Superchannel of Bacteria: Biological Significance and New Horizons

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Sphingomonades are Gram-negative bacteria. They contain no cell surface lipopolysaccharides (LPSs), but have glycosphingolipids (GSLs) with long-chain base dihydrosphingosine in the outer leaflet of the outer membrane in place of LPSs.1) Due to the presence of GSLs, the genus Sphingomonas is classified independently from pseudomonades and is grouped phylogenetically into the α-4 subclass of Proteobacteria.2) Some sphinomonades are characterized by the fact that their cells are covered with many large pleat-like substances3) and show extraordinary metabolic abilities to degrade various refractory environmental pollutants.4) These characteristics, inherent to bacteria of this genus, call attention to an unexpected structure and function of the cell surface.

The cell surface of Sphingomonas sp. strain A1, a soil isolate with alginate-degrading ability, is covered with many large pleats, and when assimilating alginate, it forms a mouth-like pit on the cell surface through reorganization and/or the fluidity of the pleats (Fig. 1).3) So far as we are aware, this is the first description of a pit-forming bacterium in the history of microbiology. The pit functions as a concentrator of alginate, and the polymer targeted there is transported into the periplasm. The alginate introduced into the periplasm is captured by periplasmic alginate-binding proteins, transferred to an ATP-binding cassette (ABC) transporter (importer) in the inner membrane,5) and then transported into the cytoplasm, where it is depolymerized into its constituent monosaccharides through reactions catalyzed by exo- and endotype alginate lyases (Fig. 2).6,7)

In case of alginate engulfment, dynamic structural and functional fluctuations leading to the formation of the pit occur on the cell surface of strain A1. This indicates that substantial structural and functional fluctuations occur frequently on the cell surface in other than extreme cases

Fig. 1. Time Course of Pit Formation.
Cells were cultured in the presence of alginate for (upper, from left) 0, 7, and 10 h, and (lower, left) 24 h. The magnified picture of the pit (lower, middle, and right) indicates the disappearance and rearrangement of pleat molecules and substantial accumulation of alginate gel particles.
such as cell division and spore formation. Formation of the pit may be considered to be the original model of the mesosome of Gram-positive bacteria and/or endo/phagocytosis by eukaryotes, and this conception is applicable to the import of other macromolecules, such as DNA, RNA, and proteins, across the cell membrane.

I. Superchannel

Alginate is a linear polysaccharide produced by seaweed and certain bacteria such as *Pseudomonas aeruginosa* (mucoid type) and *Azotobacter vinelandii*. It is composed of α-1-guluronate (G) and its C5 epimer, β-2-mannuronate (M). The cells of *Sphingomonas* sp. strain A1 directly incorporate alginate into the cytoplasm through the pit-dependent transport system, the superchannel, and the genes for the superchannel are located in a single cluster. Here, we review the overall picture of the channel and argue for its biological significance.

1. Genome sequence

The complete genome sequence of strain A1 and its properties have been reported. This is the first genome structure of a sphingomonad to be determined. The genome consists of 4,622,788 base pairs with a GC content of 62%, and it has the capacity to contain approximately 4,800 genes, the functions of 42% of which have been predicted. Thirty percent of the genes exhibit significant homology with those in the genome of *P. aeruginosa*, indicating that sphingomonades and pseudomonades evolved or diverged from a common ancestor.

Other properties of the genome are described in reference 10 together with those of plasmid DNA (pA1: 46,558 base pairs, GC content 65%, more than 40 genes).

2. Cell surface flagellin

The pit formation in strain A1 is reversible. It appears in the presence of alginate and disappears in its absence. Comprehensive analyses, including transcriptomic and proteomic ones, have indicated that cells with pits induce abundant amounts of eight proteins (p1-p8) [p1 (75 kDa), p2 (75 kDa), p3 (74 kDa), p4 (75 kDa), p5 (41 kDa), p6 (31 kDa), p7 (27 kDa), and p8 (20 kDa)] in their outer membrane. p1-p8 are homologous to bacterial proteins: p1-p4, chelator-dependent transporters for ferric ions; p5 and p6, flagellin; p7, alginate-binding protein, and p8, granule-binding protein. The genes encoding p1-p8 are mapped separately in the bacterial genome, and are independent of the gene cluster for the superchannel.

*Transports.* A mutant with disruption of *p1, p2, p3,* or *p4* shows apparent growth retardation on alginate as a carbon source. Bacterial outer membrane transporters homologous to p1-p4 are known to incorporate the iron-siderophore complex using energy generated through transient association with an inner membrane complex (TonB-ExbB-ExbD). Since alginate exhibits an ability
to chelate ferric ions, p1-p4 might function as outer membrane transporters for the import of ferric iron-chelated alginate as an iron-siderophore complex. This finding indicates that the TonB-dependent receptor is responsible for the transport of various molecules, including iron-siderophores, alginate, and aryl- or alkylsulfate esters. In homology modeling, the transporters (p1-p4) designated AlgR appear to constitute a tunnel-like β-barrel structure spanning the outer membrane (Fig. 2).

Flagellins. One of the unexpected findings of this study on the function of the outer membrane concerns the localization and function of the flagellin homologs (p5 and p6). The two proteins resemble each other in primary structure, have significant homology with flagellin, a component of the flagella of Gram-negative bacteria, and are expressed in the outer membrane in the presence of alginate, although neither flagellation nor motility has been confirmed for strain A1 cells. Both p5 and p6 of strain A1 exhibit high similarity with bacterial flagellins in the N- and C-terminal domains, although little homology is observed in the central domain. Furthermore, part of the central domain (approximately 100 amino acid residues) of p5 and of p6 is missing in comparison with the structures of Escherichia coli and Salmonella typhimurium flagellins, resulting in the formation of shaft-type flagella with no propeller. Since certain pseudomonads that form a polar flagellum produce flagellins lacking part of the central domain, the deletions observed in p5 and p6 are irrelevant to the fact that strain A1 has no flagella.

A p6-disruptant showed significant growth retardation in the presence of alginate and the cell surface changed from a pleat structure to a network structure, thereby losing the ability to form pits. The double disruption of p5 and p6 caused cell death. This evidence indicates that p5 and p6 are essential to the formation of the pit and the maintenance of cell surface structure and that they participate in the determination of cell destiny. Flagellins are exported from the cytoplasm to the cell surface through the type-III exporter encoded by the flagellum cluster, supporting the notion that the type-III secretion system evolved from the flagellar export apparatus, but the expression of p5 is suppressed in mutant cells with a disrupted peptidoglycan hydrolase gene, suggesting the presence of an alternative pathway to export flagellin to the cell surface and to localize it at the cell envelope. If this is the case, flagellins destined for the outer membrane must cross the inner membrane and periplasm and be assembled in the outer membrane containing GSL, a more hydrophobic lipid molecule than LPS, in their appropriate conformation, possibly in the absence of a biochemical energy supply.

In addition to localization on the cell surface, p5 and p6 can bind specifically to alginate with high affinity (dissociation constant, $K_a$, about nm). This fact suggests that the two flagellin homologs are involved in alginate signaling as CD44-like receptors, a mammalian transmembrane receptor ($K_d = nm$) for hyaluronan, rather than in the harvesting of alginate on the cell surface. E. coli flagellin can also bind to alginate with a $K_d$ on the nm level, indicating that alginate-binding is an inherent property of the protein. Flagellin might first be utilized as a cell surface protein and then undergo a change into a flagellar protein, or vice versa.

The crystal structure of p5, the first of a flagellin containing the flagellin_IN motif (unpublished), is helpful in elucidating the molecular basis of alginate-binding and its localization on the cell surface. p5 is divided into two structural domains, α and β, connected by two short peptide linkers. The α-domain resembles domain D1 of S. typhimurium flagellin, and consists of N- and C-terminal regions further divided into two sub-domains, α1 and α2. Alginate binds in a cleft called the spoke region at the boundary of the α1- and α2-domains. The β-domain is rich in β-strands, which constitute the central region, and its structure is closely similar to that of bacteriophage T4 gene product 11 (T4gp11). T4gp11 is a hinge protein connecting the baseplate to the short or long tail fibers, which is necessary for the irreversible attachment of the phage to the cell surface of host cells. A metalloprotease (50 kDa, M16 family) on the cell surface of strain A1 has been tentatively identified as a protein that can interact with the β-domain of p5 and retain p5 on the cell surface. The finding of structural similarity between the central β-rich domain of flagellin and bacteriophage T4gp11 should give information regarding not only the function of the central β-domain in keeping flagellin in a mobile state suitable for pit formation, but also the origin and/or evolution of molecular segments constituting flagella and phages.

Generally, flagellin forms a flagellar fiber outside the cells and helps in their planktonic movement, although in spirillum (Spirochaetales), flagellin exists in the periplasm of the cells and is believed to be involved in the formation of specific cell shapes. In Campylobacter jejunii TGH9011, a Gram-negative spiral bacterium that perhaps lacks a type-III secretion apparatus, flagellin is excreted from the cells and is used for the recognition of host cells. On the other hand, in strain A1, flagellin localizes to the cell surface and functions as a receptor for alginate and a regulator of the structure and function of the cell surface, both of which influence cell viability in the alginate medium. A question will be raised as to why flagellin-like protein shows specific and extremely high alginate-binding ability. Some researchers suggest that alginate was the first polysaccharide synthesized in the prebiotic environment. If this is so, then ancient bacteria must have had a chemotactic ability to get near the polysaccharide using flagellin.

In any case, flagellin is involved in various physiological phenomena in that it changes their localization sites and functions, and hence bacterial cells regulate the proper use of proteins and confine the biochemical energy necessary for protein synthesis to a minimum. As
the finding that the β-domain of flagellin superimposes onto the gp11 protein of bacteriophage T4 suggests, the results obtained as to structural proteomics confirm that flagella have developed as a modular system gathering many components from other biological and primordial systems essentially irrelevant to cell motility. This also suggests that each flagellar protein or module has evolved independently from other components constituting the flagella. Indeed, bacterial flagella are different from one to another in construction, and they share only about half of the proteins constituting them.

**Alginate-binding proteins.** Although p7 shares significant homology with the proteins listed on protein databases as function-unknown lipoproteins, it has no lipobox and contains no lipids, but shows alginate-databases as function-unknown lipoproteins, it has no significant homology with the proteins listed on protein domain, 23, 24) although the extent of opening of the two domains in ligand-free AlgQ1 is greater than in AlgQ2 (rotation angle, 7°). Three linker loops (loops 1–3) connect the N- and C-domains, and the cleft formed between the two domains is the alginate-binding region, which is larger than that observed in other substrate-binding proteins, such as maltose-binding proteins. 25, 26) The calcium ion in the C-domain has no function in alginate binding, since the location of the ion is far (about 40 Å) from the cleft. AlgQ1 and AlgQ2 bind to alginate in the deep cleft formed on closing (37° for AlgQ1 and 30° for AlgQ2) of the N- and C-domains, and release the polymer on opening of the two domains. 23, 27) On going from the open-cleft, ligand-free structure to the closed structure, there is a concerted shift (about 2.8 Å) of the Glu396 side chain, which moves up into the cleft as a result of sugar binding. 24, 27)

This ligand-induced movement of Glu396 might be the trigger for the motion that enables the other domain to participate in ligand binding and ultimately to engulf the bound alginate. The major driving force for hinge-closing in alginate-binding proteins is probably the exclusion of a water molecule from the binding site, which induces different hydrogen bond formation between loop 1 and loop 2. The exclusion of exactly one water molecule for domain movement is the first example among binding proteins, for in these proteins several molecules of water are usually excluded. The shift in equilibrium on sugar binding might also be enhanced through the interaction of the sugar with Glu396, which causes the resulting perturbation of the hinge to favor the closed form.

The sugar-binding proteins analyzed to date have aromatic and polar amino acid residues in the cleft, 28) while in the case of alginate-binding proteins AlgQ1 and AlgQ2, many positively-charged residues as well as aromatic residues are arranged in the cleft. 23, 24, 27) Positively-charged residues in the active site enable alginate-binding proteins to bind preferentially to alginate, which is an acidic polysaccharide. The proteins bind tightly to the non-reducing terminal residues of the alginate molecule through van der Waals contacts and hydrogen bonds 24, 27) and deliver the macromolecule to the ABC transporter in the inner membrane.

### 3. Alginate-binding proteins

Two periplasmic proteins [AlgQ1 (59 kDa) and AlgQ2 (59 kDa)] mediate the transfer of alginate to an ABC transporter. 5, 23) AlgQ1 and AlgQ2 resemble each other in primary structure and bind specifically to alginate with $K_d = 2.1 \times 10^{-7}$ M (unpublished). This poses a question, as to whether the proteins listed on the protein database as lipoproteins are truly lipoproteins.

p8 is homotrimeric in its native form, 21) and it exhibits significant homology with a phasin responsible for binding to polyhydroxyalkanoic acid granules of *Ralstonia eutropha*. 22) p8 can specifically bind to alginate ($K_d = 1.3 \times 10^{-7}$ M). 21)

The results obtained with cell surface proteins represent a distinct picture, showing that they work cooperatively to harvest and accumulate alginate in the pit. Briefly, the presence of alginate is first recognized by flagellin-homolog receptors (p5 and p6) and the resulting signals are then transmitted to a putative two-component system, the gene (sph3806) of which is located downstream of the genetic cluster for alginate import and depolymerization (Fig. 2). Through signals from the two-component system, the expression of genes for the alginate import and depolymerization system is simultaneously induced, leading to the formation of the superchannel, including the pit on the cell surface, in addition to binding proteins in the periplasm and an ABC transporter in the inner membrane, as described below. The p7 and p8 harvest alginate and its granules in the pit. p1–p4 transport alginate or ferric ion-chelated alginate into the periplasm.

### 4. The ABC transporter

The transport of alginate from periplasm to cytoplasm is carried out by an ABC transporter (importer) in the inner membrane. ABC transporters typically consist of four subunits (two ABC proteins and two transmembrane domains). 29) This is true of the ABC transporter responsible for alginate import in strain A1; i.e., the ABC transporter consists of four subunits [AlgM1 (37 kDa), AlgM2 (33 kDa), and two molecules of AlgS (40 kDa)]. 5) As is often observed in bacteria, the genes for ABC transporters (AlgS, AlgM1, and AlgM2) and alginate-binding proteins (AlgQ1 and AlgQ2) are assembled into a cluster. AlgM1 and AlgM2 are homologous to the permease domain of a bacterial ABC transporter, and AlgS exhibits ATPase activity in the dimeric form, indicating that AlgM1 and AlgM2 func-
tion as permeases for alginate transport using the energy derived from ATP hydrolysis catalyzed by the AlgS homodimer.5)

The three-dimensional structure of the ABC transporter has been constructed through homology modeling with Monte Carlo minimizations. AlgM1 and AlgM2 contain seven transmembrane α-helices each. AlgM1 and AlgM2 probably form a heterodimer with a pore approximately 7 Å in diameter for the passage of alginate at the interface between them. AlgS has an α/β-structure and forms a homodimer with P-loop/Walker and ABC-signature motifs, which are involved in ATP binding and hydrolysis, arranged at the interface between the two molecules. Mutants with disruption of the ABC transporter genes form no apparent pit on their cell surfaces and fail to incorporate alginate.5) The activity of AlgS is regulated (activated) through a conformational change in AlgM1 and AlgM2, which is induced on contact with alginase-bound AlgQ1 or AlgQ2 with an AlgM1/AlgM2 heterodimer.24) Therefore, the pit on the cell surface, the binding proteins in the periplasm, and the ABC transporter in the inner membrane are reciprocally related and constitute the superchannel for the import of alginate, confirming the existence of a novel macromolecule import system in bacteria.

5. Alginate lyases

Alginate incorporated into the cytoplasm is degraded through the actions of endotype [A1-I (65 kDa), A1-II (25 kDa), and A1-III (40 kDa)] and exotype [A1-IV (86 kDa)] alginate lyases with different substrate specificities.6,7) The three endotype alginate lyases originate from a single gene aly; i.e., A1-I is autocatalytically processed into A1-II and A1-III, specific to polyG and polyM respectively,7) and release unsaturated di-, tri-, and tetrasaccharides from alginate. All unsaturated oligosaccharides thus produced are then degraded by an exotype lyase into constituent monosaccharides, which are nonenzymatically converted to α-keto acids. The genes for all alginate lyases are included in a gene cluster for alginate import, and are inductively expressed in the presence of alginate.5) In the promoter region of the alginate lyase gene aly, a sequence similar to the catabolite-responsive element (cre), an operator for the global catabolite control protein CcpA, is present, but its contribution to aly regulation has not been determined. At any rate, the superchannel is also linked with the depolymerization system, and the expression of the superchannel and depolymerization system is regulated coordinately.

Other than the alginate lyases described above, strain A1 has two endotype alginate lyases, A1-II′ (31 kDa) and A1-IV′ (90 kDa). A1-II′ and A1-IV′ have highest identity with A1-II and A1-IV respectively, but they are not expressed in strain A1. The A1-II′ and A1-IV′ genes are thought to have evolved from the A1-II and A1-IV genes respectively, through duplication, modification, and translocation. The finding of various alginate lyase genes in the strain A1 genome gives some idea as to the molecular diversity and evolution of the lyases.31)

6. Superchannel: Overview

It has been discovered that strain A1 has a highly developed cell surface structure and a dynamic import system for macromolecules, the superchannel. The superchannel consists of the pit on the cell surface, binding proteins in the periplasm, and an ABC transporter in the inner membrane, and it is closely connected with the assimilation system. An overall picture of the superchannel (Fig. 2) presents a novel concept in the assimilation of macromolecules. When microbes assimilate macromolecules, they usually secrete macromolecule-depolymerizing enzymes from the cell, and then incorporate the depolymerized low-molecular-weight products into their cells. In contrast, the superchannel allows the cells to engulf macromolecules and depolymerize them inside themselves. It is not easy to say which type is energetically and substantially more efficient and economical for the assimilation of macromolecules. The superchannel in strain A1 is different from E. coli AcrAB-TolC(52,33) in the direction of transport, the sizes of the transporter, and the substrates transported.

The most intriguing problem has to do with how pit formation is controlled during cell proliferation on alginate. The cells of strain A1 multiply on an alginate medium with a generation time of about 30 min. Therefore, the cells have to repeat opening and closing of the pit at similar intervals or more rapidly. In fact, in alginate medium, two types of cells, with and without the pits, are observed, although the cells with a pit are always more abundant than those without one and are never found in medium containing no added macromolecules. At present, we have no idea as to how pit formation is controlled in harmony with cell division, except that mutants with disruption of the ABC transporter genes lose control of pit formation.5)

Many types of membrane structure have been postulated in bacteria. Among them, the mesosome is the most prevalent type, observed mainly in Gram-positive bacteria, although it has been claimed that the structure is an artifact caused during the thin-cell section preparation process and disappears if the cells are cryofixed, and not subjected to chemical fixation, but it is thought that a mesosome-like structure is formed, even if only momentarily, during cell growth, since such a membrane structure would provide cells with many advantages. In fact, the mesosome-like structure is thought to provide a site for DNA replication.34) Study of the superchannel coupled with a pit should provide new insight not only into the novel bacterial system for macromolecule import and depolymerization, but also into the dynamics of the cell surface and the evolutionary process for many cell surface structures, including the bacterial mesosome, eukaryotic endo/phagocyt-
tosis, and the lipid raft structure, which is a dynamic heterogeneity of biological membranes.35)

II. Polysaccharide Lyases

In the carbohydrate-active enzyme (CAZY) database (B. Henrissat, P. Coutinho, and E. Deleury; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), polysaccharide lyases, including alginate lyases, are currently classified into 18 families (PL-1-18) on the basis of their primary structures. Polysaccharide lyases recognize uronic acid residues in polysaccharides, and release unsaturated saccharides with C=C double bonds at the nonreducing terminal uronic acid residues through a β-elimination reaction, indicating that they share common structural features as to their uronic-acid recognition sites and reaction modes (β-elimination reaction).

To understand the basic framework constituting these enzymes and their evolutionary processes, and eventually to contribute to the creation of sugar-related enzymes with novel functions, we have investigated the nature of common structural features of endo- and exo-type polysaccharide lyases mainly belonging to families PL-5, -7, and -8.

1. Family PL-5 lyases

Alginate lyase A1-III (endotype) preferably depolymerizes the polyM region of acetylated and non-acetylated alginites and yields di- and tri-saccharides as the main products.7) A1-III consists of 12 α-helices, and it has an α6/α5-barrel supersecondary structure as a basic frame.36,37) P. aeruginosa family PL-5 alginate lyase AlgL also has the same α6/α5-barrel structure.38) Enzyme A1-III has a tunnel-like cleft covered with a lid loop including Arg67, Tyr68, Tyr80, and Arg88. The configuration of the lid loop (amino acid residues, 57–90) shifts to capture the substrate, an alginate. In the ligand-free form of the wild-type enzyme (apo, open conformation), the loop is situated about 16.8 Å above the glycosidic linkage to be cleaved, while this distance is 8.6 Å in the enzyme complexed with a substrate (holo, closed conformation). The active cleft contains aromatic and positively-charged amino acid residues for the recognition of uronic acid residues, and the substrate is bound in the cleft through the formation of several hydrogen bonds and van der Waals interactions with protein atoms. Around the glycosidic bond to be cleaved, which is between the −1 and +1 saccharides, the side chain of Tyr246 is close to the hydrogen of C5 of the +1 saccharide and the oxygen of the glycosidic bond. Tyr68 in the lid loop is hydrogen-bonded to Tyr246. The di-Tyr pair is stabilized by Arg67 in the lid loop and Arg239 in the cleft. This conformation indicates that Tyr246 activated through the formation of the pair draws the proton from C5 of the +1 saccharide and donates it to the oxygen of the glycosidic linkage to be cleaved; i.e., single Tyr246 functions in acid and base catalysis in the A1-III reaction.

Thus the movement of the lid loop is essential for enzyme catalysis, activation of the catalytic center Tyr246 through the formation of the Tyr68-Tyr246 pair, the binding to substrates, and the release of products. In other polysaccharide lyases, such as hyaluronate and chondroitin lyases, two separate catalytic amino acid residues function in acid and base catalysis.39,40) Catalysis by A1-III with a single Tyr as an active center can probably be regarded as a novel β-elimination reaction.

2. Family PL-7 lyases

Family PL-7 alginate lyase A1-II (the endotype) acts on the polyG region of acetylated and non-acetylated alginites, degrading it into tri- and tetra-saccharides,7) A1-II′ of strain A131 and function-unknown protein PA1167 (25 kDa) of P. aeruginosa30) have been identified as endotype family PL-7 alginate lyases. PA1167 consists of two β-sheets with 15 β-helices and three α-helices, which constitute a glove-like β-sandwich structure.38) The overall structure of PA1167 is closely similar to that of A1-II′.41) The β-sandwich structure is observed in various sugar-related proteins such as 1,3,1,4-α-glucanase42) and lectin,43) suggesting that the structural features of the sugar-binding site are conserved among these proteins.

A1-II′ shows a glove-like β-sandwich structure with a rigid cleft. Two flexible loops associate with each other over the cleft through the formation of a hydrogen bond between their edges (Asn141 and Asn199). A double mutant of A1-II′ (N141C/N199C, Asn141 to Cys and Asn199 to Cys) with a disulfide bond between Cys141 and Cys199 shows little enzyme activity, although the activity is restored by the addition of dithiothreitol (unpublished data). This is evidence of the importance of the flexibility of the lid loop in the accommodation of the substrate to the active cleft.

The two sequences, YXRSELRE and YFKAGXYXQ, are highly conserved, in the N- and C-terminal regions respectively, of family PL-7 lyases, and are perhaps responsible for the catalytic actions of the enzymes.44) This is true in the cases of A1-II, A1-II′, and PA1167. In PA1167, the conserved N- and C-terminal sequences are located in two close β-strands, SA3 and SA4 respectively. Other than these two regions, a region slightly conserved in family PL-7 lyases is found in β-strand SA5, proximate to SA3 and SA4. Amino acid residues, including Tyr and His residues in SA3, SA4, and SA5, conserved in family PL-7 alginate lyases, are responsible for the catalytic actions of the enzymes, as in the cases of polysaccharide lyases such as family PL-5 alginate lyase A1-III,37,38) and family PL-8 hyaluronate,39) chondroitin,40) and xanthan45–47) lyases. In fact, His104, Tyr193, and Tyr199 of PA1167 constitute an active cleft.38) In this active cleft, there are three hydrogen bonds and two sets of stacking-like interactions, which maintain the rigidity of the cleft and contribute to substrate binding and catalytic reactions.
3. Family PL-5+7 lyases
As mentioned above (I-5: Alginate lyases), A1-I consists of N-terminal A1-III (family PL-5) and C-terminal A1-II (family PL-7), indicating that A1-I belongs to a new polysaccharide lyase family, PL-5+7.45) A1-II, A1-II', and PA1167 resemble one another in primary structure, and both A1-II' and PA1167 have a glove-like β-sandwich structure, indicating that A1-II probably adopts the β-sandwich structure as a basic frame. Therefore, the secondary structural elements of A1-I are thought to be an N-terminal α/α-barrel and a C-terminal β-sandwich. These elements are similar to those of the family PL-8 lyases described below, suggesting that A1-I and family PL-8 lyases have evolved from a common ancestral protein.31)

4. Family PL-8 lyases
Bacillus species strain GL1 xanthan lyase (81 kDa, exotype) acts on the side chains of xanthan and liberates pyruvated mannose (PyrMan) through a β-elimination reaction.45) Almost all polysaccharide lyases so far analyzed attack the main chains of polysaccharides in an endolytic manner and release oligosaccharides from the polysaccharide. Xanthan lyase is thus characteristic in that it exolytically attacks the side chains of the polysaccharide, and it might provide useful information as to the structural features causing different substrate specificities and modes of action of polysaccharide lyases.

Xanthan lyase consists of N-terminal α-helical and C-terminal β-sheet domains, which have incomplete α6/α8-barrel and anti-parallel β-sheet structures respectively.45–47) A deep cleft formed in the N-terminal α-helical domain at the interface between the two domains constitutes an active center (the substrate-binding site), in which Tyr255 is responsible for the catalytic reaction. Some aromatic and positively-charged amino acid residues are present in the active cleft, and they are responsible for the binding and depolymerization of xanthan, an acidic polymer. This feature is common to related enzymes such as glucoamylase,59) endoglucanase,60) rhamnogalacturonan lyase,61) 1,2-mannosidase,62) and maltose phosphorylase,63) as well as polysaccharide lyases. These enzymes form the α/α-toroid family in the SCOP database (http://scop.berkeley.edu/data/scop.b.b.bbc.html). Hence it appears that these sugar-related enzymes have evolved from a common ancestral protein, although they catalyze different reactions (lyase, hydrolyase, epimerase, isomerase, or phosphorylase), and no similarities have been found among their primary structures.

The following common structural features for the catalytic reactions are shared especially among family PL-5 and -8 lyases: (i) a catalytic α/α-barrel domain, (ii) a lid loop for recognition of substrates or catalytic reaction, (iii) uronic acid-binding aromatic and positively-charged amino acid residues, and (iv) a catalytic Tyr residue. Collectively, in the catalytic reaction by polysaccharide lyases, positively charged amino acid residues bind to or neutralize the carboxyl group in uronic acid residues. A general base catalyst donates the proton to the glycoside bond to be cleaved.

5. Structural features of polysaccharide lyase
Although polysaccharide lyases are currently classified into 18 families (PL-1 to -18) on the basis of their primary structures, X-ray crystallography of them has indicated that almost all of them can be grouped into six folds: parallel β-helix, lyases for pectate (families PL-1, -3, and -9),49) and chondroitin B (family PL-6);50) β-sheet abundant flattened oval disk, rhamnogalacturonan lyase (family PL-4);51) α/α-barrel, lyases for alginate (A1-III) (family PL-5)36) and pectate (family PL-10);52) α/α-barrel + anti-parallel β-sheet, lyases for hyaluronate,39) chondroitin AC,40) and xanthan (family PL-8); β-sandwich, alginate lyases PA1167 and A1-II' (family PL-7),39) and β-propeller, rhamnogalacturonan lyase YesW (family PL-11).53)

As for the α/α-barrel, we have confirmed that a porcine kidney bifunctional protein, renin-binding protein [N-acetyl-D-glucosamine 2-epimerase (AGE)],54,55) a novel glycosaminoglycan-degrading enzyme of unsaturated glucuronyl hydrolyse (UGL) of Bacillus sp. GL1,56,57) a plant cell wall-degrading enzyme (B. subtilis unsaturated galacturonyl hydrolyase) YteR,58) and an AGE structural homolog, YihS, (unpublished) of S. typhi-murium have α/α-barrel structures as a basic scaffold. The α/α-barrel structure is also found in sugar-related enzymes such as glycoamylase,59) endoglucanases,60) endo/exo-cellulase,61) α-1,2-mannosidase,62) and maltose phosphorylase,63) as well as polysaccharide lyases. These enzymes form the α/α-toroid family in the SCOP database (http://scop.berkeley.edu/data/scop.b.b.bbc.html). Hence it appears that these sugar-related enzymes have evolved from a common ancestral protein, although they catalyze different reactions (lyase, hydrolyase, epimerase, isomerase, or phosphorylase), and no similarities have been found among their primary structures.
Some of the above-described features are also found in periplasmic alginate-binding proteins (AlgQ1 and AlgQ2). Aromatic and positively-charged amino acid residues are indispensable to the interaction of proteins or enzymes with acidic sugars. Furthermore, as in the case of the lid-loop of alginate lyase A1-III or A1-II, the opening and closing of domains AlgQ1 and AlgQ2 is essential to the binding of the substrate and the release of the products. Study of the hinge structure and energetics that allow the lid-loop and domains to move freely is necessary to determine the intrinsic roles of these protein modules in catalysis.

III. Correlated Spheres

1. Alginate biofilms and bacterial infection

Although alginate is currently prepared from brown seaweed, this polysaccharide is also produced by bacterial cells such as *A. vinelandii* and mucoid-type strains of *P. aeruginosa*. In *A. vinelandii*, alginate has an essential role in encystment (desiccation-resistant cyst), a function not observed in *P. aeruginosa*. On the other hand, alginate produced by mucoid *P. aeruginosa* in the lungs of patients with cystic fibrosis (CF), when combined with extracellular polymeric substances such as polysaccharides, proteins, and nucleic acids, forms a physically, biochemically, and (socio)microbiologically complicated biofilm and causes difficulty in respiration. Alginate biofilm formation by *P. aeruginosa* is partly controlled by quorum sensing regulated virulence factors. Therefore, antimicrobial agents as well as quorum sensing inhibitors should be able to render the biofilm susceptible to polymorphonuclear leukocytes (PMN) and antibodies and to repress bacterial growth in the CF lung.

But, this approach, founded on inhibitors, is not as easy as one might imagine. Biofilms generally exert strong resistance to the penetration of antimicrobial agents and various other chemicals, although they are believed to have many channels in their matrix to circulate fluids, nutrients, waste matter, and oxygen. It is known that bacteria in a biofilm show higher resistance (usually 1,000–2,000 times higher) to antimicrobial agents than those proliferating outside biofilms. Furthermore, the growth rate of bacteria in a biofilm is generally low, and this further increases resistance to various chemicals. Hence destruction of biofilms and dispersion of bacterial cells proliferating in their biofilms or the discovery of antibiotics that attack resting cells should be effective in repressing the development of biofilms.

It is clear that *Pseudomonas* alginate contributes significantly to the increased viscosity of CF sputum. The ability to depolymerize this alginate might help clear the CF patient’s airways and facilitate the delivery of other drugs by the aerosol route. Success with aerosolized preparation of DNase in CF supports the possibility that aerosolization of bacterial alginate lyase is feasible. Alginate lyase A1-III of strain A1 is exceedingly powerful in liquefying viscous alginate produced in the lungs of CF patients and in dispersing microcolonies of *P. aeruginosa* proliferating in the alginate biofilm, facilitating access by antimicrobial agents, PMN, and antibodies to the organisms. The antigenicity of the alginate lyase must be considered before the enzyme can be developed for clinical use. Recently, it was reported that ultrasonic vibrations block bacterial biofilms from forming on medical devices, although physical processes must be constructed to maintain continuous vibration to prevent biofilm formation.

Certain streptococci, such as *Streptococcus pyogenes* and *Streptococcus pneumoniae*, cause infectious diseases, *e.g.*, pneumonia, bacteremia, sinusitis, and meningitis. They produce polysaccharide lyases, which function as virulence factors in the degradation of host extracellular matrix glycosaminoglycans. Plant pathogenic and/or saprophytic bacteria such as *Erwinia* and *Bacillus* produce polysaccharide lyases that degrade plant cell-wall polysaccharides. We found two novel hydrolases, unsaturated glucuronol hydrolase (UGH) in *Bacillus* sp. GL1 and unsaturated galacturonol hydrolase (UGH) in *B. subtilis*. These enzymes show an ability to hydrolyze host-cell surface matrices, including unsaturated oligosaccharides formed from mammalian glycosaminoglycans, such as chondroitin, hyaluronan, heparin, and plant cell-wall pectin and rhamnogalacturonan, and are thought to be another virulence factor facilitating invasion by pathogenic bacteria of mammalian and plant cells in cooperation with polysaccharide lyases. In fact, putative genes coding for UGL and UGH are found in the genomes of pathogenic streptococci, bacilli, clostridia, and vibrios that produce polysaccharide lyases. Inhibitors of polysaccharide lyases UGL and UGH might be useful in the treatment of bacterial infectious diseases caused by the degradation of host cell-surface matrices.

Glycoside hydrolases usually have two catalytic amino acid residues as general acid and base/nucleophile catalysts. Most catalytic residues consist of acidic amino acids, Asp or Glu. The catalytic mechanisms of glycoside hydrolases are generally divided into two groups, based on the anomeric configuration of the substrate in reaction products, *viz.*, retention and inversion. UGL and UGH have *α*6/*α*6-barrel structures as a basic scaffold, and hydrate the vinyl ether group (C4=C5-O5), triggering the hydrolysis of the glycosidic bond. That is, both hydrolases hydrate C=C double bonds in unsaturated guluronic acid, but not glycoside bonds, using one Asp residue as an acid/base catalyst. The CAZy database divides glycoside-related enzymes with a *α*6/*α*6-barrel structure into two clans, GH-L (glucoamylase and maltose phosphorylase) and GH-M (cellulases), based on the active site architecture. UGL and UGH are thus the first *α*6/*α*6-barrel enzymes that hydrate vinyl ether groups, and hence may be classified into a new clan.
2. Molecular transplantation

In strain A1, pit formation is controlled by components of the ABC transporter. This suggests that the superchannel of strain A1 can be transplanted to other Sphingomonas strains by introduction of the genes for the ABC transporter. When the genes (from *aly* to *sph3806* in the cluster) described in Fig. 2 were introduced into *Sphingomonas wittichii* RW1, which has dioxin-degrading ability, the components of the ABC transporter were expressed and pits were produced on the cell surfaces of the transformed cells, even in the absence of alginate.

It is noteworthy that dibenzofuran removal was greatly improved in pit-transplanted cells of RW1. For example, when the engineered cells were cultivated in a medium containing dibenzofuran (10 mM, 1.68 g/l) as a sole carbon source, the substrate was completely depleted after 2 d cultivation, while RW1 (the wild type) required 4 d for the complete removal of dibenzofuran. In a microcosm study in which dibenzofuran-contaminated soil was used, engineered RW1 cells showed effective remediation of this compound. The increased remediation activity of the engineered cells is presumably due to increased uptake of dibenzofuran. Using the same technique, the polypropylene glycol-degrading activity of *Sphingomonas subarctica* IFO 16058 and the polydextrose-degrading activity of *Sphingomonas sunguis* IFO 13937 were also improved. Both engineered strains expressed the components of the ABC transporter and formed huge pits on their cell surfaces, as observed in RW1.

Hence we present a new biological approach to improve the import activity of bacteria by transplantation of an organ-like superchannel present in strain A1 into *S. wittichii* RW1 and other strains of sphingomonads. This new engineering method is promising, and feasible for the molecular breeding of microbial degraders of various compounds. Although molecular transplantation is currently restricted to sphingomonads strains, and the molecular basis explaining how the transplanted organs can be formed in heterologous host cells and how their expression is controlled is unclear, this engineering method might well be expanded to other microbes belonging to other species. *Agrobacterium tumefaciens* is an immediate target in this regard, since the bacterium has a gene set similar to that forming the superchannel, as in strain A1. The superchannel and pit might be horizontally transferred among bacteria.

3. α/α-Barrel enzyme engineering

In the pharmaceutical, chemical, and food industries, the discovery and development of new enzymes has largely relied on screening methods. Although these methods require time-consuming and labor-intensive processes, and more comprehensive screening methods, such as meta-genome analysis, based on bioinformatics, have been developed, it is clear that they have a good success record and will continue to be used in the future, but the recent development of protein structural biology is about to turn our attention to the use of rational design strategies for engineering new enzymes. In order to design new enzymes, there must exist reasonable, structure-based, explicit rules by which enzymes can be engineered. These include the basic frame or scaffold of proteins, the structure and movement of functional motifs, and, if possible, evidence of past evolutionary processes. Our study of lyases and hydrolases offers the possibility of engineering and/or designing novel sugar-related enzymes.

Based on primary structure, the sugar-related hydrolases are currently classified into about 110 families (GH-1 to -110). The tertiary structures of hydrolases in 69 families have been determined, and their structural and functional relationships analyzed. About 20% of all hydrolase families have a (α/β),-barrel (or TIM barrel) structure as a basic frame, but hydrolases with (α/β),-barrels show no common reaction modes, and employ various chemicals as substrates. On the other hand, enzymes with an α6/α6-barrel structure, the second most common basic frame after the (α/β),-barrel enzymes, use sugar-related compounds as substrates, although they show no similarity in their primary structures and catalyze different reactions.

Sugar-related α6/α6-barrel enzymes, such as hydrolases, transferases, epimerases, isomerases, and polysaccharide lyases, have evidently evolved from a common ancestor protein. This technique, which we call reverse evolution, should be useful in the attempt to understand the discovery and development of new enzymes. In order to design new enzymes, there must exist reasonable, structure-based, explicit rules by which enzymes can be engineered. These include the basic frame or scaffold of proteins, the structure and movement of functional motifs, and, if possible, evidence of past evolutionary processes. Our study of lyases and hydrolases offers the possibility of engineering and/or designing novel sugar-related enzymes.

Modification or change of the catalytic function of enzymes is also important in creating useful enzymes. Based on crystal structure, we have succeeded in the conversion of *B. subtilis* exo-type rhamnogalacturonan (RG) lyase into an endo-type enzyme through the excision of a single loop specifically responsible for recognizing the terminal saccharide of the substrate (unpublished data). Similar strategies will be adapted to the molecular conversion of ATP-dependent enzymes to polyphosphate-dependent enzymes. Success in co-substrate conversion from ATP to polyphosphate of ATP-dependent enzymes is important in many industries, since at present the use of expensive ATP is not advantageous and ATP-dependent enzymes inevitably require coupling with ATP-regeneration systems for reaction efficiency and process economy. An attempt in this direction is in progress with respect to bacterial NAD kinases, viz., conversion of ATP-dependent NAD kinase to polyphosphate-dependent NAD kinase, as a model. This technique, which we call reverse evolution, should be useful in the attempt to understand the evolutionary process of biochemical energy sources, and even minimum cell technologies should fall under this technique.
4. Biomass engineering

Bacterial exopolysaccharides usually consist of two to four different kinds of monosaccharides, some of which have adducts on the backbone as side chains. Due to this structural heterogeneity, bacterial exopolysaccharides and their depolymerization products are expected to show potent biochemical and biotechnological characteristics. Enzymes acting on bacterial polysaccharides, including alginate, xanthan, and gellan, should be useful in the molecular design of poly- and oligosaccharides with novel biotechnological functions. Although some of the oligosaccharides produced by polysaccharide lyases are now applicable in the food, agricultural, chemical, and medical industries, this field has not yet matured.

Bioethanol production from lignocellulosic feedstocks is currently of increased interest around the world, and batch and continuous pilot-plant scale fermentation trials are now under way corn-fiber-derived sugars. Seaweeds comprise a biomass available on a large scale at low cost, and might prove useful in the production of bioethanol. They contain alginate, gellan, and fucoidan as major polysaccharides. Polysaccharide lyases, including alginate lyase, gellan lyase, and fucoidan lyase, will perhaps be effectively applied in the conversion of these biopolymers to low-molecular-weight sugars directly available for the production of ethanol.

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References


48) Huang, W., Lunin, V. V., Li, Y., Suzuki, S., Sugiura, N.,


76) Itoh, T., Ochiai, A., Mikami, B., Hashimoto, W., and Murata, K., A novel glycoside hydrolase family 105: the structure of family 105 unsaturated rhamnogalacturonol hydrolyase complexed with a disaccharide in comparison with family 88 enzyme complexed with the disaccharide.


