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Special cell surface structure, and novel macromolecule transport/depolymerization system of *Sphingomonas* sp A1

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A bacterium isolated from soil as an alginate lyase producer shows characteristic morphological and taxonomical properties consistent with being classified in the genus *Sphingomonas*. The bacterium utilizes high molecular weight (HMW)-alginate for growth by depolymerization of the polymer with intracellular alginate lyases, which are generated from a common precursor protein through autoregulated post-translational modifications. Electron microscopic observations of the cell surface and of thin sections of cells grown on HMW-alginate revealed dynamic changes in both cell surface and membrane structures. The most remarkable change is recognized in the formation of mouth-like pits which open and close depending on the presence or absence of HMW-alginate. Enzymatic and genetic analyses of HMW-alginate incorporation processes confirmed the presence of a pit-dependent and macromolecule-specific ABC transporter system in cells of *Sphingomonas* species A1. This is the first description of a bacterium with a pit on the cell surface and a pit-dependent endocytosic uptake system for macromolecules.

Keywords: *Sphingomonas*; macromolecule transport; pit; polysaccharide lyase; ABC transporter

Introduction

Alginate is a polymer composed of β-D-mannuronate and the C5 epimer α-L-guluronate; the polysaccharide is biosynthesized by brown seaweeds and certain microbes. The two uronic acids are usually arranged in three different ways within the alginate molecule to form block structures. They are homopolymeric [poly-β-D-mannuronate (MM blocks) and poly-α-L-guluronate (GG blocks)] and heteropolymeric (MG blocks) regions. In MG blocks, there is a random arrangement of the monomers G and M [22]. GG blocks tightly bind calcium ions and can form firm but brittle gels. The 'egg-box model' has been proposed to explain this specific interaction between GG blocks and calcium ions [46]. Regions rich in MM or MG blocks non-specifically bind divalent metal ions, and form elastic gels. The viscosity of alginate is proportional to its molecular size [13].

Because of its metal-chelating and viscous properties, alginate is used widely in the food and pharmaceutical industries [15]. However, methods for depolymerization are now being sought, not only to prepare low viscosity alginates with novel physiological and food technological functions, but also to exploit new areas of application in biopolymer-based industries. Besides structural and rheological properties, bacterial exopolysaccharides have attracted a great deal of attention in recent years because they play important functional roles in the process whereby microorganisms adapt to selection pressures of different environments. The typical ecological function of bacterial exopolysaccharides is observed in many bacterial adhesion and infection processes, particularly seen in cystic fibrosis (CF) [16]. In CF, alginate produced by *Pseudomonas aeruginosa* and related bacteria form part of the biofilms that protect bacterial cells from the attack of phagocytes, antibodies or chemotherapeutic agents (antibiotics), inevitably leading to bacterial infectious diseases which are highly resistant to medical treatments [11]. The repression of alginate formation and/or removal of alginates is one of the promising approaches to conquer biofilm-dependent bacterial infectious diseases [18]. Therefore, enzymes that can liquefy alginate are required.

For food technological and therapeutic purposes, the use of an enzyme, alginate lyase, is promising, since the enzyme splits alginate intramolecularly and can produce oligomers with their inherent properties. An alginate lyase producer isolated from soil was identified as a bacterium in the genus *Sphingomonas*, since: (i) the bacterium contains no lipopolysaccharide, which is a major component of the outer membrane of other Gram-negative bacteria; and (ii) the bacterium contains glycosphingolipid, which is a ubiquitous component of the plasma membrane of eukaryotes and usually is not found in prokaryotes.

This review focuses primarily on the depolymerization mechanism of alginate by alginate lyase, and our special interests are concentrated on the cell surface structure of the bacterium *Sphingomonas* sp (strain A1), to understand the molecular basis underlying regulation of macromolecule (alginate) transport.

Properties of bacterial alginate lyase producer

A highly potent alginate lyase producer isolated from soil is a yellow-pigmented, Gram-negative rod (Figure 1-I: A–F) with a G+C content of 62–63 mol%. The bacterium is polymixin B-resistant, and contains mono- and oligosaccharide-type glycosphingolipids and isoprenoid quinone (ubiquinone 10). Major cellular fatty acids in the strain are palmitoleic acid 16:1, heptadecanoic acid 17:1, *cis*-11-octadecanoic acid 18:1, 2-hydroxytetradecanoic acid 14:0, and

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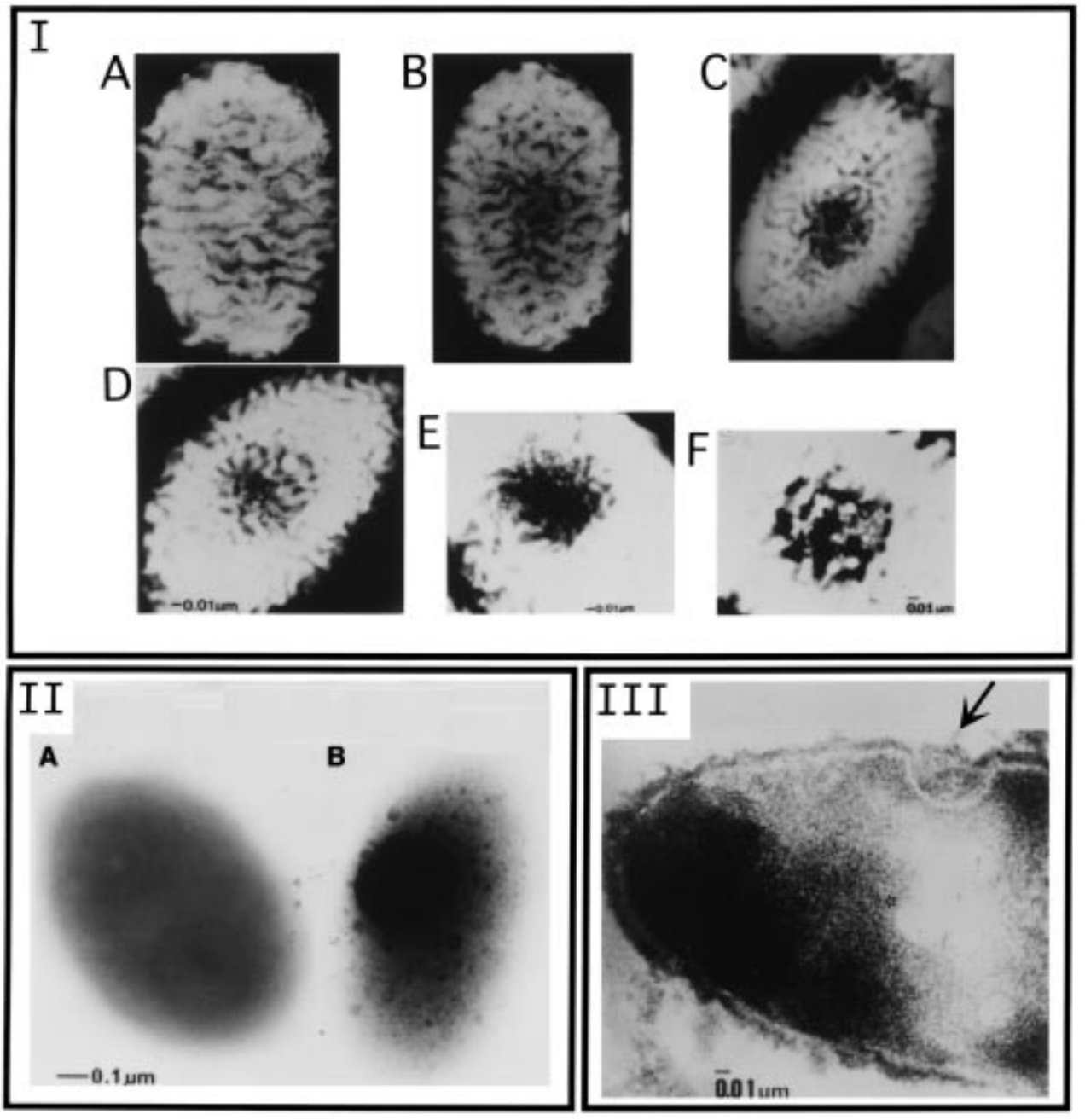

Figure 1 Cell surface structure of *Sphingomonas* sp A1. (I) Pit formed on the cell surface in the presence of alginate (culture time: A, 0 h; B, 2 h; C, 7 h; D, 14 h; E, 20 h; F, 24 h). (II) Alginate localization on the cell surface (A, cells grown in the absence of alginate; B, cells grown in the presence of alginate). Cell surfaces were stained with ruthenium red. (III) Thin section of alginate-grown cells. An arrow indicates the position corresponding to the pit.

2-hydroxypentadecanoic acid 15:0. 3-Hydroxy fatty acids are not found. Based on these and other taxonomic and physiological properties, the bacterial alginate lyase producer was placed in the genus *Sphingomonas* [27], members of which have been reported in patients and clinical materials [50], in ears of rice and other plants of the family Gramineae [30] and in other natural environments [45,49,51]. Among the bacteria in this genus, the isolate is similar to *Sphingomonas terrae* in cellular fatty acid composition.

Sphingomonas sp A1 exhibits strict nutritional requirements for carbon and nitrogen sources, and can specifically use polyuronic acids (alginate and pectin) and their depolymerization products. Glucose and pyruvate can be utilized as a carbon source, but with far less efficiency than polyuronic acids. Among the amino acids, only L-glutamate, L-aspartate and their amides (L-glutamine and L-asparagine) are utilized as nitrogen sources. Yeast extract is poorly utilized as a carbon and nitrogen source. Other compounds tested are not assimilated: mono- and oligosaccharides (mannose, rhamnose, fructose, galactose, galactosamine, xylose, arabinose, maltose, lactose, trehalose), polysac-

charides (gellan, fucoidan, inulin, arabic gum, levan, carrageenan, carboxymethyl-cellulose, pullulan, dextran, heparin, condroitin, xylan, galactan, xanthan, chitin, cellulose, chitosan, curdlan), organic acids (malate, oxaloacetate, citrate, isocitrate), and sugar alcohols (glycerol, mannitol, sorbitol).

Properties of alginate lyase

Formation and specificity

Only in the presence of alginate does *Sphingomonas* sp A1 produce alginate lyases which catalyze the depolymerization of alginate. The bacterial cells flocculate during growth on a medium containing alginate [52]. The activity of alginate lyase reaches maximum in mid-log phase and then declines in association with the progress of flocculation.

Three kinds of monomeric alginate lyases [A1-I (66 kDa), A1-II (25 kDa), A1-III (40 kDa)] are produced intracellularly [23,24,52] (Table 1). These alginate lyases are classified into three types with respect to their substrate specificity: (i) lyases active on both brown seaweed (non-acetylated) and bacterial (acetylated) alginates—A1-I, isoelectric point (pI) = 9.0, is of this type; (ii) lyases specifically active on non-acetylated alginates—A1-II (pI = 6.8) belongs to this type; and (iii) lyases highly active on acetylated alginates—A1-III (pI = 10) is categorized as this type. All of these alginate lyases depolymerize sodium-, potassium-, and propylene glycol-alginates with various M/G (β-D-mannuronate/α-L-guluronate) ratios most efficiently at pH 7.5–8.5, 70°C.

Another interesting feature of these alginate lyases lies in the amino acid sequences of their N-terminal regions (Table 1). The sequence of the first 20 amino acids of A1-I [40] is consistent with that of A1-III, whereas that of A1-II [40] matches a sequence of 20 amino acids located in an internal region of A1-I. The significance of the N-terminal amino acid sequences is discussed below in connection with processing of the A1-I molecule.

Overexpression of A1-III

Among the alginate lyase species, A1-III is exceedingly powerful in liquefying viscous alginate produced by mucoid *P. aeruginosa* (Table 1), and can be used as a therapeutic agent in CF and other biofilm-dependent bacterial infectious diseases. In order to prepare large amounts of A1-III, the region of nucleotide sequence corresponding to A1-III (from ^{54}His to ^{412}Ser in Figure 2) was excised from a gene encoding A1-I, inserted in a downstream faced to a signal peptide sequence (33 amino acids derived from an extracellular α-amylase of *Bacillus subtilis*) in a vector pISA412 and transcription of the sequence was put under the control of a penicillinase gene expression system [25]. *B. subtilis* ANA-1 (Npr$^-$, Apr$^-$, Amy$^-$) harboring the hybrid plasmid produces a large amount of A1-III (0.3 g L^{-1}) extracellularly [25]. The specific production of A1-III justifies our conclusion that A1-III, as well as A1-II, is generated through the processing of A1-I [41,55] as analyzed later (Figure 3).

Reaction mechanism of A1-III

A1-III of *Sphingomonas* sp A1 is a poly-β-D-mannuronate lyase and cleaved the glycosidic linkage of poly-β-D-mannuronate (MM blocks) and heteropolymeric region (MG blocks), but was inert on poly-β-D-guluronate (GG blocks). The enzyme was observed to act endolytically, to interact with tetrasaccharide in alginate, and to form di- and trisaccharides as final products [19]. This suggests that the enzyme recognizes the unit of tetrasaccharide in alginate and cleaves the middle linkage of the tetrasaccharide. The substrate specificity of A1-III resembles those of alginate lyases of *P. aeruginosa* [33] and *Azotobacter vinelandii* [14], but differs from that of *Klebsiella pneumoniae* [10]. The enzyme of *K. pneumoniae* is a poly-α-L-guluronate lyase and specifically cleaves linkages in poly-α-L-guluronate (GG blocks). The reaction mode of A1-III is similar to that of alginate lyases from *Azotobacter chroococcum* [17] and *Turbo cornutus* [39], but distinct from that of *P. aeru-*

Table 1 Properties of alginate lyases in *Sphingomonas* sp A1

Properties	A1-I	A1-II	A1-III
MW (subunit)	66 kDa (monomer)	25 kDa (monomer)	40 kDa (monomer)
Localization	Cytoplasm	Cytoplasm	Cytoplasm
Isoelectric point	9.03	6.82	10.16
Optimal pH (reaction)	8.0	8.0	7.6
Substrate specificity[a]			
Non-acetylated alginate:			
Na-salt M/G[b] = 0.4–0.5	134	146	30.1
M/G = 1.4–1.5	142	138	25.5
K-salt M/G = 1.2–1.3	122	132	23.5
Acetylated alginate:			
Alginate (10–91–25)[c]	68.3	0	125
Alginate (10–91–19)	62.1	0	132
Alginate (2–92–28)	59.8	0	108
N-Terminal amino acid sequence	HPFDQAVVKDPTASYV	APAAAHSSIDLSKWKLQ	HPFDQAVVKDPTASY

[a]Specific activity: Δ E_{235} min^{-1} mg^{-1} protein.
[b]Molar ratio of M and G in alginate.
[c]Alginate produced by *P. aeruginosa*-infected lungs of CF patients (parenthesis: classification number of patients in Columbia University).

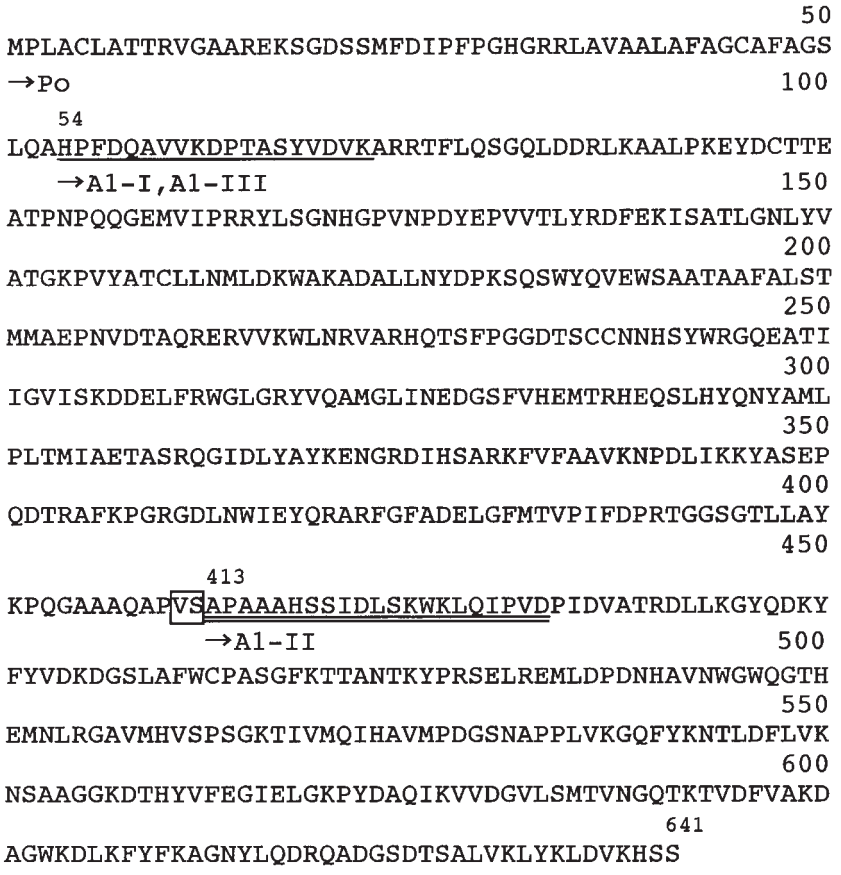

Figure 2 Deduced amino acid sequence of alginate lyases. The N-terminal amino acid sequence of A1-I and A1-III is underlined. The N-terminal amino acid sequence of A1-II is doubly underlined. The C-terminal amino acid sequence of A1-III is boxed. Arrows indicate the N-terminals of precursor protein Po, A1-I, A1-III and A1-II.

ginosa [33] and *Dendryphiella salina* [47]. The lyases from *P. aeruginosa* and *D. salina* can act on trisaccharides.

Chemical modification of A1-III

The activity of A1-III is completely lost after treatment with phenylglyoxal, diethylpyrocarbonate (DEP), or 2,4,6-trinitrobenzenesulfonic acid, although the effect of DEP is reversed by the addition of hydroxylamine. Sulfhydryl agents (*N*-ethylmaleimide, iodoacetamide and *p*-chloromercurybenzoate) show no effects on activity of the enzyme. These results indicate that the catalytic site of A1-III contains arginine, histidine and lysine, but not cysteine, as functional groups (Hisano *et al*, unpublished results).

Three-dimensional structure of A1-III

Alginate lyase (A1-III) overexpressed in *B. subtilis* was crystallized by the vapor-diffusion method with ammonium sulfate as a precipitant [38]. The crystal belongs to the space group *C2* with cell dimensions of a = 48.9, b = 92.4, and c = 81.6 Å, and one molecule constitutes an asymmetric unit. The crystal structure of A1-III has been revealed by multiple isomorphous replacement and refined at 1.78 Å to a conventional crystallographic *R*-factor of 0.180 (Figure 4) [56]. The enzyme is composed of only α-helices and contains no β-sheet structures. The enzyme has a novel α_6/α_5-barrel supersecondary structure and the topology of A1-III resembles that of glucoamylase. Since a 'tunnel-like' cleft exists in the barrel consisting of helices, alginate possibly penetrates into the cleft and interacts with the catalytic center of A1-III.

Structure of the alginate lyase gene

Gene structure

Alginate lyase genes have been isolated from *K. pneumoniae* [10] and *Pseudomonas* sp [35,37]. However, these genes are for extracellular alginate lyases and are distinct from those for the intracellular enzymes produced by *Sphingomonas* sp A1. The gene responsible for the alginate lyase of *Sphingomonas* sp A1 has been cloned in *Escherichia coli* DH1 as a nucleotide fragment with 2176 base pairs [53,55]. The open reading frame (ORF) consists of 1923 base pairs which can encode a polypeptide of 641 amino acids (Figure 2). The N-terminal 20-amino acids sequence of the lyase produced by *E. coli* DH1 is consistent with the first 20 amino acids (from ^{1}Met to ^{20}Asp) in the deduced sequence, indicating that in *E. coli* DH1 cells, the lyase is synthesized as a precursor protein (Po: 71 kDa) with an apparent alginate lyase activity.

In *Sphingomonas* sp A1 cells, all alginate lyase species are exclusively localized in the cytoplasm [53]. However, in *E. coli* DH1, the enzyme is equally distributed between both periplasm and cytoplasm [55], indicating that the alginate precursor (Po) has a region required for translo-

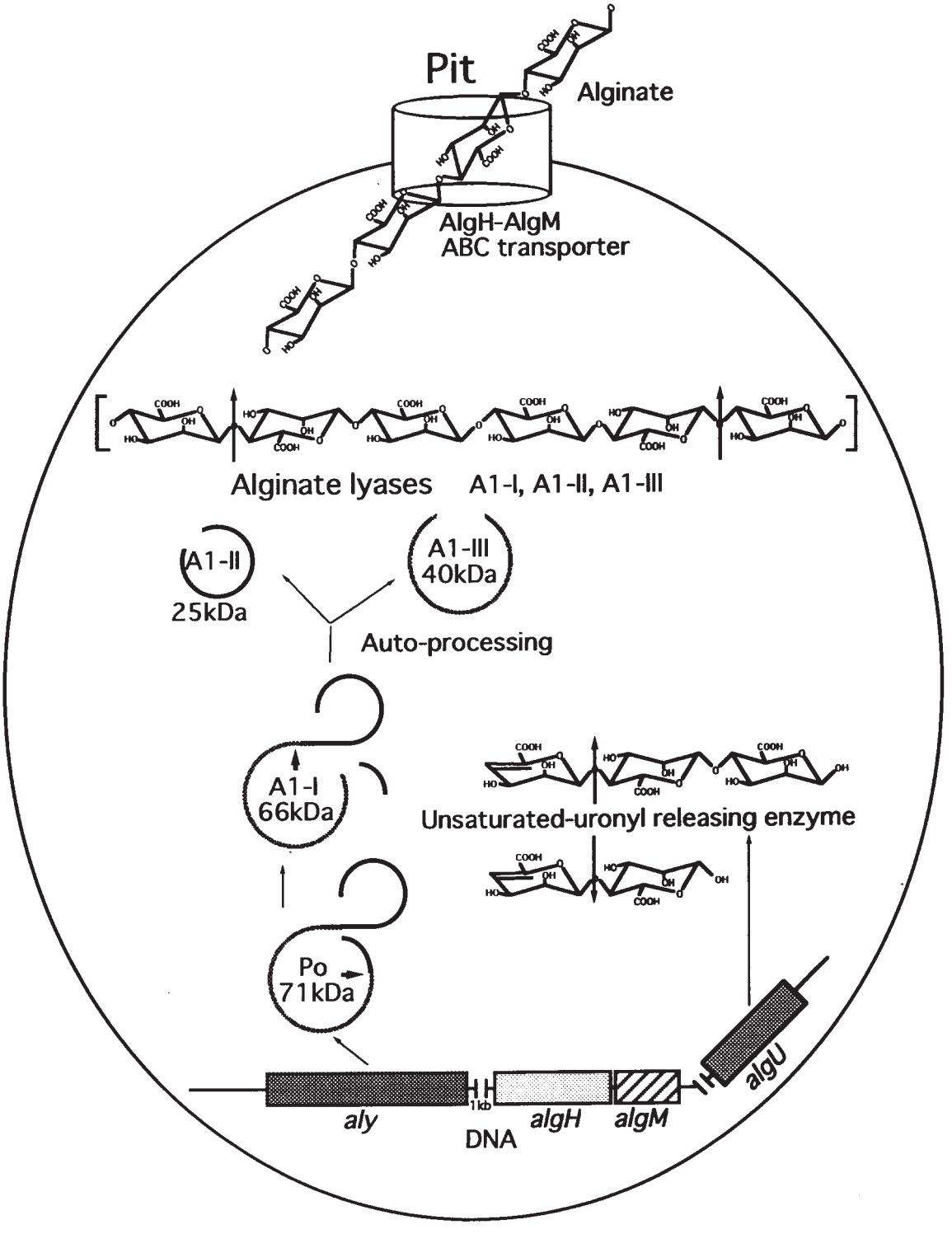

Figure 3 Post-translational processing for generation of alginate lyase species and the alginate depolymerization route. Alginates concentrated in the pit are incorporated into cells through the ABC transporter system consisting of AlgH and AlgM. Alginate lyase is synthesized as a precursor protein (Po), followed by the transformation to A1-I by releasing N-terminal peptide. A1-I is autocatalytically processed to A1-II and A1-III. Three kinds of lyases (A1-I, A1-II, and A1-III) depolymerize alginate to tri- and disaccharides with an unsaturated uronyl residue at the non-reducing terminal. The resultant oligosaccharides are converted to a monosaccharide by unsaturated-uronyl releasing enzyme.

cation of the protein across the cell membrane. Polypeptide with a molecular weight of 5367 (^{1}Met to ^{53}Ala) may function as a signal peptide for the translocation of the A1-I precursor. However, the polypeptide is quite long compared with a typical signal peptide, and the hydropathy of the precursor protein shows no hydrophobic environments typical for the amino terminal flanking region of the translated proteins. The translocation process for the A1-I precursor is

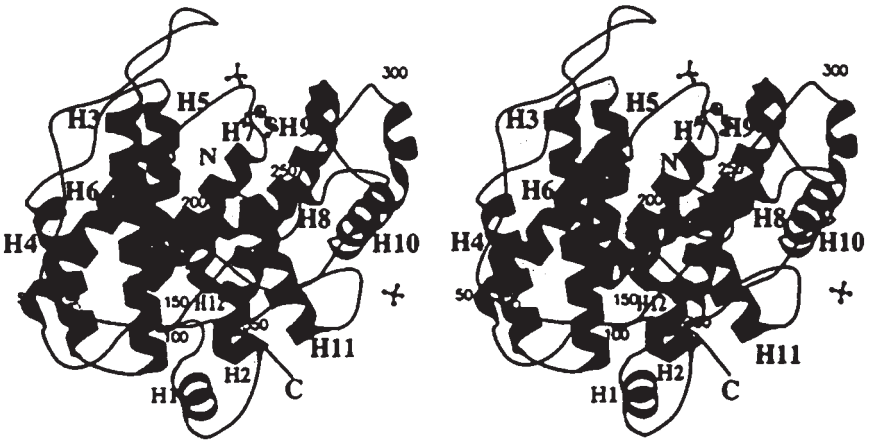

Figure 4 Crystal structure of alginate lyase A1-III. H1–H12, α-helix; balls, S–S bond.

now being analyzed, including the C-terminus hydrophobic region as an anchor.

Consensus sequences for promoter and ribosome-binding sites are not confirmed in the upstream region of the ORF of the A1-I precursor. A palindrome-like sequence is located just 10 base pairs downstream of stop codon TGA. Further biochemical and genetic analyses are needed to identify the promoter, transcriptional start point and potential termination sequences. A homology search using the EMBL Data Bank found no genes homologous with the A1-I gene.

Processing of alginate lyase

As indicated above, *Sphingomonas* sp A1 produces three different types of intracellular alginate lyases: A1-I (66 kDa) [52], A1-II (25 kDa) [23] and A1-III (40 kDa) [24] (Table 1). The sum of molecular weights of A1-II and A1-III roughly corresponds to the molecular weight of A1-I. The N-terminal amino acid sequence of A1-I is consistent with that of A1-III (Figure 2). The amino acid sequence from ^{413}Ala to ^{432}Val in the deduced amino acid sequence of A1-I is identical with the predetermined N-terminal amino acid sequence of A1-II. Furthermore, the last two C-terminal amino acid sequences of A1-III are determined to be Val-Ser (boxed in Figure 2), indicating that the peptide bond between ^{412}Ser and ^{413}Ala is cleaved.

Based on the facts presented above, we can postulate a possible molecular cascade for the generation of alginate lyase species in *Sphingomonas* sp A1 (Figure 3). The A1-I is first synthesized as a precursor protein Po (71 kDa), the presence of which has been confirmed [40], and then the protein Po is processed to generate a mature form of A1-I (66 kDa) by removing the N-terminal peptide (5 kDa). The elimination of a 5-kDa peptide from precursor protein Po is necessary to induce protease-like activity in the A1-I molecule. The native A1-I is subsequently processed at a presumed site (between ^{412}Ser and ^{413}Ala) to yield A1-II (25 kDa) and A1-III (40 kDa). The processing of A1-I is attained by protease-like activity of the A1-I molecule itself [26], and this step is rate-limiting or regulated, probably by the oxygen supply [23,24], since A1-I, usually present as the major component among alginate lyase species, is promptly processed when the culture is vigorously agitated. Therefore, A1-I is a peculiar enzyme in that it has three different catalytic centers for two alginate lyases (A1-II and A1-III) and a protease. A1-II and A1-III generated through the processing of A1-I can reveal novel functions (Table 1). Briefly, although A1-I is active on both brown seaweed (non-acetylated) and bacterial (acetylated) alginates, A1-II and A1-III are almost specific to the former and the latter alginates, respectively.

Several bacteria produce alginate lyase [48]. *P. aeruginosa* [33] and *A. vinelandii* [14], which are typical bacteria producing the alginate biofilm or cyst wall, produce periplasmic alginate lyases with a molecular weight of 39–40 kDa, and their genes are located in a cluster involved in the biosynthesis and transport (excretion) of the alginate molecule [7,34]. Thus, the alginate lyase of *P. aeruginosa* shows a significant amino acid sequence identity (63%) to that of *A. vinelandii* [14]. In contrast, *K. pneumoniae* produces alginate lyase (28 kDa) extracellularly [10]. No peculiar post-translational modification pathways for alginate lyase biosynthesis, as evidenced in the case of *Sphingomonas* sp A1, have been observed in any of the other bacterial strains so far examined. Thus, the *Sphingomonas* sp A1 lyases are quite different from those of other bacteria in maturation processes as well as in localization, and exhibit low identity scores in comparison with those of *P. aeruginosa* [accession No. L14597-1; 25% identity in 280 amino acid (aa) overlap], *A. vinelandii* (AF027499-3; 24% identity in 332 aa overlap), and *K. pneumoniae* (L19657-2; 27% identity in 294 aa overlap).

Regulation of alginate lyase gene expression

The alginate lyases in *Sphingomonas* sp A1 are induced in the presence of alginate and the induction is repressed when glucose is included in the medium. Other nutritional (carbon or nitrogen sources and metal ions) and physicochemical (temperature, pH or oxygen supply) conditions show marked influences on formation of the alginate lyases. However, when *E. coli* DH1 clones carrying the alginate lyase gene of *Sphingomonas* sp A1 are grown in the presence of sorbitol (0.5–1.0 M) or sucrose (0.6 M), the induction of alginate lyase by alginate is substantially repressed (Murata *et al*, unpublished results), suggesting regulation

of the alginate lyase gene expression by an external osmolarity.

Alginate uptake mechanism

Sphingomonas sp A1 has particular cell surface features that facilitate the translocation of macromolecules. An opening, which we call a 'pit', is the most fascinating and peculiar surface structure of the bacterium. To the best of our knowledge this is the first description of such a structure in bacteria [27,28].

Cell surface

The surfaces of cells grown in the presence (Figure 1-I: B–F) or absence (Figure 1-I: A) of alginate are covered with many large plaits. An apparent difference between the two kinds of cells is the occurrence of a pit (0.02–0.1 μm in diameter) on the surface of cells grown in the presence of alginate. Formation of the pit is determined by the presence or absence of alginate, and the pit produced in the presence of alginate disappears when the cells are returned to medium without alginate.

Alginate staining

A number of globular particles found in and around the pit (Figure 1-I: B–F) are alginate gels. In the presence of alginate, a specific region and its neighborhood of cell surface are stained intensely with ruthenium red (Figure 1-II: B), an agent used for the staining of mucopolysaccharides, suggesting that alginate molecules are concentrated in a specific cell surface region, which may correspond to the pit.

Cell sections

Thin sections of cells grown on alginate show a specific region where the cell membrane sinks into the cytosol (Figure 1-III); no such membrane structure feature is observed in thin sections of cells grown in the absence of alginate. The average pit size is approximately 0.02–0.1 μm in width and 0.05 μm in depth. Alginate molecules may be concentrated in the pit, as manifested by alginate staining (Figure 1-II: B), and then incorporated into cells.

Pit-deficient mutant

Mutant AL-L derived from *Sphingomonas* sp A1 can not assimilate high molecular weight (HMW)-alginate (average MW 27 kDa) and fails to induce alginate lyases even in the presence of polymer (Momma *et al*, submitted for publication). However, when depolymerized low molecular weight (LMW)-alginate (average MW 1 kDa) is used in place of HMW-alginate, AL-L resumes growth with a sufficient induction of alginate lyase. The two results on the mutant AL-L indicate the presence of an HMW-alginate transport system in *Sphingomonas* sp A1. Transmission electron microscopy of the mutant shows the absence of a clear pit structure as seen in Figure 1.

The results argued in this section indicate the presence of a novel and unique pit-dependent mechanism for uptake of macromolecules, which is supported by the following facts: (i) cells of *Sphingomonas* sp A1 can use HMW-alginate present in medium by using alginate lyase, which is exclusively localized in the cytoplasm and not excreted even in the periplasmic space [40]; (ii) formation of the pit is dependent on the presence of HMW-alginate in medium; (iii) the pit closes or opens depending on the presence or absence of HMW-alginate; (iv) alginate is concentrated in a specific region on the cell surface; (v) HMW-alginate-grown cells have an irregular membrane region where the membrane sinks into the cytosol; (vi) a mutant unable to depolymerize HMW-alginate fails to form a pit. Thus, a novel and pit-dependent direct uptake system for macromolecules is considered to be functioning in cells of *Sphingomoas* sp A1, and it can be hypothesized that HMW-alginate is first concentrated in the pit and then directly taken into the cells, probably in an unexplored endocytosic-like fashion.

Genes responsible for transport of HMW-alginate

The 9-kb *Hin*dIII DNA fragment that enables the mutant AL-L cells to grow on HMW-alginate has been cloned and analyzed (Momma *et al*, submitted for publication). Two possible ORFs (ORF1 and ORF2) are responsible for the complementation and designated *algH* and *algM*, respectively. About 1.3 kbp and 0.2 kbp upstream from *algH*, the alginate lyase gene, *aly* [55], and the catabolite control gene, *ccp*, respectively, are located in opposite directions to *algH* and *algM*.

The gene *algH* consists of 1089 nucleotides encoding a polypeptide of 363 amino acids with a molecular mass of 39.5 kDa. The deduced amino acid sequence (AlgH) is 52% identical to the ATP-binding domain of the ABC (ATP-binding cassette) transporter of *E. coli*, UgpC [44], and contains a completely conserved ATP-binding region. The second gene, *algM*, consists of 621 nucleotides which can code for a protein of 207 amino acids with a molecular weight of 23.3 kDa. The deduced amino acid sequence (AlgM) is 42% and 26% identical to the LPLB protein from *B. subtilis* (P39128) and UgpA from *E. coli* [44], respectively; both are transmembrane domains of the ABC transporter. Hydropathic analyses suggest that AlgH is a soluble protein and AlgM is a membrane protein having three transmembrane helices. The proteins AlgH and AlgM show homology with UgpC and UgpA, respectively, both of which are members of the ABC transporter involved in glycerol 3-phosphate uptake [44]. UgpC has been reported to be functional as a supplier of energy through hydrolysis of ATP, and UgpA as a transmembrane protein of the ABC transporter. Therefore, AlgH is suggested to be an ATPase for the supply of energy required for incorporation of HMW-alginate, and AlgM functions as a transporter protein. In fact, AlgH is confirmed to be expressed in *Sphingomonas* sp A1 and purified AlgH shows high ATPase activity. Furthermore, disruption of either *algH* or *algM* results in failure to incorporate HMW-alginate (Momma *et al*, submitted for publication).

Although the cells of *Sphingomonas mali* have a similar cell surface structure to that of *Sphingomonas* sp A1, they cannot use alginate for their growth. When transformed with the above-described 9-kb *Hin*dIII fragment, the cells of *Sphingomonas mali* produce the pit-like structure and acquire capabilities to incorporate HMW-alginate and to use the polymer for growth. This result supports our assumption that AlgH and AlgM, possibly including a pro-

tein coded by an unknown gene (*norf4*) located downstream of AlgM, play an important role in the incorporation of extracellular HMW-alginate into cells. The location of alginate lyase (*aly*) and catabolite control (*ccp*) genes in the vicinity of genes involved in the uptake of alginate indicates that the uptake and assimilation/depolymerization of HMW-alginate are closely regulated.

Although experimental evidence is still not available, based on the above-described analyses, we can present a putative pit-dependent transport/depolymerization mechanism of HMW-alginate in *Sphingomonas* sp A1 cells (Figure 5). Briefly, HMW-alginate concentrated in the pit is incorporated into the cells by an ABC transporter system, and then presumably accumulated in a vacuole, where the polymers are depolymerized to the constituent monosaccharides by alginate lyase in combination with an unsaturated-uronyl releasing enzyme (AlgU) (Miyake *et al*, unpublished results).

In the generally accepted bacterial ABC transporter systems, the substrates to be incorporated are first bound to binding proteins and then the substrates are transferred to ABC transporters [2], as has been typically shown in the transport of maltose in *E. coli* [43] and histidine in *Salmonella typhimurium* [12]. However, in the cells of *Sphingomonas* sp A1, HMW-alginate-binding protein has not yet been identified. Alginate may be concentrated in the pit produced on the cell surface. Namely, the pit on the cell surface functions as a funnel in place of HMW-alginate-binding proteins.

In addition to alginate, the cells of *Sphingomonas* sp A1 can use polygalacturonate (pectin) as a carbon source for their growth. However, during growth on pectin, the cells excrete no pectin-depolymerizing enzymes into the medium, and only by incubation with intracellular enzymes, is the polymer completely depolymerized (Hashimoto *et al*, unpublished results). These facts indicate that, as in the case of alginate, pectin is incorporated directly into cells. Although a detailed uptake system for the polymer has not been elucidated, the examples of alginate and pectin suggest that the cells of *Sphingomonas* sp A1 have a characteristic apparatus to take up macromolecules.

Applications of alginate lyase

Food tailoring

The depolymerized products of A1-I activity on sodium alginate (27 kDa) derived from an edible seaweed *Eisenia bicyclis* have an average molecular size of 1800, consisting of ten sugar residues of β-D-mannuronate and/or α-L-guluronate [54]. The oligosaccharides can still form an egg-box structure, in which various divalent metal ions are chelated. The amount of magnesium, ferrous, and calcium ions incorporated into the box reaches 40, 80 and 50 mg per g (dry) of depolymerized preparation, respectively [42]. As for unmodified alginate, the low viscosity alginate and alginate-metal complexes might be useful in certain food applications.

Biomodulator

The oligosaccharides described above have an elicitor activity and can stimulate the proliferation and differentiation of some plants such as rice, komatsuna (*Bassica rapa* ver *pervidis*) and tobacco, although the oligosaccharides repress the proliferation of HeLa cells and an alga, *Chlamidomonas reinhardtii* [54]. Proliferation of *Bifidobacterium*, a Gram-positive anaerobe, is also enhanced by

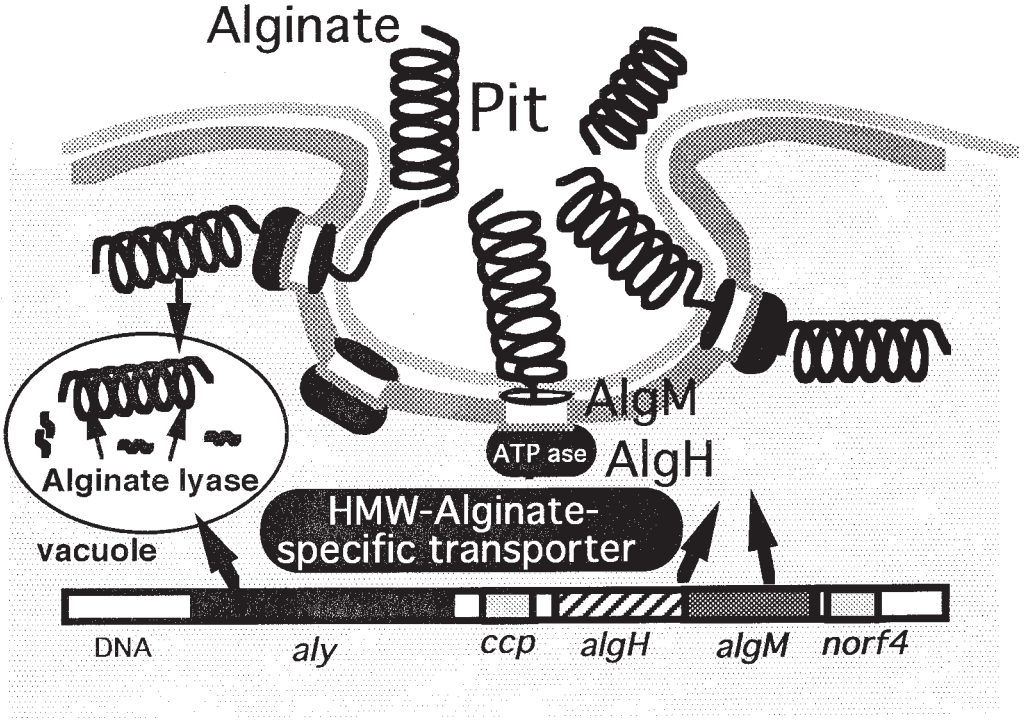

Figure 5 Model of alginate uptake in *Sphingomonas* sp A1. Genes of *aly*, *ccp*, *algH*, *algM*, and *norf4* encode alginate lyase, catabolite control protein, AlgH (ATP-binding domain of ABC transporter), AlgM (transmembrane domain of ABC transporter), norf4 (unknown protein), respectively.

the oligosaccharides; the effects are comparable with commercially obtainable growth factors of *Bifidobacterium* [1].

Therapeutic agent

Although alginate is currently prepared from brown seaweeds, the polymer is also produced by such bacteria as *A. vinelandii* [31] and mucoid strains of *P. aeruginosa* [32]. Alginate formation by mucoid *P. aeruginosa* in the lungs of patients with cystic fibrosis (CF), a common and fatal genetic disorder among Caucasians [6], leads to respiratory difficulties [3,36]. Although antimicrobial agents can repress bacterial growth in the CF lung, problems with resistance and toxicity limit their utility, particularly in older patients. *Pseudomonas* alginate significantly contributes to the increased viscosity of CF sputum. The ability to depolymerize this alginate may help clear the CF patients' airways and facilitate the delivery of other drugs *via* the aerosol route. Recent success with an aerosolized preparation of deoxyribonuclease in CF [29] supports the possibility that aerosolization of bacterial alginate lyase may become feasible in therapy of CF and other infectious diseases caused by *P. aeruginosa*.

Antigenicity of microbial alginate lyase A1-III is the most important problem in its practical use in medical areas. Polyethylene glycol (PEG)-modification is one of the promising ways to create non-antigenic enzymes. In fact, A1-III modified with succinimidyl succinate PEG (SS-PEG, MW 12 000) efficiently liquefies the alginate biofilm produced by *P. aeruginosa* isolated from CF patients, and is stable in rabbit blood for more than 4 days without appreciable loss of lyase activity (Suzuki *et al*, unpublished results). However, modification of enzymes with PEG is tedious and intricate in addition to the difficulty in preparation of PEG-modified enzymes with high enzymatic and low antigenic activities. The availability of the cloned gene should facilitate the modification by protein engineering techniques. X-ray crystallographic and biochemical analyses (Figure 4) [20] indicated the sequence of ^{27}Ser~^{44}Cys in A1-III is responsible for the antigenicity of the enzyme. The excision of the epitope is now being carried out to create a non-antigenic and more compact alginate lyase. The feasibility of the use of bacterial alginate lyase for clinical treatment of CF patients has also been considered by Bayer *et al* [4], Berry *et al* [5] and Hatch *et al* [21]. The mucoid *P. aeruginosa* can be isolated from various sources, such as lungs of patients with CF, the urinary tract, infected burn sites, contact lenses, and infected middle ear. It seems likely that alginate biofilms are formed in each site of the infection, and a non-antigenic alginate lyase produced by molecular manipulation may be utilized as an agent to eliminate chronic problems caused by alginate biofilms.

Concluding remarks

We have summarized experimental results on the transport/depolymerization system of macromolecules (alginate) in *Sphingomonas* sp A1. The most spectacular findings include: (i) the special cell surface structure (pit) of the bacterium; (ii) pit-dependent macromolecule (alginate) incorporation; and (iii) autoregulated post-translational modification processes for enzymes (alginate lyases). Here, we limit our discussion to the pit-dependent macromolecule incorporation system of the bacterium.

Generally, Gram-negative bacteria contain stereospecific solute transport systems in their inner membranes and non-stereoselective porins in their outer membranes that allow the passage of small ions, nutrients, and metabolic products across these lipid bilayer structures [18,27,50]. Although hydrophobic and large hydrophilic molecules can not pass through the outer membrane porin channels, numerous proteins, peptides, carbohydrates, and hydrophobic drugs are actively exported directly from the cytoplasm of these bacteria to the external milieu. However, no data have been accumulated as to the incorporation of macromolecules across the cell membranes, except for the incorporation of DNA by natural transformation systems [8,9]. The analysis of alginate utilization by *Sphingomonas* sp A1 indicated that an ABC transporter (traffic ATPase) is responsible for the incorporation of macromolecules.

The ABC transporters are involved in the transport of a variety of compounds across membranes of both prokaryotes and eukaryotes. ABC transporters are composed of two structural elements: an integral membrane domain (permease) and a hydrophilic domain (ATPase) that contains a conserved nucleotide-binding motif. In prokaryotic systems, the two domains usually consist of separate polypeptides interacting with each other and forming a membrane-bound complex. The prokaryotic systems also contain a soluble receptor (periplasmic substrate-binding protein) that binds the substrate in the periplasm and delivers it to the membrane-bound complex for translocation.

Analogous to the best studied ABC transporter in many prokaryotes, that in *Sphingomonas* sp A1 also consists of two elements, an integral membrane domain (AlgM, a product of *algM*) and a hydrophilic domain (AlgH, a product of *algH*). However, the molecular machinery of *Sphingomonas* sp A1 is different from typical prokaryotic ABC transporter systems: (i) the system allows incorporation of high molecular weight alginate; (ii) the system is linked with a pit formed on the cell surface; and (iii) the system does not require a periplasmic substrate-binding protein. Based on these facts, we can postulate a novel model for the alginate uptake mechanism (Figure 5). Briefly, alginate in the external medium is concentrated in the pit, and then incorporated into the cytosol by a membrane-bound complex consisting of AlgM and AlgH. AlgH (ATPase) generates energy through ATP hydrolysis and transfers it to AlgM (permease).

Thus, we for the first time showed the presence of a novel system for uptake of macromolecules in bacteria. In the utilization of macromolecules, microbes usually depolymerize them by extracellular enzymes and then take up the depolymerized low molecular weight products through the cell membrane. The direct uptake of alginate presented here is, therefore, a unique system that may constitute a novel concept in the transport (import) of macromolecules. However, we can not say which system of macromolecule utilization is energetically more efficient, since this is a matter of the economy of individual cells. Likewise, on the basis of the results presented here, it is not possible to state

whether the direct incorporation system mediated by the pit structure is a common mechanism in microbes.

The goal of our genetic and biochemical studies on the fascinating macromolecule incorporation system found in *Sphingomonas* sp A1 will be a clear view of the mechanism that controls the assembly of the structural elements (products of *algM* and *algH*) and an explanation of the function of the complex in macromolecule incorporation. The much anticipated X-ray crystal structure determinations of *algM* and *algH* products and their complex should greatly enhance our knowledge of the mechanism of action of these proteins. The crystal structure of AlgH will soon be reported elsewhere. The cell surface structure of *Sphingomonas* sp A1 is of great interest. The large plaits surrounding the cell surface are re-organized and/or re-constituted to generate a pit. Clarification of the signal transduction route that leads to the formation of a pit on the cell surface appears absolutely necessary for understanding the regulatory mechanism of incorporation and assimilation of macromolecules. If this information is combined with the novel pit-dependent ABC transporter system, our understanding of macromolecule incorporation and utilization in *Sphingomonas* sp A1 will probably expand rapidly.

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