorporated into the periplasm and is delivered to the ABC transporter by alginate-binding proteins. The ABC transporter for alginate import is a heterotetramer comprising transmembrane domains (heterodimer of AlgM1 and AlgM2) and an ATP-binding domain (homodimer of AlgS). After import into the cytoplasm by the ABC transporter, alginate is depolymerized to monosaccharides by endo- and exotype alginate lyases. Moreover, the genetic cluster for import and degradation of alginate is expressed in the presence of the polysaccharide under the control of the transcription factor AlgO (13).

Characteristics of ATPase and transport activities of reconstituted AlgM1M2SS in liposomes and crystal structures of the transporter have mainly been determined using alginate oligosaccharides with polymerization degrees (PDs) of <4 (14–16). In contrast with the *E. coli* maltose ABC transporter, interactions of AlgM1M2SS with AlgQ2 result in the formation of an alginate-binding tunnel that is accessible to solvent. In the present study, *in vitro* assays of the ATPase and transport activities of AlgM1M2SS were performed using long alginate oligosaccharides (PD ≈ 5), and non-transport ligand–bound AlgQ2 in the closed conformation interacted with the ABC transporter in the inward-facing conformation. These X-ray crystallography structure analyses were performed using the humid air and glue-coating method and demonstrated that the solute-binding protein in the closed conformation induces ATP hydrolysis in this bacterial ABC transporter.

**Results**

**Purification of alginate oligosaccharides**

M-rich polysaccharides (M-block) were obtained from alginate, and unsaturated M oligosaccharides with C=C bonds in their non-reducing ends were prepared by treatment of M-block with alginate lyase. Saturated M oligosaccharides were then prepared by acid hydrolysis of M-block, and unsaturated M oligosaccharides were further fractionated by anion exchange chromatography into unsaturated M disaccharide (Δ2M), unsaturated M trisaccharide (Δ3M), unsaturated M tetrasaccharide (Δ4M), unsaturated M pentasaccharide (Δ5M), unsaturated M hexasaccharide (Δ6M), and a mixture of unsaturated M hepta- and octasaccharides (Δ7-8M). Saturated M oligosaccharides were also divided into mixtures of saturated M hexa-, hepta-, and octasaccharides (6–8M) and of saturated M hepta-, octa-, ennea-, and decasaccharides (7–10M) using anion exchange chromatography. PDs in each oligosaccharide were determined using fluorophore-assisted carbohydrate electrophoresis (FACE) (17) (Fig. 1A), and the alginate oligosaccharides and their pyridylamino (PA)-saccharides fluorescence-labeled with 2-aminopyridine were used for subsequent experiments.

**Co-immunoprecipitation of AlgQ2 by AlgM1M2SS**

Co-immunoprecipitation assays were conducted to analyze interactions between AlgM1M2SS and AlgQ2. In these analyses, recombinant AlgM1M2SS and AlgQ2 were expressed in *E. coli* cells and were purified to homogeneity (Fig. 1B). After interactions of anti-His tag antibody conjugated Dynabeads Protein G (Veritas) and AlgM1M2SS containing a histidine tag at the C terminus of AlgM2, AlgQ2 was added in the presence or absence of ATP and Δ3M. Dynabeads Protein G was then collected, and bound proteins were subjected to SDS-PAGE, followed by Western blotting using anti-AlgQ2 antiseraum (18) (Fig. 2). AlgQ2 was observed only in the presence of both ATP and Δ3M (Fig. 2, lanes 2 and 3), and no interactions between AlgM1M2SS and AlgQ2 occurred in the absence of Δ3M (Fig. 2, lane 1). No AlgQ2 was detected in the immunoprecipitation assay using other His tag protein as a negative control in place of AlgM1M2SS (data not shown). Because AlgQ2 comprises N- and C-terminal domains and adopts substrate-free open and substrate-bound closed conformations through domain dynamics (16, 19), these co-immunoprecipitation assays suggested that Δ3M-bound AlgQ2 interacts with AlgM1M2SS in the closed conformation. However, the substrate-free AlgQ2 in the open conformation did not interact with AlgM1M2SS. Moreover, following the addition of vanadate, the intensity of
The AlgQ2 band increased slightly in the presence of ATP and Δ3M (Fig. 2, lane 3). The phosphate analogue vanadate bound the ATP-binding site with ADP and inhibited ATP hydrolysis. Hence, the increase in AlgQ2 band intensity probably reflects inhibited dissociation of AlgQ2 from AlgM1M2SS, suggesting that this dissociation event is coupled with ATP hydrolysis.

ATPase and transport activities of AlgM1M2SS

ATPase and transport activities of reconstituted AlgM1M2SS in liposomes were measured in the presence of various alginate oligosaccharides and AlgQ2 (Fig. 3). Specific ATPase activity of AlgM1M2SS in the absence of saccharide was determined to around 30 nmol/min/mg. In these experiments, all alginate oligosaccharides enhanced ATPase activity, and saccharide chain lengths, structures of non-reducing ends (saturated or unsaturated), and modifications at reducing saccharide residues with PA in alginate oligosaccharides had limited effects on ATPase activities. In contrast, transport activities varied with saccharide chain lengths, with the highest transport activity in the presence of alginate tetrasaccharide as a substrate, and almost no transport of alginate oligosaccharides with PD of >5. In addition, transport of these long oligosaccharides was comparable with that of the non-alginate oligosaccharide chitotetraose, indicating that long alginate oligosaccharides with PD of >5 were not transport substrates of AlgM1M2SS in this assay system.

X-ray diffraction experiments using the humid air and glue-coating (HAG) method

Previously, the crystal structure of AlgM1M2SS was determined in complex with Δ3M-bound AlgQ2 at a resolution of 3.2 Å (14), and similar crystals were frozen using the HAG method (20) to improve X-ray diffraction images. The HAG method is a novel technique for crystal mounting, in which crystals are coated with a water-soluble polymer and are then exposed to controlled humid air using humidifier HUM-1F (RIGAKU). AlgM1M2SS crystals in complex with Δ3M-bound AlgQ2 were obtained in drop solutions comprising 22% PEG 3000, 0.1 M ADA-NaOH (pH 6.6), 0.15 M NaCl, and 16 mM CHAPSO. Crystals were then picked up using a loop covered with 10% polyvinyl alcohol (PVA) 4500 and were subjected to X-ray diffraction experiments with air controlled at 86–92% relative humidity. In these experiments, the crystals had optimal mosaicity (0.607) at 90% relative humidity, and after freezing at 90% relative humidity, crystals were exposed to X-rays and provided diffraction data at 3.4 Å resolution, allowing determination of crystal structure by molecular replacement. The overall structure of AlgM1M2SS adopted the inward-facing conformation (Fig. 4A), and the electron density map that was obtained using the HAG method included an additional electron density at C-terminal regions of AlgM1 and AlgM2. In contrast with the previous structure, three residues (from Thr-321 to Val-323) in AlgM1 and seven residues (from Gly-285 to Val-291) in AlgM2 were accurately traced in the electron density map, indicating improved X-ray diffraction data after crystalline freezing of AlgM1M2SS in complex with AlgQ2 using the HAG method.

To obtain a snapshot of the structure of the ABC transporter in complex with the non-transport ligand-bound solute-binding protein, AlgM1M2SS and AlgQ2 were crystallized in the presence of long alginate oligosaccharides. In these experiments, crystals of AlgM1M2SS, AlgQ2, and 6–8M were
obtained in a drop solution comprising 22% PEG 3000, 0.1 M ADA-NaOH (pH 6.6), 0.15 M sodium formate, 8 mM CHAPSO, and 4 mM ATP and were picked up using a loop covered with 10% PVA 4500 and 5% ethylene glycol for X-ray diffraction experiments with controlled humid air. Dehydration of the crystal at 85% relative humidity for 10 min significantly improved mosaicity (1.714–0.732), which was reduced to 0.846 at 83% relative humidity. The crystal was finally frozen and subjected to X-ray diffraction experiments at 85% relative humidity. Subsequently, the crystal structure of AlgM1M2SS in complex with long alginate oligosaccharide-bound AlgQ2 was determined at 3.6 Å using molecular replacement. In the resulting complex, AlgM1M2SS and AlgQ2 adopted the inward-facing and closed conformations, respectively (Fig. 4C). However, although crystallization was conducted in the presence of ATP, no density map of ATP was observed. Diffraction data and refinement statistics are shown in Table 1.

Alginate recognition by AlgQ2 in the complex

In addition to AlgM1M2SS in complex with long alginate oligosaccharide-bound AlgQ2, crystal structures of AlgQ2 complexed with 3M and 7–10M were determined to analyze configurations of oligosaccharides bound to AlgQ2. The crystal of Δ3M-bound AlgQ2 was then generated in drop solution comprising 30% PEG 4000, 0.1 M Tris–HCl (pH 8.5), and 0.2 M LiSO₄. AlgQ2 was also co-crystallized with 7–10M in drop solution comprising 25% PEG 4000, 0.1 M Tris–HCl (pH 8.5), and 0.2 M calcium chloride. Finally, crystal structures of Δ3M- and 7–10M-bound AlgQ2 were determined by molecular replacement at 1.55 and 2.0 Å resolution, respectively, and overall structures of both were shown to adopt the closed conformation (Fig. 4, B and D).

The present structures of AlgM1M2SS in complex with Δ3M and 6–8M-bound AlgQ2 were essentially the same as the previously determined structure (Protein Data Bank entry 4TQU), with root mean square deviations (RMSDs) of <0.6 Å for 1641 Cα atoms. Moreover, no significant differences between structures of AlgM1M2SS in complex with Δ3M and 6–8M-bound AlgQ2 were observed at the AlgM1M2SS–AlgQ2 interface, suggesting that saccharide chain lengths had no effects on structures and conformations of AlgM1M2SS and AlgQ2.

The electron density map corresponded to four saccharide residues of AlgQ2 in the complex of AlgM1M2SS and Δ3M-bound AlgQ2, whereas three saccharide residues in Δ3M were bound to AlgQ2 in a monomer form (Fig. 4, A and B). These observations suggest two binding modes of Δ3M in the AlgQ2 interaction with AlgM1M2SS. However, the present data were insufficient for analyses of occupancy and conformations of the two trisaccharides in the complex of AlgM1M2SS and Δ3M-bound AlgQ2, and the coordinates corresponding to a tetrasaccharide were included for convenience. AlgQ2 recognizes the first to fourth saccharide residues from the non-reducing end of the substrate at subsites 1–4 (15, 16, 19). In the structure of AlgM1M2SS complexed with Δ3M-bound AlgQ2, Δ3M was accommodated at subsites 1–3 or subsites 2–4 in AlgQ2, and in the latter scenario, Δ3M was considered a translocation product of Δ3M at subsites 1–3 with the slide of one residue.

**Figure 4. Overall structure.** The images on the right show the Fo – Fc map contoured at 2.8σ around the alginate oligosaccharide. A, AlgM1M2SS/AlgQ2 + Δ3M; B, AlgQ2 + Δ3M; C, AlgM1M2SS/AlgQ2 + 6–8M; D, AlgQ2 + 7–10M.
This translocation of Δ3M was probably carried out through docking of Δ3M-bound AlgQ2 and the ABC transporter AlgM1M2SS.

In structures of the AlgM1M2SS and AlgQ2 complex and AlgQ2 alone, the electron density map corresponded to only five saccharide residues of AlgQ2, despite the addition of 6–8M to the crystallization drop solution (Fig. 4, C and D). Hence, AlgQ2 may strictly recognize five saccharide residues of alginate, and the sixth or later saccharide residue is flexible due to limited interactions with AlgQ2.

Structures of the substrate-binding site of AlgQ2 in four kinds of crystals are presented in Fig. 5. Tables 2 and 3 show amino acid residues of AlgQ2 interacting with saccharide residues via hydrogen bonds (<3.5 Å) and C–C interactions (<4.4 Å), respectively. The interaction of AlgQ2 with AlgM1M2SS produced little effect on the structure of the subsite in AlgQ2 (Fig. 5) or on the substrate-binding mode (Tables 2 and 3). Saccharide residues are represented as M1, M2, M3, M4, and M5 in that order from the non-reducing end. As reported previously (16), the number of hydrogen bonds between residues of amino acids and saccharides was greatest in M1, confirming that AlgQ2 strongly recognizes the saccharide of the non-reducing end. M5 also formed C–C interactions with multiple amino acid residues, suggesting that AlgQ2 recognizes alginate at subsites 1–5.

Discussion

ABC importers are classified as type I or II based on their structures. Type I ABC transporters include the maltose transporter MalFGK2 and AlgM1M2SS, whereas the vitamin B12 transporter BtuCD is categorized as a type II ABC transporter. MalFGK2 exhibits significant ATPase activity in the presence of both maltose-binding protein (MalE) and maltose, whereas the MalE mutant (sMBP) accommodates maltose and sucrose, which is a non-transport substrate. MalFGK2 reportedly exhibited similar ATPase activity in the presence of sMBP and either maltose or sucrose (8), suggesting that ATP hydrolysis is induced by interactions between the ABC transporter and the solute-binding protein with any substrate, but not by translo-
Figure 5. Cross-eyed stereo views of the alginate-binding site of AlgQ2. A, AlgM1M2SS/AlgQ2 + Δ3M; B, AlgQ2 + Δ3M; C, AlgM1M2SS/AlgQ2 + 6–8M; D, AlgQ2 + 7–10M. ΔM and M are shown as yellow and brown saccharides, respectively. Water molecules are shown as blue spheres. Dotted lines, hydrogen bonds.
cation of the substrate by the ABC transporter. However, the structure of the MalFGK₂ and sucrose-bound sMBP complex has not been determined, and the initial interaction mode between the ABC transporter and solute-binding protein remains unknown. In contrast, when present in excess of ATP (ATP/ABC transporter activity of MalFGK₂ in the presence of MalE was higher than that in the presence of both MalE and maltose (9). These observations suggest that interactions between MalE and the ABC transporter and solute-binding protein triggers ATP hydrolysis in AlgM1M2SS. Accordingly, X-ray crystallography demonstrated that long alginate oligosaccharide-bound AlgQ2 in the closed conformation interacts with AlgM1M2SS. To the best of our knowledge, this is the first structural characterization of the complex of the ABC transporter with the non-transport ligand-bound solute-binding protein.

Although the structure of the AlgM1M2SS and Δ3M-bound AlgQ2 complex was determined previously, it remains unclear which step of the transport cycle was structurally characterized, because Δ3M was used as a transport substrate of AlgM1M2SS. In contrast, the complex structure of AlgM1M2SS with non-transport 6–8M-bound AlgQ2 is indicative of the initial moment of the interaction between AlgQ2 and AlgM1M2SS. This structure indicates that ATP binding and hydrolysis occur after the formation of the complex, because no ATP is included in the complex structure (Fig. 6). In conclusion, substrate-bound AlgQ2 in the closed conformation initially interacts with AlgM1M2SS, and the AlgM1M2SS–AlgQ2 complex forms subsequently, followed by ATP hydrolysis.

**Experimental procedures**

**Preparation and fluorescence labeling of alginate oligosaccharides**

M oligosaccharides were prepared from alginate as reported previously (15). Briefly, alginate was hydrolyzed using HCl and was divided into M-block– and G-rich saccharides (G-block)

| Table 2 | Hydrogen bond between AlgQ2 and alginate oligosaccharide |
|---------|----------------------------------------------------------|
| AlgM1M2SS/AlgQ2 + 6–8M | AlgQ2 + 7–10M | AlgM1M2SS/AlgQ2 + Δ3M | AlgQ2 + Δ3M |
| Sugar | Atom | Amino acid | Atom | Distance | Sugar | Atom | Amino acid | Atom | Distance | Sugar | Atom | Amino acid | Atom | Distance |
|-------|------|------------|------|---------|-------|------|------------|------|---------|-------|------|------------|------|---------|
| M1 | O2 | Tyr-379 OH | 3.0 | 3.0 | O2 | Asn-375 ND2 | 3.3 | 3.3 | O2 | Asn-375 ND2 | 3.1 |
| M1 | O2 | Tyr-379 OH | 3.4 | 3.1 | O2 | Tyr-379 OH | 2.9 | 2.9 | O2 | Tyr-379 OH | 3.1 |
| M1 | O3 | Tyr-379 OH | 2.2 | 2.2 | O3 | Glu-391 NE2 | 3.2 | 2.3 | O3 | Glu-391 NE2 | 3.3 |
| M1 | O4 | Glu-391 NE2 | 2.5 | 2.5 | O3 | Tyr-379 OH | 2.2 | 2.2 | O3 | Tyr-379 OH | 2.6 |
| M1 | O6A | Ser-273 OG | 3.0 | 3.0 | O6A | Gly-396 OE1 | 3.0 | 3.0 | O6A | Gly-396 OE1 | 3.0 |
| M1 | O6A | Ser-273 N | 3.4 | 3.5 | O4 | Gly-396 OE1 | 3.5 | 3.5 | O4 | Gly-396 OE1 | 3.5 |
| M1 | O6B | Ser-273 OG | 3.2 | 3.4 | O6B | Ser-273 OG | 3.4 | 3.4 | O6B | Ser-273 OG | 3.4 |
| M1 | O6B | Ser-273 N | 3.0 | 3.0 | O6B | Ser-273 N | 3.0 | 3.0 | O6B | Ser-273 N | 3.0 |
| M2 | O2 | Arg-137 NH2 | 3.2 | 3.2 | O2 | Arg-137 NH2 | 3.2 | 3.2 | O2 | Arg-137 NH2 | 3.2 |
| M2 | O2 | Trp-270 NE1 | 2.6 | 2.6 | O2 | Trp-270 NE1 | 2.6 | 2.6 | O2 | Trp-270 NE1 | 2.6 |
| M2 | O3 | Arg-186 NH2 | 3.0 | 3.0 | O3 | Arg-186 NH2 | 3.0 | 3.0 | O3 | Arg-186 NH2 | 3.0 |
| M2 | O4 | Tyr-379 OH | 2.9 | 2.9 | O4 | Tyr-379 OH | 2.9 | 2.9 | O4 | Tyr-379 OH | 2.9 |
| M2 | O6B | Arg-20 NH2 | 3.5 | 3.5 | O6B | Arg-20 NH2 | 3.5 | 3.5 | O6B | Arg-20 NH2 | 3.5 |
| M2 | O6B | Tyr-129 OH | 2.6 | 2.6 | O6B | Tyr-129 OH | 2.6 | 2.6 | O6B | Tyr-129 OH | 2.6 |
| M3 | O2 | Asp-74 OD2 | 3.3 | 3.3 | O2 | Asp-74 OD2 | 3.3 | 3.3 | O2 | Asp-74 OD2 | 3.3 |
| M3 | O3 | Arg-313 NH2 | 2.9 | 2.9 | O3 | Arg-313 NH2 | 3.0 | 3.0 | O3 | Arg-313 NH2 | 3.0 |
| M3 | O6A | Arg-313 NH2 | 3.0 | 3.0 | O6A | Arg-313 NH2 | 3.0 | 3.0 | O6A | Arg-313 NH2 | 3.0 |
| M3 | O6B | Lys-22 NZ | 3.3 | 3.3 | O6B | Lys-22 NZ | 3.3 | 3.3 | O6B | Lys-22 NZ | 3.3 |
| M4 | O3 | Asp-21 OD2 | 3.4 | 3.4 | O3 | Asp-21 OD2 | 3.4 | 3.4 | O3 | Asp-21 OD2 | 3.4 |
| M5 | O1 | Thr-443 OG1 | 3.0 | 3.0 | O1 | Thr-443 OG1 | 3.0 | 3.0 | O1 | Thr-443 OG1 | 3.0 |
| M5 | O2 | Arg-442 NE | 3.4 | 3.4 | O2 | Arg-442 NE | 3.4 | 3.4 | O2 | Arg-442 NE | 3.4 |
| M5 | O6B | Asn-53 ND2 | 2.5 | 2.5 | O6B | Asn-53 ND2 | 2.5 | 2.5 | O6B | Asn-53 ND2 | 2.5 |
based on differences in the solubility at pH 2.85. Treatment of neutralized M-block with alginate lyase provided unsaturated M oligosaccharides, and acid hydrolysis of M-block with HCl provided saturated M oligosaccharides. M oligosaccharides were then labeled with 2-aminopyridine as reported previously (14) and were then eluted through a HiTrap Q HP column (GE healthcare). Remaining reagents and unreacted oligosaccharides were excluded using anion exchange chromatography, and purified PA-saccharides were obtained.

**FACE**

FACE (17) was conducted to determine the purity of alginate oligosaccharides, which were then mixed with labeling reagent containing 75 mM 8-amino-1,3,6-naphthalenetrisulfonic acid, 0.5 M sodium cyanoborohydride, 7.5% (v/v) acetic acid, and 50% (v/v) dimethyl sulfoxide and were incubated at 37 °C for 16 h in the shade. Labeled alginate oligosaccharides were then freeze-dried and resolved in 10% glycerol and were then subjected to electrophoresis in 30% polyacrylamide gels with running buffer containing 25 mM Tris-HCl (pH 8.5) and 0.192 M glycine. Bands of labeled oligosaccharides in gels were detected using an ultra-violet lamp.

**Assays of ATPase and transport activities**

Expression and purification of AlgM1M2SS was conducted as reported previously (14). Briefly, *E. coli* BL21(DE3)Gold

**Table 3**

| Sugar | Atom | Amino acid | Atom Distance | Sugar | Atom | Amino acid | Atom Distance | Sugar | Atom | Amino acid | Atom Distance |
|-------|------|------------|---------------|-------|------|------------|---------------|-------|------|------------|---------------|
| M1 C4 | Trp-399 | CH2 | 4.4 | M1 C1 | Trp-270 | CD2 | 4.0 | M1 C1 | Trp-270 | CE3 | 4.1 | M1 C1 | Trp-270 | CD2 | 4.3 |
| M1 C4 | Trp-399 | CZ2 | 4.3 | M1 C1 | Trp-270 | CZ3 | 4.1 | M1 C1 | Trp-270 | CD2 | 4.2 | M1 C1 | Trp-270 | CD2 | 4.0 |
| M1 C5 | Trp-270 | CB | 4.2 | M1 C1 | Trp-270 | CE2 | 4.1 | M1 C1 | Trp-270 | CD2 | 4.1 | M1 C1 | Trp-270 | CD2 | 3.9 |
| M1 C5 | Trp-270 | CD2 | 4.0 | M1 C1 | Trp-270 | CZ3 | 3.7 | M1 C1 | Trp-270 | CD2 | 3.9 | M1 C1 | Trp-270 | CD2 | 3.9 |
| M1 C6 | Trp-270 | CB | 3.9 | M1 C3 | Glu-396 | CD | 3.9 | M1 C3 | Glu-396 | CD | 4.2 | M1 C3 | Glu-396 | CD | 4.0 |
| M1 C6 | Trp-270 | CD2 | 3.8 | M1 C3 | Glu-396 | CD | 3.9 | M1 C3 | Glu-396 | CD | 4.0 | M1 C3 | Glu-396 | CD | 4.0 |
| M1 C6 | Trp-270 | CE2 | 4.0 | M1 C3 | Glu-396 | CD | 4.1 | M1 C3 | Glu-396 | CD | 4.1 | M1 C3 | Glu-396 | CD | 4.2 |
| M1 C6 | Trp-270 | CE2 | 4.0 | M1 C3 | Glu-396 | CD | 4.2 | M1 C3 | Glu-396 | CD | 4.2 | M1 C3 | Glu-396 | CD | 4.2 |
| M1 C6 | Trp-270 | CB | 3.9 | M1 C3 | Glu-396 | CD | 4.3 | M1 C3 | Glu-396 | CD | 4.3 | M1 C3 | Glu-396 | CD | 4.3 |
| M1 C6 | Trp-270 | CB | 3.9 | M1 C3 | Glu-396 | CD | 4.4 | M1 C3 | Glu-396 | CD | 4.4 | M1 C3 | Glu-396 | CD | 4.4 |
| M1 C6 | Trp-270 | CE3 | 4.1 | M1 C3 | Glu-396 | CD | 4.5 | M1 C3 | Glu-396 | CD | 4.5 | M1 C3 | Glu-396 | CD | 4.5 |
| M1 C5 | Trp-270 | CG | 4.2 | M1 C3 | Glu-396 | CD | 4.6 | M1 C3 | Glu-396 | CD | 4.6 | M1 C3 | Glu-396 | CD | 4.6 |
| M1 C5 | Trp-270 | CD2 | 4.0 | M1 C3 | Glu-396 | CD | 4.7 | M1 C3 | Glu-396 | CD | 4.7 | M1 C3 | Glu-396 | CD | 4.7 |
| M1 C5 | Trp-270 | CZ3 | 3.9 | M1 C3 | Glu-396 | CD | 4.8 | M1 C3 | Glu-396 | CD | 4.8 | M1 C3 | Glu-396 | CD | 4.8 |
| M1 C5 | Trp-270 | CH2 | 3.9 | M1 C3 | Glu-396 | CD | 4.9 | M1 C3 | Glu-396 | CD | 4.9 | M1 C3 | Glu-396 | CD | 4.9 |
| M1 C5 | Trp-270 | CE2 | 3.9 | M1 C3 | Glu-396 | CD | 5.0 | M1 C3 | Glu-396 | CD | 5.0 | M1 C3 | Glu-396 | CD | 5.0 |
| M1 C5 | Trp-270 | CE2 | 3.9 | M1 C3 | Glu-396 | CD | 5.1 | M1 C3 | Glu-396 | CD | 5.1 | M1 C3 | Glu-396 | CD | 5.1 |
| M1 C5 | Trp-270 | CH2 | 4.0 | M1 C3 | Glu-396 | CD | 5.2 | M1 C3 | Glu-396 | CD | 5.2 | M1 C3 | Glu-396 | CD | 5.2 |
| M1 C5 | Trp-270 | CE2 | 4.0 | M1 C3 | Glu-396 | CD | 5.3 | M1 C3 | Glu-396 | CD | 5.3 | M1 C3 | Glu-396 | CD | 5.3 |
| M1 C5 | Trp-270 | CH2 | 4.1 | M1 C3 | Glu-396 | CD | 5.4 | M1 C3 | Glu-396 | CD | 5.4 | M1 C3 | Glu-396 | CD | 5.4 |
| M1 C5 | Trp-270 | CE2 | 4.1 | M1 C3 | Glu-396 | CD | 5.5 | M1 C3 | Glu-396 | CD | 5.5 | M1 C3 | Glu-396 | CD | 5.5 |
| M1 C5 | Trp-270 | CH2 | 4.2 | M1 C3 | Glu-396 | CD | 5.6 | M1 C3 | Glu-396 | CD | 5.6 | M1 C3 | Glu-396 | CD | 5.6 |
| M1 C5 | Trp-270 | CE2 | 4.2 | M1 C3 | Glu-396 | CD | 5.7 | M1 C3 | Glu-396 | CD | 5.7 | M1 C3 | Glu-396 | CD | 5.7 |
| M1 C5 | Trp-270 | CH2 | 4.3 | M1 C3 | Glu-396 | CD | 5.8 | M1 C3 | Glu-396 | CD | 5.8 | M1 C3 | Glu-396 | CD | 5.8 |
| M1 C5 | Trp-270 | CE2 | 4.3 | M1 C3 | Glu-396 | CD | 5.9 | M1 C3 | Glu-396 | CD | 5.9 | M1 C3 | Glu-396 | CD | 5.9 |
| M1 C5 | Trp-270 | CH2 | 4.4 | M1 C3 | Glu-396 | CD | 6.0 | M1 C3 | Glu-396 | CD | 6.0 | M1 C3 | Glu-396 | CD | 6.0 |
| M1 C5 | Trp-270 | CE2 | 4.4 | M1 C3 | Glu-396 | CD | 6.1 | M1 C3 | Glu-396 | CD | 6.1 | M1 C3 | Glu-396 | CD | 6.1 |
| M1 C5 | Trp-270 | CH2 | 4.5 | M1 C3 | Glu-396 | CD | 6.2 | M1 C3 | Glu-396 | CD | 6.2 | M1 C3 | Glu-396 | CD | 6.2 |
| M1 C5 | Trp-270 | CE2 | 4.5 | M1 C3 | Glu-396 | CD | 6.3 | M1 C3 | Glu-396 | CD | 6.3 | M1 C3 | Glu-396 | CD | 6.3 |
| M1 C5 | Trp-270 | CH2 | 4.6 | M1 C3 | Glu-396 | CD | 6.4 | M1 C3 | Glu-396 | CD | 6.4 | M1 C3 | Glu-396 | CD | 6.4 |

**ATP hydrolysis by bacterial ABC importer**

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cells were transfected with the expression plasmid pET21b-
AlgM1(d0)M2(H10)SS(WT), in which a histidine tag is
attached to the C terminus of AlgM2, and AlgM1 and AlgS are
encoded by wild-type sequences. The same strain was used as a
host for the expression plasmid pET21b_AlgM1(d24)M2
(H10)SS(E160Q), in which the encoded AlgS lacks ATnPase
activity and 2–24 residues of the N terminus of AlgM1 have
been truncated. Membrane fractions were prepared by
ultracentrifugation from cell extracts of E. coli. After sol-
ubilization with the surfactant n-dodecyl-β-D-maltoside,
AlgM1M2SS was purified using a nickel-nitrilotriacetic acid
column (Qiagen) and a Hi Load 16/60 Superdex 200 PG col-
umn (GE Healthcare), and expression and purification of
AlgQ2 were conducted as reported previously (19). E. coli
cell extracts were eluted through a HiTrap SP HP column
(GE Healthcare) and a Hi Load 16/60 Superdex 200 PG
column.

Proteoliposomes were prepared using L-α-phosphatidylcholine (Sigma-Aldrich) and phospholipid derived from soybean as
reported previously (14). ATPase assays were performed using
type II-S, whereas transport assays were performed using type
IV-S. AlgM1M2SS was added to liposomes that were destabi-
lized in n-octyl-β-D-glucoside and were diluted to less than crit-
ical micelle concentrations to produce proteoliposomes. Mag-
nesium ions and ATP were confined in proteoliposomes for
transport assays (14).

Assays of ATPase and transport activity were conducted as
reported previously (14). Briefly, reactions were conducted in
the presence of 0.1 µM AlgM1M2SS, 1 µM AlgQ2, 5 mM Mg-
ATP, and 20 µM oligosaccharides. ATPase activity was deter-
mined as the amount of inorganic phosphate released per min,
and transport activity was measured in the presence of PA-sac-
ccharides. The reaction mixture comprised 0.1 µM AlgM1M2SS,
1.3 µM AlgQ2, 2 mM ATP, 10 mM MgCl2, 20 µM PA-saccharide,
and 20 mM Tris-HCl (pH 8.0). Liposomes were collected by
ultracentrifugation after reactions, and transport activities
were determined based on fluorescence intensities of imported
PA-saccharides.

Co-immunoprecipitation assays

Interactions between AlgM1M2SS and AlgQ2 were deter-
mined using co-immunoprecipitation assays with binding/ 
washing buffer comprising 10% glycerol, 0.1 M NaCl, 0.05% 6-
cyclohexyl-hexyl-β-β-maltoside, 0.25% CHAPSO, and 20
mM Tris-HCl (pH 8.0). AlgM1M2SS exhibits significant
ATPase activity in this surfactant solution. Precipitates were
collected from 30 µl of Dynabeads Protein G using DynaMag-2
(Life Technologies) and were then mixed with 200 µl of binding
buffer containing 0.5 µg of rabbit anti-His-tag antibody
(Cosmo Bio). After incubation at 4 °C for 15 min, precipitates
were washed once with wash buffer, and 200 µl of binding
buffer containing 20 µg of AlgM1(d24)M2(H10)SS(WT) was
added. After incubation at 4 °C for 15 min, precipitates were
washed once with wash buffer, and 200-µl aliquots of binding
buffers containing 20 µg of AlgQ2, 0 or 2 mM Mg-ATP, 0 or 0.2
mM Δ3M, and 0 or 5 mM sodium orthovanadate were added.
After incubation at 4 °C for 15 min, precipitates were washed
four times in wash buffer and were then dissolved in 10-µl
aliquots of binding buffer and 10 µl of 2× SDS buffer compris-
ing 4% SDS, 12% 2-mercaptoethanol, 20% glycerol, 0.1 M Tris-
HCl (pH 6.8), and bromphenol blue. After boiling for 3 min,
mixtures were centrifuged, and supernatants were subjected
to SDS-PAGE analyses. AlgQ2 was then detected in Western
blots using rabbit anti-AlgQ2 antiserum as a primary anti-
body and donkey anti-rabbit IgG as a secondary antibody
(GE Healthcare).

Crystallization and X-ray diffraction experiments

Crystallization and crystal freezing of AlgQ2 (15) and crys-
tallization of the AlgM1(d24)M2(H10)SS(E160Q) and AlgQ2
complex (14) were conducted as reported previously. Crystals
of AlgM1(d24)M2(H10)SS(E160Q) and AlgQ2 complexes were
frozen using the HAG method (20). Crystals were picked up
using a loop covered with 10% PVA 4500 with or without eth-
ylene glycol and were frozen in a cold nitrogen gas stream at
optimized humidity.
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Analysis of diffraction data

Diffraction data from frozen crystals were collected at a wavelength of 1.000 Å using a BL38B1 or BL26B2 beamline in SPring-8 (Hyogo, Japan) and were processed using HKL2000 (22). Phases were solved by molecular replacement using Molrep (23) in CCP4 interface (24) with the structure of the AlgM1M2SS and AlgQ2 complex (Protein Data Bank entry 4TQU or 4XIG) or the structure of AlgQ2 as initial search models. Structures were refined using Refmac5 (25) and phenix. refine (26), and model construction was performed using WinCoot (27). The figures of structures were produced using PyMOL (28). Interactions between oligosaccharides and proteins were investigated using the program Contact in the CCP4 interface. RMSD values were calculated using PyMOL.

Author contributions—A. K., K. U., and S. B. performed the experiments. A. K., K. U., Y. M., N. M., S. B., T. K., B. M., K. M., and W. H. analyzed the data. Y. M., K. M., and W. H. designed the study. A. K., K. U., Y. M., K. M., and W. H. wrote the manuscript.

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