Review

Bacteria with a mouth: Discovery and new insights into cell surface structure and macromolecule transport

By Kousaku MURATA,[*1,†] Shigeyuki KAWAI[*2] and Wataru HASHIMOTO[*3]

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Abstract: A bacterium with a "mouth"-like pit structure isolated for the first time in the history of microbiology was a Gram-negative rod, containing glycosphingolipids in the cell envelope, and named *Sphingomonas* sp. strain A1. The pit was dynamic, with repetitive opening and closing during growth on alginate, and directly included alginate concentrated around the pit, particularly by flagellins, an alginate-binding protein localized on the cell surface. Alginate incorporated into the periplasm was subsequently transferred to the cytoplasm by cooperative interactions of periplasmic solute-binding proteins and an ATP-binding cassette transporter in the cytoplasmic membrane. The mechanisms of assembly, functions, and interactions between the above-mentioned molecules were clarified using structural biology. The pit was transplanted into other strains of sphingomonads, and the pitted recombinant cells were effectively applied to the production of bioethanol, bioremediation for dioxin removal, and other tasks. Studies of the function of the pit shed light on the biological significance of cell surface structures and macromolecule transport in bacteria.

Keywords: *Sphingomonas*, alginate, cell surface structure, mouth-like pit, flagellin, ABC transporter

Introduction

The main function of the mouth is to allow the passage of food in any form into the body of animals and form the beginning of the digestive system. Is the mouth a special device for multicellular organisms? Does a mouth exist in unicellular organisms? Bacteria, as single-celled organisms, are in principle unable to directly incorporate macromolecules into their cells. Therefore, bacteria frequently excrete enzymes externally from the cell, which depolymerize macromolecules extracellularly, and the resulting low-molecular-weight products are incorporated into the cell through specialized transport systems on their cell surface. However, in open systems, depolymerized products and extracellular enzymes may dissipate through diffusion, so a macromolecule utilization strategy like this is not always economic and convenient.

To overcome these disadvantages in macromolecule utilization, in evolutionary processes, bacteria must develop ingenious, well-organized, and unconventional macromolecule-utilizing systems that are beyond our expectations. In this study, emphasis was placed on the macromolecule alginate so that such a macromolecule utilization mechanism in bacteria can be identified. Alginate is a viscous macromolecule carbohydrate produced by seaweed[1)] and microbes such as *Azotobacter vinelandii*[2)] and *Pseudomonas aeruginosa* (mucoid type).[3)] Seaweed alginate is employed in various bio-industries, particularly in the food industry, because the polymer is biocompatible and biodegradable in addition to its

[*1] Professor Emeritus, Kyoto University, Kyoto, Japan.
[*2] Research Institute for Bioresource and Biotechnology, Ishikawa Prefectural University, Nonoichi, Ishikawa, Japan.
[*3] Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto, Japan.
[†] Correspondence should be addressed to: K. Murata, 53-11 Ichibanwari, Gokasho, Uji, Kyoto 611-0011, Japan (e-mail: kuki3.aya@gmail.com).

Non-standard abbreviation list: ABC: ATP-binding cassette; Arg: arginine; Asn: asparagine; Asp: aspartic acid; ATPase: adenosine triphosphatase; DEH: 4-deoxy-L-*erythro*-5-hexoseulose uronate; Gln: glutamine; Glu: glutamic acid; GSL: glycosphingolipid; HAP: hook-associated protein; Ile: isoleucine; K_d: dissociation constant; Leu: leucine; LPS: lipopolysaccharide; Lys: lysine; PVA: polyvinyl alcohol.

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excellent biophysical and biochemical properties such as thickening, gelling, and film-forming.[4] In bacterial ecology systems, alginate mainly functions in biofilm formation, comprising a thick coating with slime-like properties.[5]

During a study on bacterial alginate degradation, we unexpectedly found a bacterium able to grow on alginate without extracellular alginate-depolymerizing enzymes. Furthermore, the bacterium was identified as a member of the *Sphingomonas* and named *Sphingomonas* species strain A1 (herein referred to as *Sphingomonas* sp. A1 or strain A1). *Sphingomonas* sp. A1 was shown to have a "mouth"-like aperture, which we call a pit, on the cell surface.[6),7)] This discovery of a "mouth"-like structure is the first ever report in bacteria, suggesting that the mouth might not be uniquely a possession of multicellular organisms.

The investigation of this unconventional cell surface structure and alginate transport system is expected to produce original insights into cell surface biology, including cell shape, cell envelope function, cell economy, and macromolecule transport systems, and suggests novel biological and physiological areas for future studies, particularly on the evolution and origin of bacterial cell structure. Furthermore, characteristic and innovative macromolecule processing biotechnologies and bioindustries are also expected to be developed, with pitted cells, such as *Sphingomonas* sp. A1 playing a central role.

Here, we review the biological significance of the "mouth"-like pit on the cell surface of *Sphingomonas* sp. A1 with a particular focus on recent advances in macromolecule alginate recognition and uptake systems in cell, as well as on biotechnological applications of the pit for biofuel generation and pollutant bioremediation. The content will be retrospectively described from our own perspective, first on the discovery of bacteria with a "mouth"-like pit and then on the unique alginate uptake system involving flagellin molecules in detail, covering research on alginate dating from about 30 years ago.

1. Discovery of bacteria with a "mouth"-like pit

Alginate is a linear, negatively charged heteropolysaccharide composed of two isomer residues, β-D-mannuronate (M) and its C5 epimer, α-L-guluronate (G),[1)] which are linked homo- or heterogeneously by glycosidic bonds to form three different blocks, polyM, polyG, and polyMG.[8),9)] This polymer produced by seaweed and certain microbes is depolymerized by enzymes, alginate lyases, yielding non-viscous oligoalginates with various physiological and chemical functions.[10)] Alginate lyase is an important biocatalyst that is used in many new applications. To further the application of this enzyme in a broad range of fields, including the biomedical, environmental, and food industries, bacteria with alginate lyase were screened.

A bacterium isolated from a rice paddy as an alginate degrader was a Gram-negative rod with a G + C content of 62–63 mol%, yellow-pigmented, and strictly aerobic.[6),7)] The bacterium was polymyxin B-resistant and contained mono- and oligosaccharide-type glycosphingolipids (GSLs) and isoprenoid quinone (ubiquinone 10),[11)] which are characteristic components of *Sphingomonas*. Members of this genus are characterized by the presence of GSL instead of lipopolysaccharides (LPSs) and are classified independently from Pseudomonadales.[12)] Although Gram-negative cells are surrounded by outer and inner membranes, the outer membrane of *Sphingomonas* strains contains GSL and is different from that of other Gram-negative bacteria, which contain LPSs in their cell envelope. Because of this peculiar property, the cell surface of *Sphingomonas* strains is more hydrophobic than that of other Gram-negative bacteria and shows high affinity toward hydrophobic chemicals such as dioxin and polypropylene glycol (see section 3: Applicability of the "mouth"-like pit). Based on these taxonomic and physiological properties, the isolated alginate lyase producer was classified into the genus *Sphingomonas* and named *Sphingomonas* sp. A1.[6),7)] Species of this genus have frequently been found in patients and clinical samples,[13)] in the ears of rice and other plants of the family Gramineae,[14)] and in other natural environments.[15)–17)]

The strain A1 cells exclusively use polyuronates (alginate and pectin) and their depolymerization products as carbon sources for their growth. Glucose and pyruvate can be utilized as carbon sources, but with far less efficiency than polyuronates.[18)] Strain A1 cells grew well on alginate and oligoalginates with different M/G ratios at pH 6–7, 30 °C in aerobic conditions, with a doubling time of approximately 25 min. However, unlike almost all of the alginate-degrading bacteria analyzed to date,[19)] the cells of strain A1 contained most of their alginate lyases in the cytoplasm.[6),11),20)] This means that alginate in the medium has to enter the cells in order to make contact with alginate lyases. The uptake system of macromolecule alginate into cells, however, has not yet been elucidated.

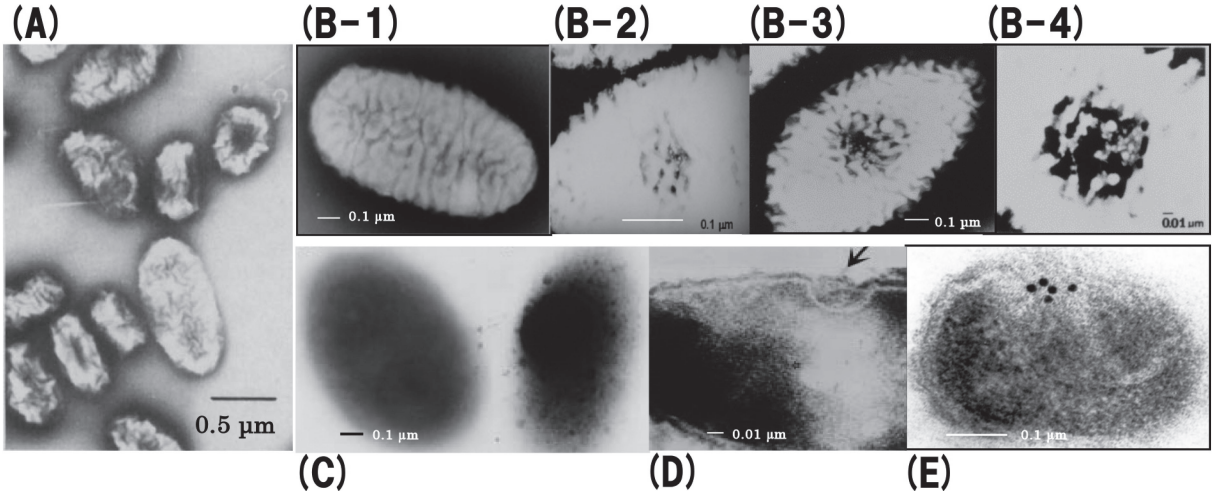

Fig. 1. Pit structure of *Sphingomonas* sp. A1.[6),7),21)] Cells were grown aerobically on nutrient medium with or without alginate and all the pictures of cells in exponential growth phase were taken with a transmission electron microscope. **(A)** Cell shapes growing in the presence of alginate. **(B)** Time course of change in cell surface structure (from left to right: **B-1, -2, -3, and -4**) grown in the presence of alginate. The globular particles in the pit **(B-3, 4)** are alginate gel. **(C)** Ruthenium red staining of cells grown in the presence (right) or absence (left) of alginate. **(D)** Thin section of alginate-grown cells. An arrow indicates the irregular position corresponding to the pit. **(E)** Immunoelectron microscopy of ABC transporter using an anti-ABC transporter antibody. Figures were cited from Refs. 20–23.

The morphological characteristics of the cell surface were examined with cells of strain A1 grown in the presence or absence of alginate. Transmission electron microscopic investigations[6),7),20),21)] revealed the following morphological observations: (i) cells grown on alginate were of two types that always coexisted in the medium: cells with or without a pit, a "mouth"-like structure on the cell surface **(Fig. 1, A)**, and this feature was not observed in the absence of alginate. (ii) The surface of cells grown in the absence of alginate showed a pleat-like structure without a pit **(Fig. 1, B-1)**. (iii) Cells grown in the presence of alginate produced pits on their cell surface **(Fig. 1, B-3 and B-4)**. The pits contained even globular particles, some of which were insoluble forms (granules) of alginate. (iv) When the alginate-grown cells were treated with ruthenium red, an agent used to stain mucopolysaccharides, the pit periphery was strongly and specifically stained **(Fig. 1, C)**, suggesting that alginate was concentrated in the pit. (v) The thin section of cells grown on alginate showed a specific region where the cell surface sunk into the cells **(Fig. 1, D)**. No such structures were observed in cells grown in the absence of alginate. (vi) The average pit size was 0.02–0.1 µm in diameter **(Fig. 1, D)** and less than ∼150 nm in depth, the latter of which was determined using tunnel scanning electron microscopy (unpublished).

These lines of evidence suggest that: (a) the pit is formed only in the presence of alginate, (b) the pit functions as a concentrator of alginate, and (c) strain A1 cells have a pit-dependent alginate assimilation system, which differs from the alginate import and degradation pathway of other alginate-degrading microbes. Therefore, to the best of our knowledge, this is the first description of a pit-forming bacterium in the history of microbiology and it indicated that cell surfaces are highly fluid and/or plastic, anisotropic in function and structure, and have numerous hidden possibilities to construct highly organized structures. How dynamic is the cell surface? How is such a huge structure (pit) formed? How is the pit formation process regulated to match with cycles of DNA replication and cell division at intervals of approximately 25 min, which is the doubling time of strain A1 cells in alginate medium? The exquisite regulation of cellular events is central problem of biology to be addressed.

At present, the pit formation process and mechanism are only a matter of speculation. In the early stage of growth on alginate, a few tiny holes begin to open near the center of the cell surface layer **(Fig. 1, B-2)**, which increase in number, and, in association with the degradation of cell surface pleats, eventually develop into a funnel- or concentrator-like structure, namely a pit **(Fig. 1, B-3 and**

B-4). The pit is wide at the top and narrow at the bottom **(Fig. 1, D)** and guides alginate and/or alginate granules into periplasmic space.

In order to clarify these exquisite regulatory mechanisms of cellular events comprehensively, the strain A1 genome, which was the first genome structure of a sphingomonad to be determined,[21] was analyzed. The genome comprises 4,622,788 base pairs with approximately 4,800 genes, and 30% of these genes show significant homology with those of *P. aeruginosa*, suggesting that sphingomonads and pseudomonads have evolved or diverged from a common ancestor, although the former and the latter, at present, belong to alpha- and gamma-proteobacteria classes, respectively.

2. Alginate recognition and transport systems

2.1. Overview of alginate transportation. Enzymatic and genetic analyses indicated that the cells of *Sphingomonas* sp. A1 are equipped with an unconventional macromolecule (alginate) transport system[20),22)–27)] **(Fig. 2)** comprising a pit and cell surface proteins described in the following section, solute-binding proteins (AlgQ1 and AlgQ2) in the periplasm, and ATP-binding cassette (ABC) transporter (AlgM1M2SS) in the inner membrane, as well as alginate depolymerization enzymes (alginate lyases) in the cytoplasm. ABC transporter complexes, which seem to be arranged just below the pit **(Fig. 1, E: unpublished)**, comprise four proteins: AlgM1 and AlgM2 that function as permeases, and two molecules of AlgS that function as adenosine triphosphatases (ATPases) for the generation of energy required for alginate transport.[28] Alginate accumulated in the pit is delivered into the periplasm and then transported to the cytoplasm by the ABC transporter. Alginate is finally decomposed into constituent monosaccharides by alginate lyases present in the cytoplasm and then metabolized. The cells of strain A1 contain two different types of alginate lyases; endo-type (A1-I, -II, -III, -II′ and -IV′) and exo-type (A1-IV).[20),26),29)–31)] The details of the molecular structure, substrate specificity, and reaction conditions of these lyases, including post-translational modification processes, are described elsewhere.[32)–37)] Genes encoding the proteins involved in alginate uptake and depolymerization form a single cluster **(Fig. 2)**. The expression of genes involved in alginate import and degradation is regulated by transcription factor AlgO.[38] Similar to LacI, ligand-free AlgO depresses the expression of the gene cluster through binding to the vicinity of the promoters, whereas alginate oligosaccharide-bound AlgO is released from the strain A1 genome and the genetic cluster is subsequently transcribed. A similar gene cluster has also been found in the genome of *Agrobacterium tumefaciens*[39] and *Chania multitudinisentens*,[40] suggesting that the pit observed in

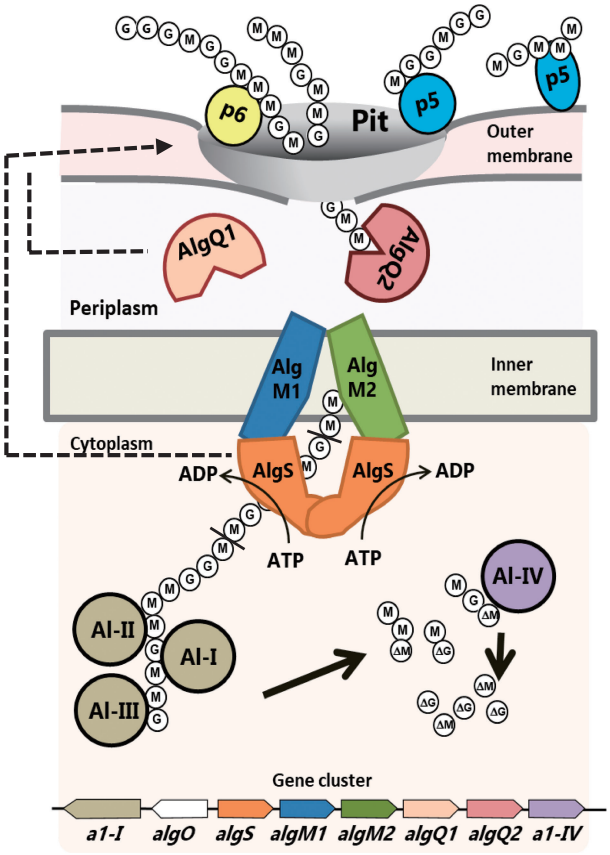

Fig. 2. Overall picture of alginate import and depolymerization systems in *Sphingomonas* sp. A1.[27] M and G represent the β-D-mannuronate and α-L-guluronate residues of alginate, respectively. p5 and p6 are cell surface flagellins with alginate-binding ability. AlgQ1 and AlgQ2 (encoded by *algQ1* and *algQ2*, respectively) are solute-binding proteins that pass alginate to the ABC transporter. AlgM1, AlgM2, and AlgS (encoded by *algM1*, *algM2*, and *algS*, respectively) form a heterotetramer (ABC transporter, AlgM1M2SS) and transport alginate across the inner membrane. A1-I (encoded by *a1-I*), -II, and -III (both are generated from A1-I) are endo-type alginate lyases and A1-IV (encoded by *a1-IV*) is an exo-type lyase. Other cytoplasmic alginate lyases are not included in the scheme. A series of actions by these lyases produce unsaturated monosaccharides (ΔM and ΔG), which are structurally the same. All of these proteins, together with a transcriptional regulator, AlgO (encoded by *algO*), are encoded by genes in the alginate-related gene cluster shown below. Dotted lines indicate the regulation of pit formation by AlgQ1 or AlgQ2 and/or AlgS. Figure was cited from Ref. 27 with slight modifications.

strain A1 cells may be latent in several bacteria with LPS-based cell walls.

Thus, the above-mentioned alginate import system is unique because strain A1 cells directly incorporate alginate into the periplasm through the pit without depolymerization.[21)–27)] In the following sections, the properties of each component (cell surface-localized flagellin, AlgQ1 or AlgQ2, and AlgM1M2SS) are described to understand the overall function of this alginate import and depolymerization system.

Cell surface-localized flagellin. Proteomics-based analysis of outer-membrane proteins responsible for the import of macromolecule alginate into the cells of strain A1[41)] led to the identification of eight cell surface proteins (p1–p8) whose expression is alginate-dependent.[41),42)] Homology analysis indicated that proteins p1–p4 are TonB-dependent transporters[43)] and may be functioning in the transport of alginate ferric chelates as a siderophore. Protein p7 with an alginate-binding ability was first considered as a lipoprotein by homology analysis but later identified to be one of the components constituting alginate and/or metal transporter system.[44)] Protein p8 is an alginate granules-binding protein and gathers insoluble alginates in the periphery of pit (globular particles found in the pit: **Fig. 1, B-3 and B-4**).[42)] The gene encoding p8 was identified in the genome of strain A1 and shown to be similar to that for the polyhydroxyalkanoate granule-associated protein of *Ralstonia eutropha*.[45)] However, p8-disrupted cells showed significant growth retardation of strain A1 cells in the alginate medium.

Unexpectedly, proteins p5 and p6 turned out to be homologs of a bacterial flagellar protein, flagellin, and they were experimentally confirmed to have extremely high-affinity interactions with alginate (dissociation constant [K_d]: $\sim 10^{-9}$ M).[41)] Furthermore, immunoelectron microscopy using an anti-p5 antibody indicated that strain A1 flagellin homologs are exclusively localized on the cell surface. Judging from the high affinity to alginate (∼nM level), it was suggested that p5 and p6 recognize external soluble alginate as receptors on the cell surface, similar to the human receptor CD44 binding to acidic polysaccharide hyaluronan,[46)] and, together with protein p8, facilitate the concentration of alginate in the pit. Flagellins p5 and p6 seemed to be distinct in their functions from ordinary bacterial flagellins that constitute helical flagellar filaments.

Usually, flagellin forms a flagellar fiber outside the cell and helps in planktonic movement, although in spirillum (Spirochaetales) cells, flagellin has been shown to localize in the periplasm.[47)] Alternatively, in strain A1 cells, flagellins p5 and p6 influence the cell surface structure and alginate metabolism. In fact, disrupting flagellin p6 changed the cell surface structure from a pleat form to a network one associated with incomplete pit formation, thereby inducing deteriorated growth of strain A1 cells on alginate medium. Furthermore, although flagellin is usually exported from the cytoplasm to the cell surface through a type-III exporter encoded by the flagellum cluster,[48),49)] in strain A1 bacterium, an alternative pathway may be functioning in flagellin export and localization (unpublished). This observation is contrasted with the fact that in *Campylobacter jejuni* TGH9011, a Gram-negative spiral bacterium possibly lacking in a type-III secretion apparatus, flagellin is excreted from the cells and is used for host cell recognition.[50)]

Other than flagellin homologues p5 and p6, strain A1 cells have an additional flagellin homologue p5′, although it is not discussed in this review because of its low expression level compared with that of p5 or p6.[51)] The unusual behaviors of flagellins in strain A1 cells suggested that these proteins have unique roles in alginate assimilation. Analysis of the structure–function relationship of strain A1 flagellin homologs may contribute to elucidation of the alginate recognition mechanism and cell shape control. Therefore, in this review, we have focused on describing the biological significance of flagellin molecules.

Properties of flagellin. The bacterial flagellum is a extracellular rotary device that has evolved exclusively for bacterial locomotion. The number of flagella, helical chirality of the filament, and rotational direction of the flagellar motor determine the behavioral properties of bacteria such as motility, host adhesion, colonization, and virulence[52)] of each bacterial species. The structure of flagellin, a principal component of the flagellum, is responsible for the helical shape of the flagellar filament, which is a tubular structure composed of several thousand flagellin subunits.[53)] Newly synthesized flagellin is transported through the center hole of the filament to the tip where it is spontaneously polymerized into a part of the filament. During transport, flagellin is unfolded by FliS, a flagellar secretion chaperone, which structurally resembles Type III secretion chaperones.[54)]

In primary structures, flagellins p5 and p6 exhibit high similarity with bacterial flagellins in

the amino terminal (N-terminal)- and carboxy terminal (C-terminal)-domains, although little homology exists in the central domain of the molecules. The central domain portion, including approximately 100–200 amino acid residues, of the ordinary flagellin structure of *Escherichia coli* or *Salmonella typhimurium* (FliC)[55),56)] is missing in flagellins p5 and p6.

Generally, three major Pfam motifs, flagellin_N, flagellin_IN, and flagellin_C, are found in bacterial flagellins (https://www.ebi.ac.uk/interpro/). Most flagellins contain the flagellin_N and flagellin_C motifs. On the other hand, the presence of the flagellin_IN motif varies depending on the diversity in the flagellin central domain. Although this motif varies in sequence, an isoleucine (Ile)-asparagine (Asn) pair is present at the center and is usually used as an index for the presence or absence of the flagellin_IN motif in flagellin molecules. Furthermore, the number of flagellin_IN motifs also varies depending on the flagellar proteins. The hook-associated protein (HAP) and flagellins usually contain several (one to five) flagellin_IN motifs in their molecules, but the β-domain (described below) of flagellin p5 contains one flagellin_IN motif comprising 53 residues. Because *S. typhimurium* flagellin FliC contains no flagellin_IN motifs, the three-dimensional structure of bacterial flagellin with the flagellin_IN motif had not been determined at this time.

In order to obtain information about the structural factors of flagellins p5 and p6 involved in alginate binding and cell-surface localization, the crystal structure of flagellin p5 was determined[57)] **(Fig. 3, B)** and compared with that of *S. typhimurium* flagellin FliC[56)] **(Fig. 3, A)**.

Crystal structure of flagellin. The typical three-dimensional structure of *S. typhimurium* flagellin FliC has been determined by X-ray crystallography and electron cryomicroscopy[55),56)] **(Fig. 3, A)**. Bacterial flagellin usually consists of four structural domains, D0, D1, D2, and D3. The N-terminal chain starts from D0, passes through D1 and D2, and reaches D3. It then returns through D2 and D1, and the C-terminal chain ends in D0[56)] **(Fig. 3, A)**. The D0 and D1 are indispensable for self-assembly. The N- and C-terminal regions, which contain ~50 residues each, constitute D0 and are rich in hydrophobic residues and thus interact hydrophobically with other flagellin subunits in the filament.[55),56)] Because of the restrictions on filament formation and molecular passage inside the filaments, amino acid sequences in D0 and D1 are highly conserved among bacterial flagellins, whereas the solvent-exposed central D2 and D3 domains vary in amino acid sequence and chain length. These situations are true in strain A1 flagellin homologues p5 and p6 **(Fig. 3, B)**; however, the two homologs are different from ordinary bacterial flagellated flagellins in function and localization.

The ribbon diagram of the overall structure and topology of the secondary structure elements of p5 were determined[57)] **(Fig. 3, B, C, and D)**. Two short-peptide linkers connect the two flagellin p5 structural domains, α and β. The α-domain comprises N- and C-terminal regions and resembles the D1/D0 of FliC in *S. typhimurium*.[56)] The α-domain is further divided into α_1- and α_2-subdomains **(Fig. 3, B)**. α_1 and α_2 correspond to D1 and D0 in FliC, respectively. The terminal domain (α-domain) consists of residues 62–167 and residues 283–326. The N-terminal part of the α-domain forms two long α-helices (H1 and H2) that are arranged in an antiparallel fashion and are followed by two β-hairpins (S1 and S2, and S3 and S4), and the C-terminal part forms an α-helix (H5), which closely resembles D1 of *Salmonella* flagellin FliC.

On the other hand, the central domain (β-domain, residues 173–281) between the N- and C-terminal parts of the α-domain significantly differs from the D2 and D3 of *Salmonella* flagellin FliC, although each consists mainly of β-strands. The β-domain contains two antiparallel β-sheets, *i.e.*, sheets A and B **(Fig. 3, B)**, which sandwich an α-helix (H3). Each β-sheet consists of four strands (SA1–SA4 and SB1–SB4). A longer helix (H3) is present between the two β-sheets, and a shorter helix (H4) connects two strands (SB2 and SB1) of sheet B **(Fig. 3, C and D)**.

Role of β-domain. To structurally characterize the β-domain, we searched for homologous structures to the flagellin p5 β-domain using the DALI program (https://www.ebi.ac.uk/msd-srv/ssm/). Unexpectedly, significant similarity was found only in the structure of the finger domain of gp11 (T4gp11), a baseplate protein of bacteriophage T4[57)] functioning in the control of the host cell recognition, attachment, tail sheath contraction, and phage DNA ejection, with a Z value of 4.6 and a reference amino acid length of 90[57),58)] **(Fig. 3, E)**.

The topologies of the flagellin p5 β-domain and T4gp11 finger-domain are similar, although their latter half slightly differs. However, their primary structures show slight similarity, including the region where the three-dimensional structures are most similar. Furthermore, although the flagellin p5 β-

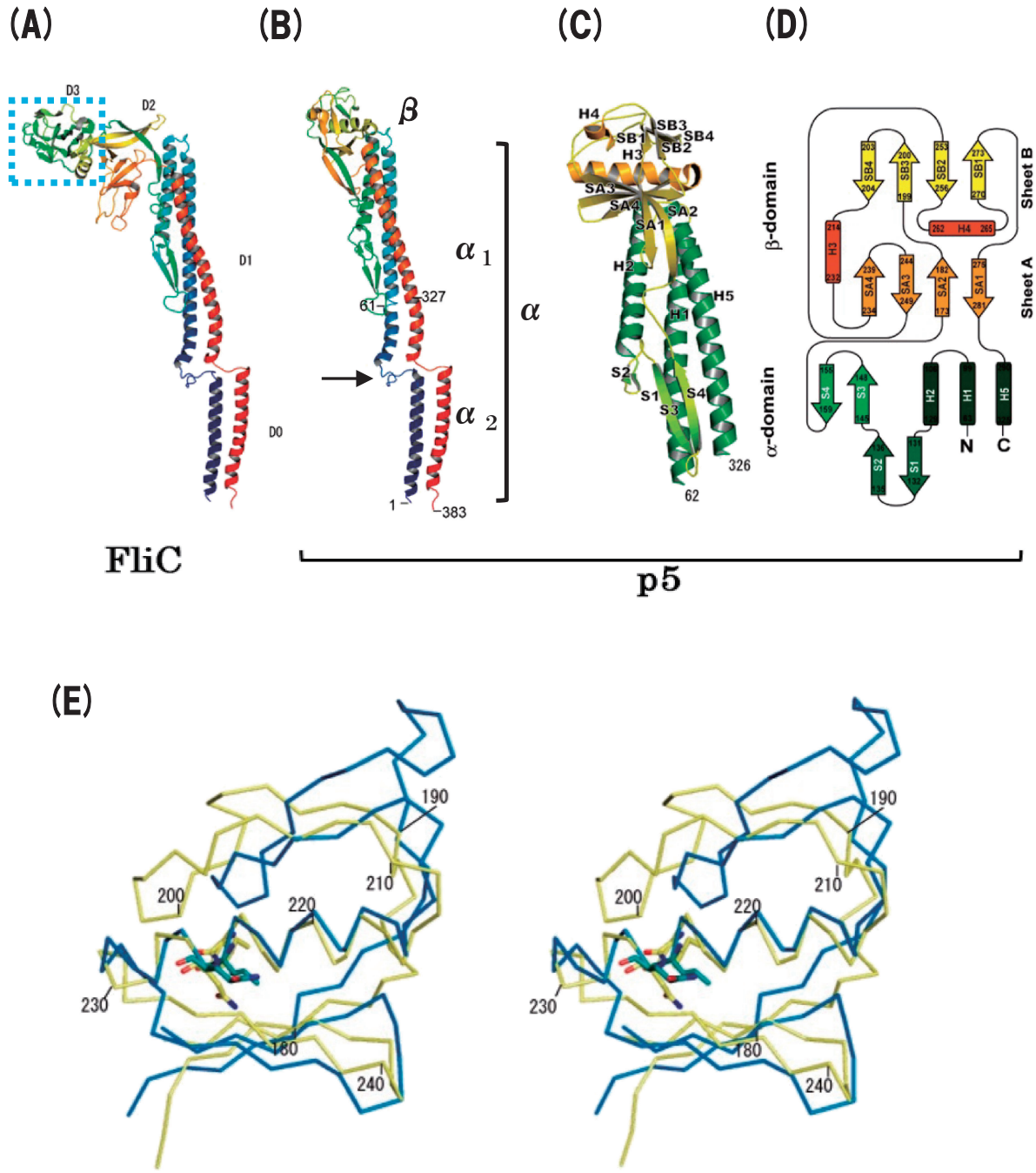

Fig. 3. Crystal structure of flagellin p5 and structural comparison with T4-phage gp11 finger domain.[57] *Top*: Crystal structure of flagellin p5. The structure was determined using truncated p5:$\Delta N_{53}C_{45}$ with deletion of 53 amino acids at the N-terminal end and 45 amino acids at the C-terminal end. **(A)** Ribbon diagram of *Salmonella* flagellin FliC (PDB entry 1UCU) with the domain nomenclature.[56] **(B)** Structure of flagellin p5. Spoke region is shown with an arrow, and the missing domain in p5 is boxed (dotted line) in FliC **(A)**. The chain in panels **(A)** and **(B)** is color-coded from blue to red from the N-terminus to the C-terminus. Both structures in panels **(A)** and **(B)** are shown at the same scale and in the same orientation. **(C)** Ribbon diagram of p5ΔN53C45. The terminal α-domain is colored green, and the central β-domain is colored yellow. The model starts from Thr62 and ends at Ala326. **(D)** Topology of the secondary structure of p5ΔN53C45. *Bottom*: **(E)** Wall-eye stereoview of the superimposition of flagellin p5 β-domain (yellow) and T4gp11 finger-domain (blue). Residues 173–249 of flagellin p5 and 81–160 of T4gp11 are represented in the figure. An Ile-Asn pair at the center of the flagellin_IN motif of flagellin p5, and the corresponding amino acid residues (Leu-Gln pair) in T4gp11 finger-domain are shown as a stick model (p5, yellow; T4gp11, blue). Figures were cited from Ref. 57.

domain has one flagellin_IN motif comprising 53 residues, corresponding to residues 197–249, and, the motif contains an Ile-Asn pair at the center, the T4gp11 finger-domain has no flagellin_IN motifs and contains a leucine (Leu)-glutamine (Gln) pair at the position corresponding to the Ile-Asn pair in the flagellin_IN motif of the flagellin p5 β-domain **(Fig. 3, E)**. As described above, some flagellar proteins such as HAP and flagellin contain several flagellin_IN motifs in their molecules.[59] It is normally repeated but also appears individually with a conserved Ile-Asn pair at the center of the motif. The fact that the flagellin_IN motif is abundant in flagellar proteins but absent in structurally similar T4gp11 finger-domain indicates that this motif is irrelevant to the folding of these proteins.

What kinds of biological significance are hidden behind the structural similarity between the flagellin p5 β-domain and T4gp11 finger-domain? How should it be interpreted? As described in the next section, flagellins p5 and p6 can bind to extracellular alginate in a cleft near the N- and C-terminals, for which residues 20–40 and 353–363, respectively, are crucial **(Fig. 3, B)**. Therefore, flagellin p5 must be localized on the cell surface and fastened tightly to the outer membrane. The flagellin p5 β-domain with a similar structure to that of the T4gp11 finger-domain may function as an anchor to connect flagellin p5, as well as p6, to the cell surface, because the finger-domain plays an essential role in associating with other proteins in the T4 phage.[57),58] Although more detailed structure-function studies are required, the finding on structural similarity between the p5 β- and gp11 finger domains might partly provide evidence regarding the origin or evolution of molecular segments or both, constituting cell parts or abiotic structures, such as flagella and phages, including viruses.[60)–63]

Alginate-binding site. We found that flagellin p5 of strain A1 cells and flagellin of *E. coli* cells specifically and extremely strongly bind alginate at acidic pH[41] and, for the first time, firmly established that the alginate binding is an inherent property of flagellin. In addition to the flagella shape, the crystal structure of flagellin p5 showed the molecular basis for alginate binding[57] **(Fig. 4, A and B)**.

Alginate was found to be bound in a cleft called the "spoke region" at the boundary of the α_1- and α_2-subdomains described above. In this spoke region, residues 20–40 and 353–363 are crucial for alginate-binding and several basic residues such as arginine (Arg) and lysine (Lys) are present on the surface

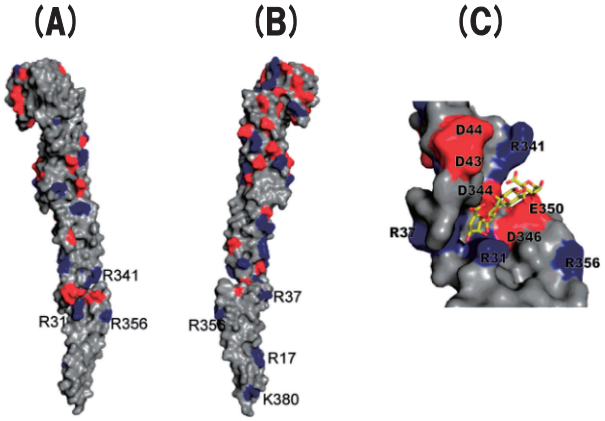

Fig. 4. Alginate-binding domain in flagellin p5.[57] **(A and B)** Surface models of flagellin p5. Panel B is rotated 180° from that in panel A. Red and blue show acidic and basic residues, respectively. **(C)** Surface of cleft. The tetrasaccharide coordinates were obtained from the Protein Data Bank (entry 1J1N). Figures were cited from Ref. 57.

around the region **(Fig. 4, C)**. These positively charged residues are indispensable for recognizing negatively charged alginate. The cleft is approximately 20 Å wide, corresponding to the alginate tetrasaccharide width **(Fig. 4, B)**, it also contains acidic residues, aspartic acid (Asp) and glutamic acid (Glu), and a few hydrophobic residues. The preference for a lower pH for flagellin p5 binding to alginate may reflect the protonation of acidic residues.[41),57] The residues forming the spoke region in the cleft are mostly conserved among flagellins derived from some bacteria, thus confirming again that alginate binding is an inherent property of flagellin. This new concept of flagellin may provoke some critical arguments: whether the occurrence of this property is inevitable or coincident, what is the physiological significance of this property, and which comes first, alginate and flagellin, in the course of molecular evolution?

Flagellum gene cluster. Although flagellins p5 and p6 were found in the non-flagellated cell outer membrane growing on a liquid medium with alginate,[25),51] on semi-solid agar plates, the cells swarmed toward alginate using newly formed flagella comprising flagellins.[51] Analyzing the regulatory mechanism of the flagellum gene expression system in the presence or absence of alginate is required to understand the chemotactic behaviors of strain A1 cells. However, information on this system is presently insufficient.

Briefly, in the strain A1 cells, flagellum-related genes are divided into small and large gene sets, -I and -II.[64] Although Set-I specifies polar flagellin p5

and p5′ discussed above,[51] Set-II includes lateral flagellin p6. Curiously, *Sphingomonas* sp. A1 cells produce only polar flagellum comprising flagellins p5 and p6. Although the significance behind this observation is unclear, and the mixed use of different flagellins in a single flagellum has been observed in several members of the α-proteobacteria such as plant pathogen *A. tumefaciens*,[65] the shared use of polar and lateral flagellins in a single flagellum is, to the best of our knowledge, a new finding among flagella analyzed to date. It seems likely that the two flagellins p5 and p6 are not redundant in function but have specific properties and/or roles and give motility to the cells of strain A1 through their synergistic interactions.

Why do flagellins exhibit extremely high alginate-binding ability? What is the advantage of this ability to strain A1 cells along with other bacteria with flagella? This important problem will be elucidated in the future. Strain A1 cells exclusively use alginate as a carbon source for their growth. They might have refined their specific molecular skills, including a special, perceptive alginate detection system, and motile organ, so that they can move quickly to obtain alginate.

2.2. Alginate transport. Pit formation by *Sphingomonas* sp. A1 cells uncovered the cell's hidden ability to induce structural and functional fluctuations on its cell surface. Is the pit a transporter, channel, or pump? Although it is an ultimate problem to be solved, we cannot answer this question at the moment. Alginate accumulating in the pit possibly by the action of flagellins p5 and p6, as well as by protein p8, is first transported into the periplasm and then incorporated into the cytoplasm by successive actions of solute-binding proteins (AlgQ1 and AlgQ2) in the periplasm and ABC transporter (AlgM1M2SS) in the inner membrane of strain A1 cells **(Fig. 2)**.

Solute-binding proteins. Alginate concentrated in the pit is transferred to the periplasm through the pit by an unknown mechanism. Two periplasmic proteins, AlgQ1 and AlgQ2, resembling each other in structure with EF-hand-like motifs and alginate-binding properties, mediate alginate transfer to the ABC transporter.[66)–70)] AlgQ2, as well as AlgQ1, comprises N- and C-domains with an α/β-structure **(Fig. 5)** and a calcium ion in the C-domain.[71] The divalent cation is located far from the alginate-binding site (approximately 40 Å) and contributes to the stabilization of protein AlgQ2 **(Fig. 6, A)**. In fact, a mutation at the calcium-binding site caused the loss of calcium-binding activity and significant disorder of the EF-hand-like motif in AlgQ2.[71]

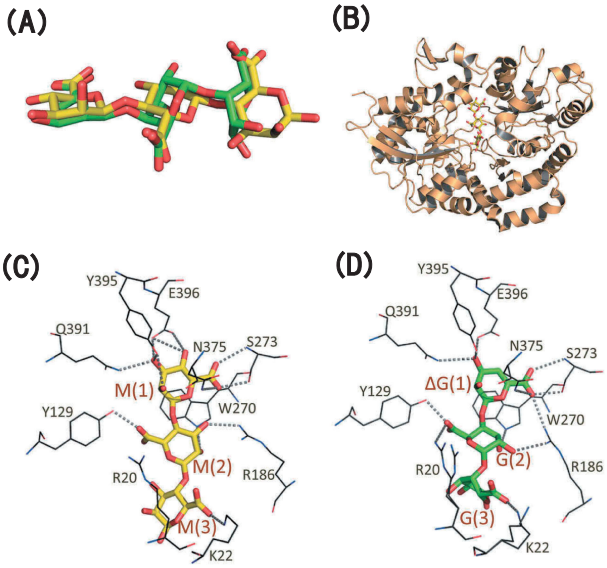

Fig. 5. Three dimensional interactions of AlgQ1 with oligoalginates MMM and ΔGGG.[27] **(A)** Structural difference between MMM and ΔGGG. Carbon atoms in MMM and ΔGGG are represented as yellow and green, respectively. **(B)** Overall structure of AlgQ1 in complex with MMM (PDB ID; 3VLU). **(C)** Alginate-binding site of AlgQ1 in complex with MMM (PDB ID; 3VLU). **(D)** Alginate-binding site of AlgQ1 in complex with ΔGGG (PDB ID; 3VLV). Hydrogen bonds formed between AlgQ1 and alginate are shown as dashed lines. Subsite numbers are shown in the parentheses in **(C)** and **(D)**. Figures were cited from Ref. 27.

The N- and C-domains are connected by three linker loops (loops 1, 2, and 3), and the cleft formed between the two domains is the alginate-binding site **(Fig. 6, A)**, which is significantly larger than that observed in other substrate-binding proteins, such as maltose-binding protein.[72] AlgQ2 binds to alginate (K_d: $\sim 10^{-7}$ M) in the deep cleft formed on closing (30°) of the N- and C-domains and releases the polymer on opening of the two domains **(Fig. 6, A)**. Upon going from the open cleft (unliganded structure) to the closed cleft (liganded structure), the Glu396 side chain moves up into the cleft as a result of alginate binding. This ligand (alginate)-induced movement of Glu396 is a trigger for the motion that enables the other domain to participate in alginate binding. The major driving force for hinge closing in alginate-binding proteins AlgQ1 and AlgQ2 was the exclusion of a water molecule from the binding site, which induces different hydrogen bond formation between loops 1 and 2. Excluding exactly one water

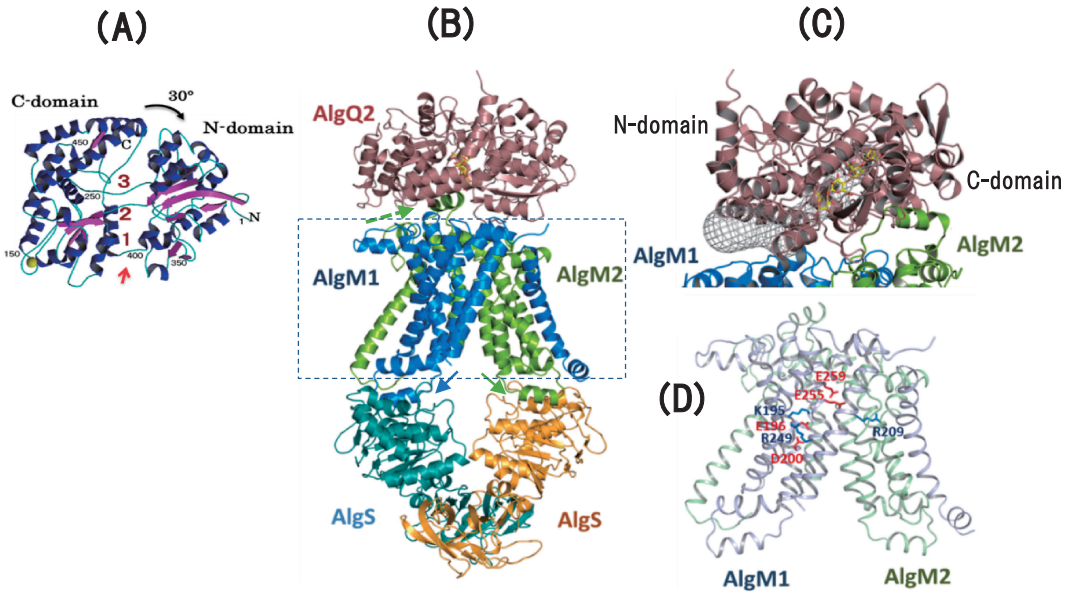

Fig. 6. Crystal structure of alginate transporter complexed with solute-binding protein.[27] **(A)** Overall structure of AlgQ2. Two globular domains (N- and C-domain) of AlgQ2 are connected by three loops 1, 2, and 3 and form a cleft (red arrow) for alginate binding. The color denotes the secondary structure elements: blue, α-helices; red, β-strands; cyan, loops and coils; yellow, calcium ion. **(B)** Overall structure of AlgM1M2SS complexed with AlgQ2. Dotted green arrow: a helix of AlgM2 protruded into the periplasm. Dotted area represents cytoplasmic membrane. Solid blue and green arrows: helices of AlgM1 and AlgM2 protruded into the cytoplasm, respectively. **(C)** The tunnel-like structure (grayed mesh). Alginate-binding cleft between the two domains of AlgQ2 is covered through the interactions between the C-terminal domain of AlgQ2 with AlgM2 and N-terminal domain of AlgQ2 with AlgM1, resulting in the formation of a long tunnel-like structure. **(D)** Charged amino acid residues in transmembrane AlgM1M2. Glu259 and Arg209 on the periplasmic side of AlgM1 and AlgM2, respectively, promote the passing of alginate. Blue and red represent basic and acidic residues, respectively. Figures were cited from Ref. 27.

molecule for domain movement is the first example among binding proteins, because in these solute-binding proteins several molecules of water are usually excluded.

A lot of knowledge of how binding proteins function in transport has been obtained through studies on maltose-binding protein.[72)–76)] The way in which alginate binds to AlgQ1 or AlgQ2 is of special interest, because alginate is a highly polar organic molecule and strain A1 cells can grow efficiently on any alginate and oligoalginates with different structures and M/G ratios. Interactions between AlgQ1 or AlgQ2 and various oligoalginates were confirmed by differential scanning fluorimetry or ultraviolet absorption difference spectroscopy,[69),70)] and they indicated that all oligoalginates examined can interact with AlgQ1 or AlgQ2, irrespective of differences in M/G composition, structure of the non-reducing end sugar (saturated or unsaturated), and sugar length (di- to tetrasaccharides). The K_d values of oligoalginates were in the range of 10^{-6} M–10^{-5} M.

These peculiar properties regarding the broad substrate specificity of AlgQ1 or AlgQ2 for oligoalginates were supported by X-ray crystal structure analyses.[27)] Note that ΔM and ΔG in oligoalginates described below indicated unsaturated terminal uronic acids with C=C double bonds between C4 and C5 in their structures. The crystal structures of AlgQ1 in complex with six types of oligoalginates (MMM, MG, ΔMMM, ΔGGG, ΔMMGM, and ΔMM)[70)] and AlgQ2 with ΔMMM and ΔMMGM,[68)] indicated that AlgQ1 and AlgQ2, both of which have at least four subsites (from terminal end: S1, S2, S3, and S4), recognize the non-reducing end sugar residue of oligoalginates most accurately and heavily, but they do not prefer saturated G residues at the S1 subsite because of steric hindrance of the C-5 carboxyl group.[27)] Furthermore, although the structures of oligoalginates differ according to their M/G compositions, AlgQ1s with different oligoalginates are structurally identical even at their ligand-binding site, as exemplified in the cases of MMM and ΔGGG **(Fig. 5)**.[27)] This is presumably because of the flexible interactions of AlgQ1 with the hydroxyl group of uronate residues at S2 and S3 subsites. C-5 carboxyl groups are recognized by specific residues of AlgQ1,

although there is a difference in the orientation of the carboxyl group between G and M residues.

This flexible mechanism for substrate recognition enables AlgQ1 and AlgQ2 to bind non-specifically to alginate oligomers, leading to the sufficient growth of strain A1 cells on various alginates with different M/G ratios. The AlgQ1 and AlgQ2 bind preferentially to the non-reducing terminal residues of alginate, delivering it to the ABC transporter in the inner membrane.

ABC transporter. ABC transporters are present in all living organisms and constitute a large protein superfamily sharing similar structural features.[77)–80)] Usually, they consist of two homologous transmembrane domains and two nucleotide-binding domains. Bacterial ABC transporters (importers) responsible for nutrient uptake are equipped with additional periplasmic or lipid-anchored extracellular solute-binding proteins specific to a particular substrate.[81)] Mainly based on the folds of their transmembrane domains, Type I, II, and III ABC transporters (importers) have been elucidated so far.[82)] Type I ABC transporters have five to eight helices in their transmembrane domain, whereas Type II ABC transporters have ten helices. On the other hand, Type III transporters require a membrane-embedded component instead of solute-binding proteins. The three-dimensional structure of a Type I ABC transporter was first revealed for the molybdate transporter.[83)] Its characteristic fold has also been confirmed in other Type I transporters such as the *E. coli* maltose transporter,[84)] methionine transporter,[85)] and amino acid transporter.[86)]

The alginate transporter AlgM1M2SS of *Sphingomonas* sp. A1 cells belongs to the Type I ABC transporters. ABC transporter AlgM1M2SS is absolutely required for alginate transport, because disrupting the transporter induces complete loss of viability of strain A1 cells on alginate medium.[66)] The components of the alginate transport system are reciprocally linked and work cooperatively to achieve efficient transport of alginate.[69),87)] Conveniently, the strain A1 ABC transporter is localized just below the pit **(Fig. 1, E: unpublished)**.

The crystal structures of AlgM1M2SS and AlgM1M2SS/AlgQ2 complexes in an inward-facing conformation were determined.[27),87)] In the AlgM1M2SS/AlgQ2 complex **(Fig. 6, B)**, AlgM1 and AlgM2 showed similar topology, with six transmembrane helices, and a dimer that is closed to the periplasm but open to the cytoplasm (*i.e.*, inward-facing conformation), thus indicating that the determined conformation of AlgM1M2SS/AlgQ2 complex is in the state before substrate alginate translocation. One helix of AlgM2 protruding into the periplasm was indispensable for the interactions with AlgQ2. Helices of AlgM1 and AlgM2 protruding into the cytoplasm were important for interactions between AlgM1 and AlgS or AlgM2 and AlgS,[88)] respectively. One of the two helices in each subunit was packed in a pocket of AlgS, which is called the coupling helix conserved among transporters.

The tertiary structures of AlgM1M2SS and AlgM1M2SS/AlgQ2 complexes were almost similar to those of maltose transporter,[89)] but the manner of their interaction with alginate was significantly different. The alginate transport process is characterized by both the presence of a "tunnel"-like structure at the AlgQ2 and AlgM1M2 interface **(Fig. 6, C)** and the charged features of the AlgM1M2 dimer inner cavity[27),87)] **(Fig. 6, D)**. These structural features are essential for the transport of alginate, an acidic polysaccharide.

Briefly, in the AlgM1M2SS/AlgQ2 complex, the C-terminal domain of AlgQ2 interacts with AlgM2, and the N-terminal domain of AlgQ2 interacts with AlgM1. Consequently, the substrate-binding cleft between the two domains of AlgQ2 is covered by AlgM1M2 and a long "tunnel"-like structure is created in the AlgM1M2SS/AlgQ2 complex **(Fig. 6, C)**. The "tunnel"-like structure formed continues to the alginate-binding site of AlgQ2, and its length reaches approximately 30 Å, corresponding to alginate heptasaccharide. By this "tunnel"-like structure, the interaction between AlgM1M2SS and AlgQ2 with alginate polymer becomes easier and alginate is smoothly transferred to the ABC transporter.

The surface of the inner cavity of AlgM1M2 contains both acidic and basic amino acid residues **(Fig. 6, D)**; it is different from that of the maltose transporter which transfers neutral small saccharides as a substrate.[72)] The charged residues in the ABC transporter inner cavity are indispensable for effective binding of alginate from AlgQ1 or AlgQ2, and its subsequent release into the cytoplasm. The negative and positive charges derived from the residues Glu259 and Arg209 on the periplasmic side of AlgM1 and AlgM2, respectively, may contribute to the effective transport of the negatively charged alginate released by the solute-binding protein through the "tunnel"-like structure.

In the AlgM1M2SS complex, the distance between the periplasmic and cytoplasmic ends of

the inner cavity is approximately 27 Å, corresponding to six residues of linear alginate.[27] The ATPase activity of AlgS is induced by AlgQ1 or AlgQ2 complexed with alginate or alginate oligomers.[28] However, probably because of the experimental condition constraints, proteoliposome-containing AlgM1M2SS complex failed, in the presence of AlgQ2 and ATP, to transport longer oligosaccharides than tetrasaccharides, although the crystal structure of the AlgM1M2SS/AlgQ2 in complex with longer oligoalginates takes the same inward-facing conformation and the longer oligoalginates obviously stimulated the ATPase activity of AlgS in the proteoliposome.[27),28),87),88] It is thus expected that alginate with a higher molecular weight than tetrasaccharide will be transported. The observation that most of the endo- and exo-types of alginate lyases are usually found in the cytoplasm supports this notion.[20] The long "tunnel"-like structure created in the AlgM1M2/AlgQ2 complex can be regarded as a molecular aperture highlighted in this review and, through collaboration with the pit on cell surface, contributes to the direct transport of alginate from outside the cell to the inside (*i.e.*, cytoplasm).

Thus, in *Sphingomonas* sp. A1 cells, extracellular alginate is first concentrated in the pit by the action of flagellins p5 and p6.[41] Even the alginate granules are gathered in the pit **(Fig. 1, B-3 and B-4)** by the action of p8 protein.[42] Alginates concentrated in the pit are transferred into periplasm by an unknown mechanism, and then transported into cytoplasm through the unique "tunnel"-like structure formed in the interface between solute-binding proteins (AlgQ1 or AlgQ2) in the periplasm and ABC transporter (AlgM1M2SS) in the inner membrane,[27] which is localized just below the pit **(Fig. 1, E: unpublished)**. In this case, any types of alginate molecules are delivered, owing to the highly flexible substrate (alginates) recognition mechanism of solute-binding proteins, AlgQ1 or AlgQ2.[27] Alginate is finally depolymerized/degraded by endo- and exo-type alginate lyases in the cytoplasm, facilitating alginate release from the ABC transporter. In that sense, the above-described proteoliposome experiments conducted in the absence of alginate lyases inside the liposome may have described the capability of alginate transport system as low.

Based on these results, an overall scheme for the transport and assimilation of alginate by the cells of *Sphingomonas* sp. A1 was constructed **(Fig. 2)**. In this scheme, the alginate mechanism of passing through the pit structure into the periplasm is an intriguing problem, which needs to be addressed in the future. The alginates transported into the cytoplasm were degraded into monosaccharides by β-elimination reactions catalyzed by alginate lyases of endo- and exo-types.[29),90)–92] The monosaccharides were converted non-enzymatically into α-keto acids (4-deoxy-L-*erythro*-5-hexoseulose uronate [DEH]).[93] Details of the DEH metabolic pathway, including evolutionary and structural aspects of alginate lyases and other enzymes involved in the pathway are described elsewhere.[26),94)–98]

3. Applicability of the "mouth"-like pit

The "mouth"-like pit formed on the bacterial cell surface has some distinct traits not found in other bacteria identified to date and is considered to be of potential use in scientific, industrial, and environmental processes, along with other related microbial tools. Here, we show possible applications of sphingomonads with a "mouth"-like pit for fermentative production of biofuel (bioethanol) from alginate and bioremediation of environmental pollutants. These studies also revealed biologically and biotechnologically important problems regarding the nature of the cell surface structures.

Marine biotechnology and biofuel. Although attempts to produce bioethanol from land biomass, starch, and cellulose, have been extensively studied in Japan, realizing this has been impeded by economic or technical issues.[99] The typical marine biomass alginate contains no lignin and is produced in large quantities by brown seaweed algae.[99),100] Genetically modified *Sphingomonas* sp. A1 cells overexpressing alcohol dehydrogenase (encoded by a gene *adhB*) and pyruvate decarboxylase (encoded by *pdc*) genes from *Zymomonas mobilis* were used to produce bioethanol from alginate and showed unprecedented success in producing large amounts of bioethanol (13 g bioethanol/60 g alginate/ℓ) from alginate as a carbon source.[101] Although the explanation of experimental strategies are omitted, this was achieved by the mobilization of advanced technologies, including metabolic and genetic engineering, transcriptome and metabolome analyses, protein structural biology, and fermentation technology, in combination with cell surface morphology of the "mouth"-like pit, and may be one of the typical multidisciplinary science. Subsequently, a group in the U.S. also reported bioethanol production from alginate using engineered *E. coli* cells.[102] The bioethanol productivity of their

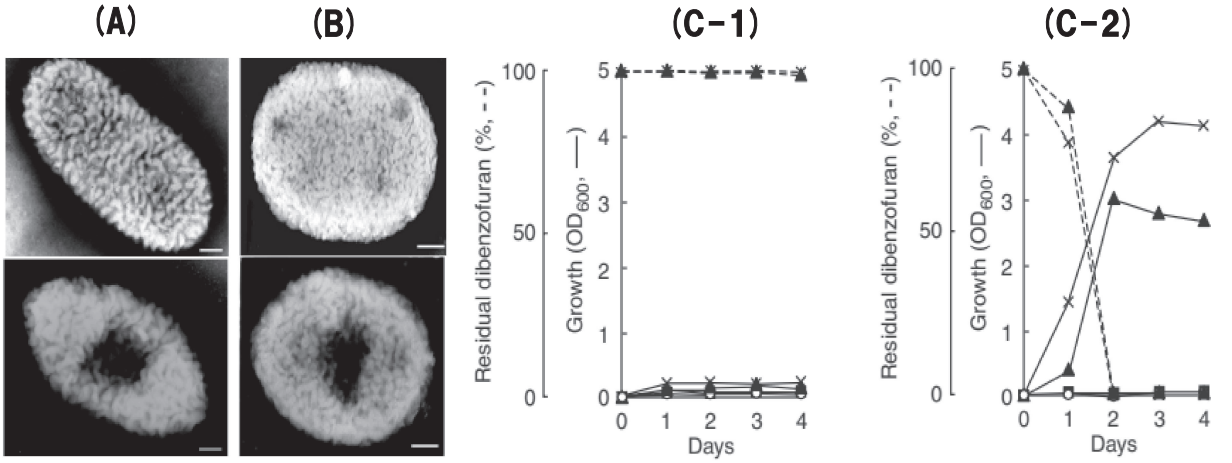

Fig. 7. Mouth-like pit transplant and improvement of bioremediation potentials of sphingomonads.[110] **(A and B)** The cell surface structures of *S. wittichii* RW1/pKS13, vector plasmid **(A, above)** and RW1/pBE11 **(A, below)** and those of *S. subarctica* IFO 16058/pKS13 **(B, above)** and IFO 16058/pBE11 **(B, below)**. The surface structures of RW1 (wild-type) and IFO 16058 (wild-type) were similar to those of RW1/pKS13 and IFO 16058/pKS13, respectively (data not shown). The white bar represents 0.1 µm. **(C)** Time courses of cell growth of *S. wittichii* RW1/pKS13 **(C-1)** and RW1/pBE11 **(C-2)** on dibenzofuran. Both cells were grown in the absence (open circle) or presence of 0.5% alginate (closed square), 10 mM dibenzofuran (closed triangle) or both (x mark). Solid line, cell growth (optical density (OD) at 600 nm); dashed line, dibenzofuran concentration. For details about cultivation conditions, see Supplementary to Ref. 110. Figures were cited from Ref. 110.

system was reported to be approximately 37.8 g bioethanol/130 g dry milled macroalgae/ℓ. In this case, it is necessary to take into account that, other than alginate, mannitol in the dried materials is also converted into bioethanol.

We have also achieved bioethanol production from mannitol using yeast *Saccharomyces paradoxus* NBRC 0259 (AKU4135)[103] or *Saccharomyces cerevisiae* BY4742 with a dysfunction of Tup1-Cyc8, a general corepressor complex that regulates many kinds of genes.[104] This method for producing bioethanol from both marine biomass (bioethanol productivity: more than 70 g/ℓ from 5% alginate and 10% mannitol, a concentration beyond practical application levels) using genetically modified strain A1 in combination with *S. paradoxus* NBRC 0259 or *S. cerevisiae* BY4742 with a dysfunction of Tup1-Cyc8 may contribute to the development of marine biotechnology and can be applied to real-life uses in the future, especially from the viewpoint of carbon neutral biofuel production.

Organ transplant and bioremediation. Sphingomonads are a group of bacteria characterized by, among other things, their wide-ranging capabilities for degrading environmentally hazardous compounds and are distributed extensively in nature, especially in soils and aquatic environments. To improve their bioremediation capabilities further, the "mouth"-like pit of *Sphingomonas* sp. A1 was transplanted into two related sphingomonads, *S. wittichii* RW1[105–107] and *S. subarctica* IFO 16058,[108] which have dioxin- and polypropylene glycol-degrading activities, respectively.

To express the pit of strain A1, a hybrid plasmid, pBE11, containing all the genes involved in alginate import **(Fig. 2, below)** was constructed using a vector plasmid pKS13 and introduced into cells of *S. wittichii* RW1 and *S. subarctica* IFO 16058.[109] In both cases, the cells transformed with pBE11 (designated as strains RW1/pBE11 and IFO 16058/pBE11) formed a pit on their cell surface **(Fig. 7, A and B)**. Western blotting analysis indicated that the AlgS and AlgQ2 proteins were expressed in RW1/pBE11 cells grown in the absence of alginate.[110] The pits formed were similar in size to those observed in strain A1 cells **(Fig. 1, B-3 and B-4)**, and the formation of the pit was constitutive and irrespective in the presence or absence of alginate.[110]

To evaluate dibenzofuran-degrading activity, RW1/pBE11 cells were cultured in a two-liquid phase system[111] in a medium containing 0.5% alginate and/or 10 mM (1.68 g/ℓ) dibenzofuran as carbon sources **(Fig. 7, C-1 and C-2)**. After only 2 days of cultivation, RW1/pBE11 cells had nearly reached the stationary phase of growth on 10 mM

dibenzofuran, with or without alginate **(Fig. 7, C-2)**, and dibenzofuran was completely depleted **(Fig. 7, C-2)**. Although the data are not shown here, RW1/pBE11 cells grew on 100 mM (16.8 g/ℓ) dibenzofuran and completely depleted it after 1 week of cultivation. The growth of the strain RW1/pBE11 increased when the cells were grown in the presence of both alginate and dibenzofuran **(Fig. 7, C-2)**; this may be because alginate acts as a solubilizer for dibenzofuran, because RW1 has no alginate-assimilating ability. In addition, RW1/pBE11 cells may also remove another dioxin analog, dibenzo-*p*-dioxin, more rapidly than RW1 (wild-type) cells.[110]

On the other hand, the growth of control cells, RW1 and RW1/pKS13, was very low, regardless of the presence or absence of alginate **(Fig. 7, C-1)**. This was presumably due to the lag time during the first few days in the medium used. In fact, after 4 days of cultivation, these control cells gradually recovered their growth by assimilating dibenzofuran (data not shown).[110] Furthermore, uptake rates of dibenzofuran were measured in resting cells of RW1 (wild-type) and RW1/pBE11. RW1/pBE11 cells showed a gradual increase in the uptake of dibenzofuran after incubation with dibenzofuran, whereas RW1 (wild-type) cells showed a constant uptake.[110] These findings clearly indicated that the increased growth rate of RW1/pBE11 on dibenzofuran was associated with the increased uptake of dibenzofuran through the transplanted pit, although there is no structural relationship between dibenzofuran and alginate.

Using the same technique, we also succeeded in improving the biodegrading activity of polypropylene glycol-degrading *S. subarctica* IFO 16058. The engineered IFO 16058/pBE11 cells produced a pit **(Fig. 7, B, below)** and removed polypropylene glycol 700 at a faster rate than cells of a non-engineered strain.[110] Although IFO 16058/pBE11 cells might metabolize alginate as well, the formation of the pit was independent of the presence of alginate, similar to engineered cells of strain RW1. The pit formation by engineered cells of RW1 and IFO 16058 strains, but not by strain A1 cells, in the absence of external alginate was considered to be due to the difference in the transcriptional regulatory mechanism of genes involved in alginate incorporation between the cells of strains A1 and RW1 (or IFO 16058), as discussed previously.[110]

Thus, we reported an approach for improved bacterial bioremediation of organic pollutants that increases the uptake capabilities of target organisms by transplanting a nonspecific pit present in *Sphingomonas* sp. A1 into related *Sphingomonas* strains. The bioremediation capabilities of dioxin-degrading *S. wittichii* RW1 and polypropylene glycol-degrading *S. subarctica* IFO 16058 were increased with this approach. This method has the potential to improve the capacity of microbial degraders suitable for the bioremediation of various pollutants. A microcosm study showed that RW1/pBE11 cells were effective in environmental remediation of dibenzofuran.[110]

If the "mouth"-like pit structure can be referred to as an organ as in mammals, this is the first successful method enabling organ transfer among bacterial strains, termed "organ transplant technology". This technology may serve as a tool to improve the import activity of microorganisms and create microbial cells with morphologically and physiologically distinct features. Although this technology is currently restricted to sphingomonad strains, this engineering method might also be expanded to other microbes belonging to other species. Gram-negative *A. tumefaciens* cells with LPS-based cell walls are an immediate target in this regard, because the bacterium has a gene set similar to that for forming the "mouth"-like pit,[39] as in *Sphingomonas* sp. A1.

Discussion and summary

The finding of apertures, such as a "mouth"-like pit, on a bacterial cell surface raised some significant biological problems concerning the cell shape, cell surface structure and function, cell economy, macromolecule transport, and metabolism, as well as functional and evolutional aspects of the aperture and cell surface components such as flagellin. A detailed study on each item mentioned above is indispensable for the overall understanding of a course of events that determine the fate of alginate. However, to avoid duplication, we have discussed only the cell surface structure, including novel and dynamic structure found in *B. subtilis* cells.

Compared with the cell shape, the cell surface structure and function are accurately and precisely controlled so that the cells can respond simultaneously and instantaneously to any cellular events. Changes in cell surface structure often tend to occur locally on a small scale. Following our finding of the pit in 1995, another "mouth"-like aperture structure designated as a "dent" was identified in 2007 on the cell surface of Gram-negative *Sphingopyxis* sp. 113P3, one of the genera of sphingomonads. As with the pit in *Sphingomonas* sp. A1, the dent has a function to directly incorporate a macromolecule,

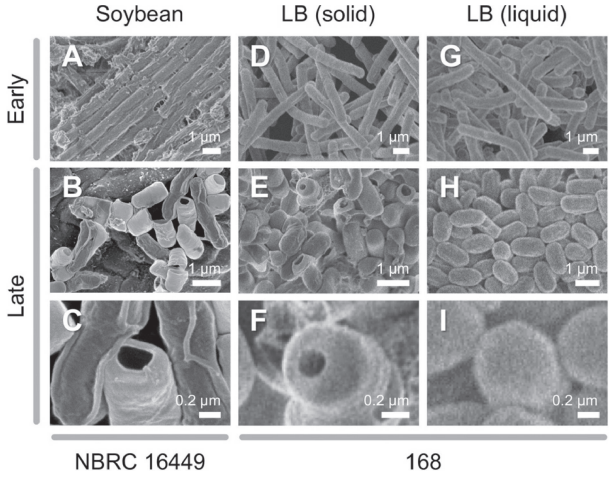

Fig. 8. Formation of a "hole"-like structure on the *B. subtilis* cell surface.[115] Cell shapes were examined using a scanning electron microscope. Left column; *B. subtilis* NBRC 16449 cells grown for 5 h **(A)** and 48 h **(B, C)** on dead soybeans. Middle column; *B. subtilis* 168 cells grown for 4 h **(D)** and 216 h **(E, F)** on Luria-Bertani (LB) medium solidified with agar. Right column; *B. subtilis* 168 cells grown for 4 h **(G)** and 239 h **(H, I)** in liquid LB medium. Bars, 1 μm **(A, B, D, E, G, H)** or 0.2 μm **(C, F, I)**. Figure was cited from Ref. 115.

polyvinyl alcohol (PVA), present extracellularly into the cells.[112)–114)] Namely, although the cells of strain 113P3 can grow on PVA as a carbon source, PVA-degrading enzymes are exclusively localized in the periplasmic space.

In 2020, we found that *Bacillus* cells can form a "hole"-like structure in their cell surface[115)] during growth on solid media. Briefly, when the cells of *B. subtilis* 168 were grown in a nutrient medium solidified with agar, the cells shrank at the end of growth **(Fig. 8, E and F)** and formed a "hole"-like structure in one end of the cell **(Fig. 8, E and F)**. The cells of *B. subtilis* NBRC 16449[116)] also showed similar morphological changes to those of *B. subtilis* 168 cells during growth on solid medium (dead soybeans) **(Fig. 8, A and B)** and formed a "hole"-like structure **(Fig. 8, B and C)**.

The cells of *B. subtilis* 168, however, never produced such the "hole"-like structure in liquid medium even after prolonged incubation for about 10 days **(Fig. 8, H and I)**, although they shrank as observed in the growth on solid medium **(Fig. 8, G and H)**. These results indicated that solid-state growth is one of the triggers for the creation of this unique "hole"-like structures on *B. subtilis* cell surfaces. Although the hole is different from the pit and dent in shape, size, dynamics (no repetition of opening/closing), and time of formation (usually in late growth phase), as far as we know, this is the first description of an aperture structure in *B. subtilis* cells.

A closely similar "hole"-like structure has also been observed in liquid medium-grown cells of *Clostridium sporogenes*, a Gram-positive, rod-shaped, spore-forming bacterium,[117)] suggesting that the hole is an exit for spores (or outgrowing cells),[117)] or a route for substances necessary to maintain spore viability.[118)] However, judging from the fact that the "hole"-like structure in *B. subtilis* formed in solid media **(Fig. 8)** and that of *C. sporogenes* in liquid media, the physiological function of the "hole"-like structure in cells of *B. subtilis* is different from that of *C. sporogenes*. Studies on the solid- and liquid-medium-dependent hole creation mechanism and the signaling pathway linked to this phenomenon will provide an acritical concept in understanding bacterial strategies for fitness or survive in aqueous and non-aqueous environments.

The aperture structures, *i.e.*, the pit of *Sphingomonas* sp. A1 **(Fig. 1)**, dent of *Sphingopyxis* sp. 113P3, and hole of *B. subtilis* 168 and NBRC 16449 **(Fig. 8)** may further our understanding regarding the control mechanism of cell surface structures, because the formation of these unique structures causes wide-range rearrangement or remodeling of Gram-negative LPS- or GSL-based lipid bilayers or Gram-positive thick peptidoglycan layers. The pit and dent, as well as the hole, might be generated through invagination of the cell surface. The cells of Gram-negative bacteria generally contain non-stereoselective porins in their outer membranes that allow the passage of small molecules, such as ions, nutrients, and metabolites, across the lipid bilayer.[119)] In that sense, the presence of the pit and dent, including a hole that may pass macromolecules and/or particles, may be considered to be created as special equipment to facilitate macromolecule processing.

Nonetheless, keeping the comparative physiological and biochemical arguments aside, the "mouth"-like aperture-dependent macromolecule transport systems might open a new field for research, such as cell morphology engineering or cell economy engineering, as exemplified through the construction of microbes with high productivity of biofuel or bioremediation capability of environmental pollutants by "organ transplant technology".[110)] Investigations of the structures and functions of the pit, dent, and hole structures will contribute to

progress in research in this direction that will surpass the borders of genera and species. The question arises as to why, when, and how these apertures are generated on the cell surface. Many biologically important problems remain unsolved.

In summary, it should be emphasized that apertures, such as the pit, dent and hole, were found during the course of studying possible applications of alginate lyase in *Sphingomonas* sp. A1,[120)–123)] PVA-degrading enzymes in *Sphingopyxis* sp. 113P3,[112)–114)] and plant cell wall (pectin)-degrading enzymes in *B. subtilis*,[115),124)–132)] respectively. In other words, applied studies led to the discovery of novel basic biological phenomena, which will expand and develop new areas of microbiology and bioscience. This development, combined with the recent dramatic changes in the scientific environment, has prompted us to adopt the exaggerated and unduly expanded title used for this review.

The results obtained indicated that microbial cell surfaces are not structurally and functionally fixed and that the cell surfaces are not isotropic in function and structure. Depending on the changing environment, microbes can be induced to undergo an unexpected, large-scale change in their cell surface, in some instances, by recruiting proteins with various functions. In this regard, the physiological significance of flagellin to bind alginate and to localize on the cell surface is a research priority, because this phenomenon may increase our understanding of the origin and evolution of the flagellin protein.

The discovery of the latent ability of microbes to reconstruct their cell surface represents a significant increase in our knowledge on the nature of the cell surface. It predicts even that the bacterial cells probably have front and back sides and the latter reflects a cellular capacity to survive in diverse environments. Knowledge on remodeling of the cell surface structure expands the realm of applied microbiology, as evidenced by the successful utilization of cells with pits and their associated enzymes in environmental processes.[99),110)] In particular, the results of a microcosm study using genetically modified microbes in environmental remediation[110)] indicated the usefulness of this microcosm system for removing dioxin contained in a defoliant. In Japan, a large amount of defoliant (approximately 26 tons) is presumed to be buried in the soil.[133)] There is now concern about its adverse health effects because its outflow is in line with environmental changes. However, biotechnological knowledge for the removal of this dioxin has not yet been recognized.

The bacterial structure resembling the mouth of multicellular animals is a remarkable feature produced through the dynamic rearrangement and/or reconstitution of cell surface molecules. It is certain that other unique systems and/or apparatus will be found in the future. However, using routine microbiological approaches, the detection of such molecular apparatus would be rare, because it is not easy to sense the time when they appear on the cell surface. In that sense, it is not so surprising that, despite the long history of morphological research regarding the differentiation mechanisms of *B. subtilis* cells, which is a model microorganism for sporulation studies, the "hole"-like structure has not been observed even to this day.[115)] The serendipitous finding of the pit, dent, and hole demonstrates the necessity of constantly questioning and not taking all observations arising from microbiological experiments for granted.

Lastly, we would like to add a few additional sentences regarding the behavior of *Sphingomonas* sp. A1 cells toward alginate. The cells can recognize a place where alginate is. Second, they acquired the means to reach there: a flagellum consisting of flagellin with alginate-binding ability. They put on flagellins to gather alginate around themselves. Third, they created a mouth (pit) to swallow alginate into the cells along with an ABC transporter. Finally, they prepared intracellular enzyme systems to depolymerize alginate (by more than six alginate lyases) and convert it to energy and cellular constituents. This series of events manifests a straight "will" of the organism – "I want to eat alginate". Although spiritualistic, it looks like the "will" directly organized the molecules to realize this unique and complete alginate-utilizing system.

Chemotaxis studies[51),132),134)] experimentally confirmed that, according to the concentration gradient of alginate on agar plates, *Sphingomonas* sp. A1 cells can swim toward alginate present 2 cm away from them. This distance represents 10,000 times the cell length (∼2 μm). Therefore, if we liken it to a person who is 2 m tall, the chemotactic behavior of strain A1 cells can be regarded as a person swimming toward a favorite dish 20 km away. Cells of strain A1 must have a good nose besides a big mouth.

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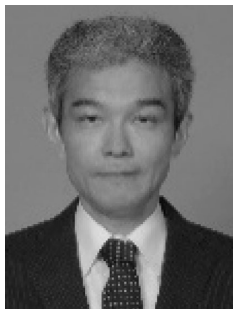
Profile

Kousaku Murata was born in Kyoto Prefecture in 1948, graduated from the Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, in 1974, and completed his graduate studies at the same university. He then moved to the Research Laboratory of Applied Biochemistry, Tanabe Pharmaceutical Co., Ltd. In 1980, he obtained the position of research associate at the Department of Applied Molecular Biotechnology, Research Institute for Food Science, Kyoto University. After receiving his Ph.D. in 1982, he worked as a postdoctoral fellow at the Department of Biochemistry, Faculty of Medicine, Cornell University, New York, U.S.A. In 1988, he was promoted to Associate Professor, and in 1995, to Professor at the Institute for Food Science, Kyoto University. In 2001, he became Professor at the Laboratory of Basic and Applied Molecular Biotechnology (of which he was a founder), Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University. During his tenure at Kyoto University, he served as an adjunct lecturer at Kyoto Bunkyo University and other colleges/universities. In 2013, he retired from Kyoto University and became Professor Emeritus. From 2014 to 2019, he worked as a Distinguished Professor of Setsunan University, Graduate School of Science and Engineering, Faculty of Life Science. He served as a vice president of the Japan Society of Bioscience, Biotechnology, and Agrochemistry between 2011 and 2013. He was honored to receive the Agricultural Chemistry Encouragement Award from the Japan Society of Agricultural Chemistry (1988), the Nagai Memorial Prize from the Nagai Memorial Foundation (1993), Agricultural Chemistry Award from the Japan Society of Agricultural Chemistry (2007), and others including many thesis awards. He was chosen as a Highly Cited Researcher in the world (International Scientist Medal of the Year) (2003), Fellow of the World Innovation Foundation (*WIF*) (2003), Deputy Director General of the International Biographical Centre (Cambridge) (2004), Fellow of the Japan Society of Agricultural Chemistry (2015), and Meritorious Member of The Vitamin Society of Japan (2013) and of The Society for Biotechnology, Japan (2020). He is currently working as an academic and technical advisor of CHA Research Center, Fukujuen Co. Ltd., Kyoto (2010–).

Profile



Shigeyuki Kawai was born in Kyoto Prefecture in 1972, graduated from the Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, in 1994, and was awarded an MSc from the Graduate School of Agriculture, Kyoto University in 1996. He left the same Graduate School without completing the Ph.D. course in 1998 and took up a role as Assistant Professor at the Institute of Food Science, Kyoto University in 1998, at Graduate School of Agriculture, Kyoto University in 2001, and an Associate Professor at the same Graduate School in 2007. He became a Professor at the Research Institute of Bioresource Engineering, Ishikawa Prefectural University in 2018, and the Director of this Institute and Assistant to the President of this University in 2019. He was awarded his Ph.D. degree of at the Graduate School of Agriculture, Kyoto University in 2002. He was honored to receive the Japan Society for Bioscience, Biotechnology, and Agrochemistry (JSBBA) Award for Young Scientist in 2007, and others including many thesis awards.

Profile

Wataru Hashimoto was born in Osaka Prefecture in 1967, graduated from the Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, in 1990, and received his Ph.D. at the same university, in 1995. He became a research associate at the Department of Food Safety and Utilization, Research Institute for Food Science, Kyoto University. In 2000, he was promoted to Associate Professor at the Institute for Food Science, Kyoto University, and in 2015 to Professor at the Laboratory of Basic and Applied Molecular Biotechnology, Division of Food Science and Biotechnology, Graduate School of Agriculture, at Kyoto University. In 2005, he was a visiting researcher at the Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL, U.S.A. He was honored to receive the Japan Society for Bioscience, Biotechnology, and Agrochemistry (JSBBA) Award for Young Scientist in 2003, and others including many thesis awards.