

DESCRIPTION

METHOD AND GENE FOR PROVIDING OR ENHANCING NONSPECIFIC ADHESIVE PROPERTY AND/OR AUTOAGGLUTINATING PROPERTY 5 FOR MICROORGANISMS

Technical Field

The present invention relates to a method for providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a target
10 microorganism, DNA used for such method, a protein encoded by such DNA, a microorganism obtained by such method, and a method for culturing such microorganism.

Background Art

15 Microorganisms have activity of conversion of substances based on excellent catalytic action. Thus, microorganisms have been extensively used in the fields of, for example, brewing, fermented food production, and wastewater or gas treatment. Recently, production of pharmaceutical products with the use of recombinant microorganisms, fermentative production of bioethanol, and synthesis of chemical
20 products with the use of microorganisms have been implemented or researched, and techniques involving the use of microorganisms are industrially important. However, production of microbial cells that play key roles in reactions is costly in terms of, for example, media and energy. In addition, microbial reactions are often carried out in aqueous solutions, which necessitate the separation of microorganisms from reaction
25 solutions after substances are produced. Microorganisms were mainly separated via centrifugation or filtration in the past.

In order to facilitate separation and continuously or repeatedly use valuable microbial cells, immobilization and autoagglutination of microbial cells were considered to be effective. Examples of conventional immobilization techniques include
30 immobilization through entrapment in a gel such as alginic acid and surface immobilization to allow microorganisms to adsorb on the surfaces of porous carriers.

However, the entrapment immobilization method is disadvantageous in that transportation of oxygen and substrates in gel is often limited in the rate, gel is brittle in mechanical strength and thus is likely to be destroyed by agitation or the like, and microbial cells leak from gel, for example. Also, a conventional technique of surface immobilization does not involve accumulation of target microorganisms on a surface, and the technique merely involves introduction of porous carriers or the like for use in the fields of wastewater processing or environmental cleanup where large quantities of many sorts of microorganisms are present, thereby allowing easily-adhering microorganisms to be carried on a surface as a biofilm. That is, there is no technique that allows microorganisms of interest to adhere to a solid surface as one likes. Regarding agglutination, a method involving the use of coagulating agents, such as high-molecular-weight polymers, is extensively used in wastewater processing. In addition, a method involving screening of agglutinating microorganisms for use thereof has been employed. However, there is no technique that enables spontaneous agglutination of non-agglutinating microbial cells, and particularly bacteria.

Disclosure of the Invention

An object of the present invention is to provide a means for providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism with weak or without nonspecific adhesive property or autoagglutinating property.

The present inventors have succeeded in isolating an adhesion-associated gene from a microorganism having nonspecific adhesive property. They discovered that such gene was the autotransporter adhesin gene and introduction thereof into the target microorganism would lead to provision or enhancement of nonspecific adhesive property and/or autoagglutinating property for the target microorganism. This has led to the completion of the present invention.

Specifically, the present invention includes the following.

(1) A method for providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a target microorganism, comprising introducing into the target microorganism a DNA encoding autotransporter adhesin from a

microorganism having nonspecific adhesive property.

(2) The method according to (1), wherein the microorganism having nonspecific adhesive property is a bacterium of the genus *Acinetobacter*.

(3) The method according to (1) or (2), wherein the DNA encoding autotransporter adhesin is the following DNA (a), (b), or (c):

(a) a DNA comprising the nucleotide sequence as shown in SEQ ID NO: 1;

(b) a DNA comprising a nucleotide sequence having 70% or more homology to the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism; or

(c) a DNA consisting of a part of the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism.

(4) The method according to any of (1) to (3), wherein the target microorganism is a bacterium of the genus *Escherichia*.

(5) The method according to (4), wherein the bacterium of the genus *Escherichia* is *Escherichia coli*.

(6) The method according to any of (1) to (5), wherein the DNA encoding autotransporter adhesin is introduced with the following DNA (a) or (b):

(a) a DNA comprising the nucleotide sequence as shown in SEQ ID NO: 3; or

(b) a DNA comprising a nucleotide sequence having 90% or more homology to the nucleotide sequence as shown in SEQ ID NO: 3.

(7) The method according to (6), wherein the following DNA (a) or (b) is introduced into the target microorganism:

(a) a DNA comprising the nucleotide sequence as shown in SEQ ID NO: 5; or

(b) a DNA comprising a nucleotide sequence having 70% or more homology to the nucleotide sequence as shown in SEQ ID NO: 5 and having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a host microorganism.

(8) Also described herein is a microorganism obtained by the method according to any of (1) to (7), for which nonspecific adhesive property and/or autoagglutinating property have been

provided or enhanced.

(9) A DNA of the following (a), (b), or (c):

(a) a DNA comprising the nucleotide sequence as shown in SEQ ID NO: 1;

(b) a DNA comprising a nucleotide sequence having 70% or more homology to the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism; or

(c) a DNA consisting of a part of the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism.

(10) A microorganism into which the DNA according to (9) has been introduced, for which nonspecific adhesive property and/or autoagglutinating property have been provided or enhanced.

(11) The microorganism according to (10), which is a bacterium of the genus *Escherichia*.

(12) The microorganism according to (11), which is *Escherichia coli*.

(13) A protein of the following (a), (b), or (c):

(a) a protein comprising the amino acid sequence as shown in SEQ ID NO: 2;

(b) a protein comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 2 by deletion, substitution, insertion, or addition of one or several amino acids and having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism; or

(c) a protein consisting of a part of the amino acid sequence as shown in SEQ ID NO: 2 and having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism.

(14) A method for culturing a microorganism, comprising causing the microorganism according to (8), (10), (11), or (12) to adhere to a carrier and/or aggregate to recover the microorganism.

In another aspect, the present invention is directed to a method for production of chemical products using a microorganism wherein the microorganism according to the present invention is used.

In another aspect, the present invention is directed to a use of the microorganism according to the present invention in the production of chemical products using a microorganism.

The present invention enables provision or enhancement of nonspecific adhesive property and/or autoagglutinating property for a microorganism with weak or

without nonspecific adhesive property or autoagglutinating property. This facilitates separation of a microorganism in the microbial reaction process and enables efficient recovery of valuable microbial cells and continuous or repeated use thereof.

5 Brief Description of the Drawings

Fig. 1 shows the positional relationship of sequence fragments obtained in Example 1.

Fig. 2 shows whole the nucleotide sequence determined as a result of cloning and sequencing in Example 1.

10 Fig. 3 shows the amino acid sequence of AadA.

Fig. 4 shows the results of preparation of multiple alignments of each of the signal peptide, the head domain, the neck domain, and the membrane-anchor domain of autotransporter adhesin with ClustalW for sequence comparison.

15 Fig. 5 shows the results of a comparison of the sequence of Tol 5-OmpA and that of an outer membrane protein having homology thereto.

Fig. 6 shows the results of optical microscopic observation of morphological changes resulting from introduction of the *aadA* gene and the *aadA-ompA* operon.

Fig. 7 shows an electron micrograph of DH5 α ::*aadA* that has adhered to a polyurethane surface.

20 Fig. 8 shows an electron micrograph of DH5 α ::*aadA-ompA* that has adhered to a polyurethane surface.

Fig. 9 shows an electron micrograph of DH5 α (WT) that exists on a polyurethane surface.

25 Fig. 10 shows photographs of liquid cultures of DH5 α ::*aadA-ompA*, DH5 α ::*aadA*, and DH5 α WT.

Fig. 11 shows the results of the autoagglutination tests of DH5 α ::*aadA* and DH5 α WT.

Fig. 12 shows a table summarizing primers used in Example 1.

30 Best Modes for Carrying out the Invention

The method of the present invention comprises introducing a DNA encoding

autotransporter adhesin from a microorganism having nonspecific adhesive property (i.e., the autotransporter adhesin gene) into the target microorganism.

Some pathogenic microorganisms adhere to certain biotic surfaces (e.g., surfaces of certain cells, organisms, or tissues thereof) and form colonies. However, the term "microorganism having nonspecific adhesive property" used in the present invention refers to a microorganism that is capable of adhering to any type of biotic or abiotic surfaces, including abiotic surfaces of carriers or the like, in addition to certain types of biotic surfaces. A microorganism having nonspecific adhesive property can adhere to, for example, glass, plastic, and metal carriers.

Examples of microorganisms having nonspecific adhesive property include Gram-negative bacteria, such as *Acinetobacter* bacteria, *Pseudomonas* bacteria, *Escherichia* bacteria, *Caulobacter* bacteria, *Xanthomonas* bacteria, *Haemophilus* bacteria, *Yersinia* bacteria, *Bartonella* bacteria, *Neisseria* bacteria, and *Actinobacillus* bacteria. In the present invention, *Acinetobacter* spp. are particularly preferable.

A specific example of a microorganism having nonspecific adhesive property is strain *Acinetobacter* sp. Tol 5. This strain is capable of degrading toluene and was isolated from a reactor for off-gas treatment. The strain is deposited at the International Patent Organism Depositary of the National Institute of Advanced Industrial Science and Technology, Incorporated Administrative Agency (Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan) under the Accession Number: FERM P-17188.

Nonspecific adhesive property of microorganisms can be evaluated by an adhesion test via crystal violet staining (the CV adhesion test). Specifically, the bacterial cell liquid culture is subjected to centrifugation, the culture supernatant is removed, an inorganic salt medium is added to the cell pellet, the cell suspension is obtained via ultrasonication, the turbidity (OD₆₆₀) of the cell suspension is adjusted to a constant level (0.5 or lower) with a medium, 1 ml each of the suspension is added to each well of a 48-well plastic plate, incubation is carried out at an optimal temperature for the relevant microorganisms for 2 hours, the suspensions are completely removed from the wells using a pipette, the wells are washed with 1 ml of inorganic salt medium, the plate is air dried, an aqueous solution of 1% aqueous crystal violet solution is added

to the wells, incubation is carried out at room temperature for 15 minutes, crystal violet is removed with the use of a pipette, the wells are washed twice with 1 ml of inorganic salt medium, the plate is air dried, 1 ml of inorganic salt medium is added to detach the stained bacterial cells from the inner wall of the wells, the cells are dispersed via
5 ultrasonication, and absorbance (A_{590}) is then measured. Microorganisms exhibiting an absorbance A_{590} of 0.15 or higher, preferably 0.18 or higher, and more preferably 0.2 or higher can be evaluated as having nonspecific adhesive property.

Autotransporter adhesins are proteins that are reported as adhesive nanofibers of Gram-negative bacteria. They are known to interact specifically with tissues, cell
10 surface molecules, and extracellular matrix of hosts, although it is not reported that autotransporter adhesins nonspecifically adhere to a variety of solid surfaces. It is said that autotransporter adhesins have functions such as adhesion, invasion, cytotoxicity, serum tolerance, and intercellular propagation. Autotransporter adhesins have a common domain organization (i.e., the N-terminal signal peptide, the internal passenger
15 domain, and the C-terminal translocator domain). In particular, the C-terminal translocator domain characterizes this protein family. Autotransporter adhesin secretion begins with an export across the inner membrane by the Sec system, which is mediated by the signal peptide. Subsequently, the translocator domain is inserted into the outer membrane and the β barrel structure is formed. In the end, the passenger
20 domain passes through the outer membrane and appears on the bacterial cell surface. Autotransporter adhesins are divided into monomeric autotransporter adhesin and trimeric autotransporter adhesins (Shane E. Cotter, Neeraj K. Surana and Joseph W. St Geme III, 2005, Trimeric autotransporters: a distinct subfamily of Autotransporter proteins, *TRENDS in Microbiology*, 13: 199-205). The translocator domain of
25 monomeric autotransporter adhesin is considered to form the β barrel structure, which is a hydrophilic pathway consisting of 14 transmembrane antiparallel β -sheets and the transmembrane α helices, from a single subunit. In contrast, it is known that the translocator domain of trimeric autotransporter adhesins forms a thermostable and SDS-resistant trimer in the outer membrane, a subunit having four β -sheets is oligomerized,
30 and thus the 12-stranded β barrel structure is formed from 3 subunits. Also, many general autotransporter adhesins have intramolecular chaperone regions, although the

trimeric autotransporter adhesin subfamily does not have the chaperone. In addition, the passenger domains of practically all monomeric autotransporter adhesins form non-covalent bonds with the translocator domains for linking to surfaces of bacteria, or are released outside the cells. However, the passenger domains of all trimeric autotransporter adhesin proteins are considered to remain linked to the translocator domains via covalent bonds.

Trimeric autotransporter adhesin is abbreviated as TAA, and it is also referred to as the Oca family (i.e., the Oligomeric Coiled-coil Adhesin Family) as a new class that forms a common oligomer structure, a coiled-coil structure (Andreas Roggenkamp, Nikolaus Ackermann, Christoph A. Jacobi, Konrad Truelzsch, Harald Hoffmann, and Jurgen Heesemann 2003, Molecular analysis of transport and oligomerization of the *Yersinia enterocolitica* adhesin YadA. J. Bacteriol. 185: 3735-3744).

In the present invention, trimeric autotransporter adhesin is preferable. Examples of trimeric autotransporter adhesins include *Yersinia enterocolitica* adhesin (YadA, *Yersinia* adhesin A) (El Tahir Y, Skurnik M. 2001, YadA, the multifaceted *Yersinia* adhesin, Int. J. Med Microbiol., 291:209-218), *Haemophilus influenzae* Hia that causes meningitis (St. Geme JW 3rd, Cutter D., 2000, The *Haemophilus influenzae* Hia adhesin is an autotransporter protein that remains uncleaved at the C terminus and fully cell associated, J. Bacteriol., 182: 6005-13), *Aggregatibacter (Actinobacillus) actinomycetemcomitans* EmaA that causes periodontal diseases (Mintz, K. P. 2004, Identification of an extracellular matrix protein adhesin, EmaA, which mediates the adhesion of *Actinobacillus actinomycetemcomitans* to collagen, Microbiology. 150, 2677-2688), *Moraxella catarrhalis* UspA1 and A2 that are major pathogens of respiratory infections (Lafontaine ER, Cope LD, Aebi C, Latimer JL, McCracken GH Jr, Hansen EJ. 2000, The UspA1 protein and a second type of UspA2 protein mediate adherence of *Moraxella catarrhalis* to human epithelial cells in vitro, J. Bacteriol., 182: 1364-73), *Bartonella henselae* BadA that causes cat-scratch diseases (Riess T, Andersson SG, Lupas A, Schaller M, Schäfer A, Kyme P, Martin J, Wälzlein JH, Eehalt U, Lindroos H, Schirle M, Nordheim A, Autenrieth IB, Kempf VA., 2004, *Bartonella* adhesin a mediates a proangiogenic host cell response. J. Exp. Med., 200: 1267-78), *Neisseria meningitidis* (meningococci) NadA (Capecci B, Adu-Bobie J, Di Marcello F,

Ciucchi L, Masignani V, Taddei A, Rappuoli R, Pizza M, Aricò B. 2005, *Neisseria meningitidis* NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells, Mol. Microbiol., 55: 687-98), and phytopathogenic bacteria; i.e., *Xanthomonas oryzae* XadA (Ray SK, Rajeshwari R, Sharma Y, Sonti RV., 2002, A high-molecular-weight outer membrane protein of *Xanthomonas oryzae* pv. *oryzae* exhibits similarity to non-fimbrial adhesins of animal pathogenic bacteria and is required for optimum virulence, Mol Microbiol., 46: 637-47).

Examples of preferable autotransporter adhesins include a protein comprising the amino acid sequence as shown in SEQ ID NO: 2 and proteins that are functionally equivalent thereto. The term "proteins that are functionally equivalent" refers to proteins having biological and biochemical functions equivalent to those of the protein comprising the amino acid sequence as shown in SEQ ID NO: 2. An example of a protein that is functionally equivalent to the protein comprising the amino acid sequence as shown in SEQ ID NO: 2 is a protein comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 2 by deletion, substitution, insertion, or addition of one or several amino acids and having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism. A further example is a protein consisting of a part of the amino acid sequence as shown in SEQ ID NO: 2 and having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism; that is, a deficient protein comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 2 by deletion of one or several regions comprising several tens to several hundreds, and optionally 1,000 or more, continuous amino acid residues and maintaining the activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism.

Deletion, substitution, insertion, or addition of one or several amino acids can be performed by modifying the sequence of DNA encoding the protein of interest (e.g., the nucleotide sequence as shown in SEQ ID NO: 1) by a conventional technique such as site-directed mutagenesis (Zoller et al., Nucleic Acids Res., 10, 6478-6500, 1982).

Side chains of amino acids constituting proteins vary in terms of hydrophobic properties, electric charge, size, and other conditions. Nevertheless, several highly

conserved relationships between amino acid residues are known on an empirical basis or as a result of physicochemical measurement, in which the highly conserved relationships means that the side chains would not substantially influence the three-dimensional structure of the entire protein (also referred to as a "conformation"). Examples of known conservative substitutions between different amino acid residues include substitutions between amino acids such as glycine (Gly) and proline (Pro); glycine and alanine (Ala) or valine (Val); leucine (Leu) and isoleucine (Ile); glutamic acid (Glu) and glutamine (Gln); aspartic acid (Asp) and asparagine (Asn); cysteine (Cys) and threonine (Thr); threonine and serine (Ser) or alanine; and lysine (Lys) and arginine (Arg). Thus, amino acid substitution is preferably conservative.

The protein comprising the amino acid sequence as shown in SEQ ID NO: 2 is trimeric autotransporter adhesin comprising a signal peptide, a head domain, a neck domain, a stalk domain, and a membrane anchor domain. Thus, amino acid mutation preferably maintains such domain structure. In the amino acid sequence as shown in SEQ ID NO: 2, the signal peptide corresponds to amino acids 1 to 57, the head domains correspond to amino acids 108 to 269 and amino acids 2997 to 3148, the neck domains correspond to amino acids 296 to 319 and amino acids 3149 to 3172, the stalk domains correspond to amino acids 406 to 2966 and amino acids 3173 to 3537, and the membrane anchor domain corresponds to amino acids 3538 to 3630.

The term "several" means usually 2 to 10, preferably 2 to 5, and more preferably 2 to 3. Functionally equivalent proteins usually have high homology at the amino acid sequence level. The term "high homology" refers to usually 70% or more, preferably 80% or more, more preferably 90% or more, further preferably 95% or more, and most preferably 99% or more homology (or identity) at the amino acid level.

Concerning a protein consisting of a part of the amino acid sequence as shown in SEQ ID NO: 2, a sequence of several tens to several hundreds continuous amino acids that can be deleted from the sequence of SEQ ID NO: 2 is preferably a sequence corresponding to either of or both the stalk domains, either of the head domains, or either of the neck domains, and one or a plurality thereof may be deleted. In the aforementioned domains, deletion of the entire region from the head domain to the stalk domain that is located more closely to the amino terminus (i.e., positions 108 to 2966) or

the entire region from the head domain to the stalk domain that is located more closely to the carboxyl terminus (i.e., positions 2997 to 3537) is more preferable. Further preferably, a plurality of repeated regions observed in the stalk domain are deleted such that each region appears only once without repetition. Most preferably, any one of the plurality of repeated regions observed in the stalk domain is deleted.

Examples of a preferable autotransporter adhesin gene (i.e., a DNA encoding autotransporter adhesin) include DNA comprising the nucleotide sequence as shown in SEQ ID NO: 1 and DNA that is functionally equivalent thereto. An example of DNA that is functionally equivalent to DNA comprising the nucleotide sequence as shown in SEQ ID NO: 1 is DNA comprising a nucleotide sequence having 70% or more, preferably 80% or more, more preferably 90% or more, further preferably 95% or more, and most preferably 98% or more homology (or identity) to the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism. Another example is a DNA hybridizing under stringent conditions to a DNA comprising a nucleotide sequence complementary to the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism. A further example is a DNA consisting of a part of the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism; that is, a DNA of a deficient gene comprising a nucleotide sequence derived from the nucleotide sequence as shown in SEQ ID NO: 1 by deletion of 1 or several regions comprising continuous nucleotides of several tens to several hundreds, and optionally 1,000 or more, and encoding a protein that maintains the activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism.

Under stringent conditions, a specific hybrid is formed, but a nonspecific hybrid is not formed. Hybridization may be carried out under low or high stringency conditions, with high stringency conditions being preferable. Under low stringency conditions, hybridization is followed by washing at 42°C in 5 x SSC and 0.1% SDS, and

preferably at 50°C in 5 x SSC and 0.1% SDS, for example. Under high stringency conditions, hybridization is followed by washing at 65°C in 0.1 x SSC and 0.1% SDS, for example.

A mutation of a nucleotide sequence preferably maintains a domain structure comprising a signal peptide, a head domain, a neck domain, a stalk domain, and a membrane anchor domain. In the nucleotide sequence as shown in SEQ ID NO: 1, the signal peptide corresponds to nucleotides 1 to 171, the head domains correspond to nucleotides 322 to 807 and nucleotides 8989 to 9444, the neck domains correspond to nucleotides 886 to 957 and nucleotides 9445 to 9516, the stalk domains correspond to nucleotides 1216 to 8898 and nucleotides 9517 to 10611, and the membrane anchor domain corresponds to nucleotides 10612 to 10890.

Concerning a DNA consisting of a part of the nucleotide sequence as shown in SEQ ID NO: 1, a sequence of several tens to several hundreds continuous nucleotides that can be deleted from the sequence of SEQ ID NO: 1 is preferably a sequence encoding either of or both the stalk domains, either of the head domains, or either of the neck domains, and one or a plurality thereof. In the aforementioned domains, deletion of the region encoding the entire region from the head domain to the stalk domain that is located more closely to the amino terminus (i.e., nucleotide positions 322 to 8898) or the region encoding the entire region from the head domain to the stalk domain that is located more closely to the carboxyl terminus (i.e., nucleotide positions 8989 to 10611) is more preferable. Further preferably, a plurality of repeated regions observed in the coding region of the stalk domain are deleted in such a manner that each region appears only once without repetition. Most preferably, any one of the plurality of repeated regions observed in the coding region of the stalk domain is deleted.

By introducing a DNA encoding autotransporter adhesin with a DNA of the nucleotide sequence as shown in SEQ ID NO: 3 into a target microorganism, nonspecific adhesive property and/or autoagglutinating property of the target microorganism can further be improved. DNA of the nucleotide sequence as shown in SEQ ID NO: 3 has homology with the OmpA gene of the outer membrane protein of *Acinetobacter* bacteria, and it thus encodes the outer membrane protein. A gene functionally equivalent to DNA comprising the nucleotide sequence as shown in SEQ ID NO: 3 may be introduced.

An example of DNA that is functionally equivalent to DNA comprising the nucleotide sequence as shown in SEQ ID NO: 3 is DNA comprising a nucleotide sequence having 90% or more, preferably 95% or more, and more preferably 98% or more homology to the nucleotide sequence as shown in SEQ ID NO: 3. Another example is DNA hybridizing under stringent conditions to DNA comprising a nucleotide sequence complementary to the nucleotide sequence as shown in SEQ ID NO: 3.

An operon comprising a DNA encoding autotransporter adhesin derived from a microorganism having nonspecific adhesive property may be introduced into a target microorganism. For example, DNA comprising the nucleotide sequence as shown in SEQ ID NO: 5 may be introduced into the target microorganism, so that DNA encoding autotransporter adhesin and DNA encoding the outer membrane protein can be introduced into the target microorganism. An operon that is functionally equivalent to the above operon may be introduced. An example of a functionally equivalent operon is an operon comprising DNA comprising a nucleotide sequence having 70% or more, preferably 80% or more, more preferably 90% or more, further preferably 95% or more, and most preferably 98% or more homology to the nucleotide sequence as shown in SEQ ID NO: 5 and having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a host microorganism.

Target microorganisms into which DNA encoding autotransporter adhesin is to be introduced include, but not particularly limited to, microorganisms with weak or without nonspecific adhesive property and/or autoagglutinating property. Examples include: bacteria of the genus *Escherichia*, such as *Escherichia coli*; bacteria of the genus *Acinetobacter*, such as *Acinetobacter calcoaceticus*; bacteria of the genus *Ralstonia*, such as *Ralstonia eutropha*; bacteria of the genus *Pseudomonas*, such as *Pseudomonas putida* and *Pseudomonas fluorescens*; bacteria of the genus *Aeromonas*, such as *Aeromonas caviae*; bacteria of the genus *Alcaligenes*, such as *Alcaligenes latus*; and bacteria of the genus *Xanthomonas*, such as *Xanthomonas campestris*.

By introducing a DNA encoding autotransporter adhesin into a target microorganism through transformation, a microorganism for which nonspecific adhesive property and/or autoagglutinating property have been provided or enhanced can be obtained. Typically, a DNA encoding autotransporter adhesin is ligated to an adequate

vector, and a target microorganism (i.e., a host microorganism) is transformed with such vector to produce a microorganism for which nonspecific adhesive property and/or autoagglutinating property have been provided or enhanced. Specifically, multiple copies of the aforementioned DNA may be introduced into the host microorganism, such
5 DNA may be ligated under the control of a constitutive expression promoter, or such DNA may be ligated under the control of a promoter in inducible enzyme systems, to produce a microorganism for which nonspecific adhesive property and/or autoagglutinating property have been provided or enhanced. The same applies to a case in which a DNA encoding the outer membrane protein is introduced with a DNA
10 encoding autotransporter adhesin and to a case in which an operon comprising a DNA encoding autotransporter adhesin is introduced.

At the outset, DNA of interest is ligated to a vector to prepare a recombinant vector. As the vector, a phage, cosmid, artificial chromosome, or plasmid vector that can autonomously replicate in a host cell can be used. When a plasmid is introduced
15 into a chromosome as an expression cassette, for example, a vector must be capable of autonomous replication in a host, which is necessary for constructing such expression cassette (e.g., *E. coli*), but a vector is not necessarily capable of autonomous replication in a host into which the expression cassette is to be introduced (e.g., a yeast host). As such recombinant vector, for example, a shuttle vector that is designed to be used in *E.*
20 *coli*, and yeast hosts can also be used.

Examples of plasmids include *E. coli*-derived plasmids (e.g., pET21a(+), pET32a(+), pET39b(+), pET40b(+), pET43.1a(+), pET44a(+), pKK223-3, pGEX4T, pUC118, pUC119, pUC18, and pUC19), *Bacillus subtilis*-derived plasmids (e.g., pUB110 and pTP5), yeast-derived plasmids (e.g., YEp13, YEp24, and YCp50), plasmids
25 for the yeast *Pichia pastoris* (e.g., pPICZ, pPICZ α , pHIL-D2, pPIC3.5, pHIL-S1, pPIC9, pPIC6, pGAPZ, pPIC9K, pPIC3.5K, pAO815, and pFLD), and λ phages such as phage DNA (e.g., λ gt11 and λ ZAP). In addition, animal virus vectors, such as vaccinia virus vectors, and insect virus vectors, such as baculovirus vectors, can be used. Further, a commercially available cloning vector, such as pCR4-TOPO[®], may be used for cloning
30 and sequencing.

In order to insert DNA into a vector, purified DNA is first cleaved with an

adequate restriction enzyme and inserted into a restriction enzyme site or multicloning site of an adequate vector DNA to ligate DNA of interest to the vector. For example, DNA of interest can be synthesized by a conventional technique. For the purpose of incorporating such DNA into a vector, the DNA may be amplified via PCR with primers, such that the resultant comprises the cleavage sites with adequate restriction enzymes at both ends. PCR conditions can be adequately determined by a person skilled in the art.

In addition to a promoter and DNA of the present invention, a *cis* element such as an enhancer, a splicing signal, a poly A addition signal, a selection marker, a ribosome binding sequence (an SD sequence), or the like may be ligated to the recombinant vector according to need. Examples of selectable markers include, but are not limited to, drug-resistant markers, such as kanamycin, ampicillin, tetracycline, and chloramphenicol, and auxotrophic markers, such as leucine, histidine, lysine, methionine, arginine, tryptophan, and uracil.

Promoters are not particularly limited, and a person skilled in the art can select adequate promoters in accordance with host microorganisms. When *E. coli* hosts are used, for example, T7 promoters, *lac* promoters, *trp* promoters, or λ -PL promoters can be used. In addition, a promoter comprising the nucleotide sequence as shown in SEQ ID NO: 28 and a promoter that is functionally equivalent thereto, such as a promoter comprising a nucleotide sequence having 90% or more, preferably 95% or more, and more preferably 98% or more homology to the nucleotide sequence as shown in SEQ ID NO: 28, can be preferably used.

In order to ligate a DNA fragment to a vector fragment, a known DNA ligase is used. The DNA fragment is annealed to the vector fragment, followed by ligation to prepare a recombinant vector. Preferably, a recombinant vector can be obtained by performing a ligation reaction with the use of a commercially available ligation kit, such as Ligation High (TOYOBO Co., Ltd.) under the indicated conditions.

Recombinant DNA techniques including cloning, ligation, PCR, or the like, described in, for example, Sambrook, J. et al., *Molecular Cloning* 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. Press, 1989 and *Short Protocols In Molecular Biology*, Third Edition, A Compendium of Methods from Current Protocols in Molecular Biology, John Wiley & Sons, Inc. may be employed.

According to need, the obtained vector may be purified via the boil method, an alkali SDS method, or the magnetic bead method, or with the use of commercially available kits based on the principles of such methods. Further, the vector may be concentrated via a means such as ethanol precipitation or polyethylene glycol precipitation.

A recombinant vector may be introduced into a target microorganism by any method without particular limitation. Examples include a method involving the use of calcium ions, electroporation, and lipofection.

A transformed microorganism comprising DNA of interest can be selected with the aid of a marker gene in the recombinant vector. For example, a transformed microorganism of interest can be selected by forming colonies on an LB agar medium plate comprising antibiotics such as ampicillin or kanamycin. In order to verify whether or not a cloned host microorganism has been transformed by a recombinant vector, a sample from the microorganism may be subjected to verification of amplification of the insert via PCR or sequence analysis by the dideoxy method using a sequencer. In addition to the introduction of an autonomously replicable plasmid, chromosomal integration may be carried out by positioning a region homologous to the chromosomal gene in a vector to induce homologous recombination, thereby introducing the target gene.

The obtained transgenic microorganism is cultured in a medium by a method that is commonly used for culture of the target microorganism. A natural or synthetic medium may be used for culturing the transgenic microorganism obtained from a microbial host, such as *E. coli* or yeast, provided that such medium comprises carbon sources, nitrogen sources, inorganic salts, and the like assimilable by the microorganism and such medium can efficiently culture the transgenic microorganism. Specific examples include M9 medium, M9G medium, BS medium, LB medium, nutrient broth medium, meat extract medium, SOB medium, SOC medium, and PDA medium.

Any assimilable carbon compounds can be used as carbon sources. Examples of carbon compounds that can be used include: saccharides such as glucose; polyols such as glycerin; alcohols such as methanol; and organic acids such as pyruvic acid, succinic acid, and citric acid. Any nitrogen compounds can be used as nitrogen sources.

Examples thereof that can be used include peptone, meat extract, yeast extract, casein hydrolysate, an alkaline extract of soy bean cake, alkylamines such as methylamine, and ammonia or a salt thereof. In addition, phosphate, carbonate, salts of sulfate, magnesium, calcium, potassium, iron, manganese, and zinc, given amino acids, given
5 vitamins, antifoaming agents, or the like may be used according to need. Further, an inducer of protein expression, such as isopropyl- β -D-thiogalactopyranoside, may be added to a medium according to need.

In general, culture is conducted under aerobic conditions, such as shake culture or aeration and agitation culture conditions, at preferably 0°C to 40°C, more preferably
10 10°C to 37°C, and particularly preferably 15°C to 37°C. During culture, the pH level of the medium can be adequately changed, provided that a host can grow therein and the activity of autotransporter adhesin is not deteriorated. The pH level is preferably between about 4 and 8. The pH level is adjusted with the use of an inorganic or organic acid, an alkaline solution, or the like. If necessary, antibiotics such as
15 ampicillin or tetracycline may be added to the medium during the culture.

Thus, a microorganism for which nonspecific adhesive property and/or autoagglutinating property have been provided or enhanced can be obtained. Nonspecific adhesive property of the resulting microorganism can be evaluated by the aforementioned adhesion test via crystal violet staining (the CV adhesion test), and
20 autoagglutinating property can be evaluated in the following manner, for example. Specifically, the bacterial cell liquid culture is centrifuged, the culture supernatant is removed, inorganic salt medium is added to the cell pellet, the cell suspension is obtained via ultrasonication, the turbidity (OD₆₆₀) of the cell suspension is adjusted to a constant level with a medium, and this OD₆₆₀ value is defined as the initial OD₆₆₀ value.
25 The cell suspension in a glass centrifuge tube is allowed to stand at room temperature, resulting in the sedimentation of aggregated cells, and changes over time in the OD₆₆₀ values in the supernatant are assayed. A microorganism exhibiting a decrease in the OD₆₆₀ value relative to the initial OD₆₆₀ value of 10% or more, preferably 15% or more, and more preferably 20% or more can be evaluated as having autoagglutinating property.

30 The resulting microorganisms are cultured, and bacteria or cells are then disrupted by a known technique, such as a mechanical technique, an enzymatic

technique involving the use of lysozyme, or chemical processing with e.g., a surfactant. Thus, autotransporter adhesin proteins can be isolated.

The present invention can be directly applied within the conventional fermentation industry or for waste treatment. In addition, the present invention is very effective for biomass energy production and green biotechnology involving the use of microbial cells. In particular, it is desirable to carry out production of energy or chemical products at low cost. Provision or enhancement of nonspecific adhesive property and/or autoagglutinating property for a microorganism leads to immobilization or aggregation of cells of the microorganisms, which directly leads to promotion of production efficiency and serves as a foundation for the development of the aforementioned industries.

Examples

Example 1: Analysis of an adhesion-associated gene

1-1: Preparation of the less-adhesive mutant T1 and primers used for inverse PCR

In order to analyze an adhesion-associated gene from *Acinetobacter* sp. strain Tol 5, less-adhesive mutants were produced via random transposon insertion. Tol 5 WT strain (Tol 5 wild-type strain) as the recipient cells were conjugated with *E. coli* S17-1 strain having transposon vector as the donor cells at 28°C for 22 to 24 hours. Selection was performed on a medium containing toluene and tetracycline, and the obtained cells were subjected to an adhesion test. As a result, several strains exhibiting less adhesive property were obtained. Genomic DNAs thereof were obtained and treated with the *Hind*III restriction enzyme, and Southern hybridization was carried out using the resultants as templates and DIG-labeled tetA as a probe. As a result, an intensive band was detected at around 5 kb in one strain, and this strain was designated as the less-adhesive strain, T1. The surface structure of the T1 strain was observed under an electron microscope, and it was found to lack appendages (i.e., cell surface projections).

Subsequently, a DNA fragment of approximately 5 kb, which was detected via Southern hybridization, was excised from agarose gel in order to analyze the transposon insertion site of the T1 strain. This fragment was designated as T1-5kb. T1-5kb was

inserted into the pUC118 vector and the resultant was cloned in *E. coli* DH5 α . Both ends of the insert portion in the resulting plasmid DNA were sequenced. As a result, a 68-bp nucleotide sequence from the Tol 5 WT strain was found (SEQ ID NO: 6), in addition to the Tn5 sequence, in T1-5kb.

1-2: Cloning of the adhesion-associated gene

In order to analyze the nucleotide sequence of the transposon insertion site in the less-adhesive mutant, T1, inverse PCR of chromosomal DNA of the WT strain was performed on the basis of the 68-bp sequence of the insertion site, which had become apparent via analysis of T1. At the outset, the Tol 5 wild-type strain was cultured in BS medium, followed by extraction of DNA and treatment with RNase. The obtained genomic DNA was treated with restriction enzymes, other than *Hind* III, of which sites were present in the pUC118 multicloning site (i.e., *Acc* I, *Hae* III, *Hinc* II *Kpn* I, *Pst* I, *Pvu* II *Sal* I, *Sau*3AI, *Sma* I, or *Xba* I) and verified via agarose gel electrophoresis (hereafter referred to as "E.P."). The restriction enzyme *Hind* III was excluded from the step because the *Hind* III site was found within the 68-bp sequence when it was used for analyzing T1 and thus use of *Hind* III was considered to be inappropriate for obtaining a target region. Based on the results of E.P., some enzymes of which bands remained at positions of high molecular weights equal to that of the genome were determined to be not appropriate because it appears that there are not a sufficient number of restriction enzyme reaction sites of the enzymes and thus resulting fragments are too long. Also, enzymes of which bands exhibiting low molecular weights were observed were also determined to be not appropriate because resulting fragments are too short and thus sequencing thereof would provide only a little sequence information. As a result, *Hinc* II that showed signals between 1,500-bp to 5,000-bp was selected. The genomic DNA was treated with *Hinc* II (Tol 5/*Hinc* II). Tol 5/*Hinc*II, which had been subjected to DNA purification, was ligated to cause self-ligation (self-cyclization) (Tol 5/*Hinc*II/circular). Subsequently, inverse PCR was carried out using the resultant as a template. Primers with the *Hinc* II linker were used herein for the ligation into a *Hinc* II-treated vector. The conditions used are described below. The resultant 5-kb PCR product was designated as Tol 5-5kb.

Inverse PCR conditions

Polymerase: KOD-plus-

Template DNA: Tol 5/*HincII*/circle

Primer F: T1-5kb R3

5 Primer R: T1-5kb RC

Annealing: 66°C

Extension: 5 minutes

The resultant PCR product was purified and treated with the restriction enzyme
10 *Hinc II*. This treatment is intended to shorten the fragment for the purpose of
improving the success rate of cloning and to cleave the linker with *Hinc II*. DNAs
were extracted from agarose gel so as to obtain separately samples of the fragments
(QIAquick Gel Extraction Kit). The resultant 2.8-kb fragment and 1.8-kb fragment
were designated as Tol 5-2.8kb and Tol 5-1.8kb, respectively. Tol 5-2.8kb and Tol 5-
15 1.8kb were ligated into vectors. Thereafter, transformation was carried out (via a
chemical procedure; blue/white colony selection). White colonies were picked up and
cultured in LB liquid medium. Thereafter, small-scale extraction of plasmid DNA was
performed. Plasmid DNA was treated with the restriction enzyme *Hinc II*, and
confirmed via E.P. As a result of restriction enzyme treatment, bands of the vector plus
20 2.8 kb and 1.8 kb were ideally obtained. The size and the orientation of the insert
fragment were verified via PCR. The conditions used are shown below.

PCR conditions

Polymerase: Blend Taq

25 Template DNA: Tol 5-1.8kb, Tol 5-2.8kb

Primer F: T1-5kb R3, pUC118F

Primer R: T1-5kb RC, pUC118R

Annealing: 62°C

Extension: 2 minutes

30

As a result of PCR, regarding Tol 5-2.8kb, a 2.8-kb fragment was amplified

with the use of the primer pair of pUC118R and T1-5kb R3 and, regarding Tol 5-1.8kb, a 1.8-kb fragment was amplified with the use of the primer pair of pUC118F and T1-5kb RC. Based on such results, the size of the inserted fragment was confirmed, and the orientation of fragment insertion was determined. These 2 strains were subjected to sequencing via a primer-walking method. However, the sequencer signal was disordered, and computational assembly also yielded the problem of inconsistent nucleotides at sites that were considered to be the overlapping regions. As a result of analysis, a plurality of long repeat sequences were found in the sequences. This also results in the problem that the lengths of the assembled sequences were less than those of the insert fragments of interest; i.e., 2.8 kb and 1.8 kb. Thus, primer designing and sequencing were repeated and, consequently, nucleotide sequences corresponding to the length of the insert fragments were determined. Because of such repeat sequences, however, reliability of the results was insufficient. In order to enhance the certainty of sequencing, accordingly, we attempted to directly clone Tol 5-5kb for sequencing it again. In order to perform TA cloning, Taq-based DNA polymerase Easy-A exhibiting high accuracy was used to perform PCR to amplify Tol 5-5kb. PCR conditions used are shown below.

PCR conditions

Polymerase: Easy-A High-Fidelity PCR Cloning Enzyme

Template DNA: Tol 5/*Hinc* II/circular

Primer F: T1-5kb R3-2

Primer R: T1-5kb RC-2

Annealing: 67.9°C

Extension: 5 minutes

TA cloning was carried out using the resultant PCR product. Transformation was carried out via a chemical procedure and the resultants were spread on LB/Km agar medium. Colonies were cultured in LB/Km liquid medium, and after culturing, plasmid DNA was extracted. In order to verify the insert, the DNA was treated with the restriction enzyme *Eco*RI. As a result, the existence of the vector and a 5-kb

fragment was observed. Further, the size of the insert fragment was confirmed via PCR. The conditions are shown below.

PCR conditions

5 Polymerase: Blend Taq

Template DNA: Tol 5-5kb plasmid No. 5

Primer F: pUC118F

Primer R: pUC118R

Annealing: 65°C to 71°C

10 Extension: 5 minutes

As a result of PCR, a 5-kb band of the insert was observed and it was subjected to sequence analysis. Sequencing was carried out by the primer walking method again, which was still very difficult because of the repeat structure. Based on the
15 aforementioned sequence information of the 1.8-kb and 2.8-kb fragments, primer designing and sequencing were carefully repeated, and a 4,907-bp sequence having a gigantic repeat structure was obtained as a result of a laborious and time-consuming procedure. The Tol 5-2.8kb sequence and the Tol 5-1.8kb sequence were completely consistent with the sequences of Tol 5-5kb. Thus, the accuracy of sequencing was
20 improved. The obtained Tol 5-5kb sequence is shown in SEQ ID NO: 7. Based on this sequence, the translated proteins were subjected to homology search via BLAST. As a result, a 4,026-bp ORF exhibiting 20% to 30% homology to the autotransporter adhesin, of which the sequence has been reported in other bacteria, was found, and transposon was found to have been inserted into this ORF. Thus, the existence of ORF
25 associated with high adhesive property and appendage production of the Tol 5 strain was demonstrated. This ORF was, however, discontinued at the 3' end of Tol 5-5kb, which necessitated further cloning of a downstream region following Tol 5-5kb, in order to elucidate the entire ORF. Even if autotransporter adhesin associated with bacteria adhesion was hit via homology search, homology was low, and the membrane anchor
30 domain at the carboxyl terminus, which defines this group, did not appear. Thus, it could have been another protein having a partially similar sequence.

In order to obtain sequential information of further downstream regions based on the resulting sequence, the southern hybridization was carried out in an attempt to obtain a target fragment. In order to prepare a DIG-labeled probe, primers were designed while avoiding the repeat portion. With the use of the primers and chromosomal DNA of the Tol 5 strain as a template, a target band of 160 bp was amplified. PCR conditions used are shown below.

PCR conditions

Polymerase: Blend Taq

Template DNA: Tol 5 genome

Primer F: Tol 5-ad-probe2F

Primer R: Tol 5-ad-probe2R

Annealing: 66°C to 72°C

Extension: 1 second

Since a plurality of bands were amplified, it was deduced that the probe sequence portions without repetition in the portion analyzed above have further repeats in a downstream region that had not been analyzed. In order to advance sequencing, the 160-bp target band alone was excised from it with the use of the QIAquick Gel Extraction Kit. The resultant was used as a template to prepare a probe via PCR. PCR conditions used are shown below.

PCR conditions

PCR DIG Probe Synthesis Kit

Template DNA: Tol 5 160bp probe Gel Extraction

Primer F: Tol 5-ad-probe2F

Primer R: Tol 5-ad-probe2R

Annealing: 67.9°C

Extension: 2 minutes

The products of digestion of chromosomal DNAs of the Tol 5 strain with a

variety of restriction enzymes were subjected to southern hybridization with the use of the resulting probes. As a result, signals representing probe hybridization were detected at 3 kb and 2.3 kb of the digestion product with the *Pst* I restriction enzyme. These two DNA fragments were excised from agarose gel and extracted (QIAquick Gel Extraction Kit). The resultant fragments were designated as Tol 5-3kb and Tol 5-2.3kb. The excised DNA was ligated to *Pst* I-digested pUC118. The resultant recombinant vector was used to transform *E. coli* DH5 α (a chemical procedure; blue/white colony selection). In addition, colony hybridization was carried out to select transformants into which such fragments had been introduced. As a result, signals were observed only in the colonies into which Tol 5-3kb had been introduced. Colonies in which signals were observed (i.e., Tol 5-CC, -C1, -C2, and -C3) were picked up and cultured in LB/Amp liquid medium, and small-scale extraction of plasmid DNA was performed. The resultants were each treated with the *Pst* I restriction enzyme. The resultants were subjected to southern hybridization, and signals were detected in Tol 5-CC, -C2, and -C3. These colonies were subjected to DNA sequencing. As a result of sequencing of the both ends of the inserts, there was no fragment, which was consistent with a known sequence. In addition, these sequences were different from each other. That is, cloning of a downstream region of the 5-kb region (Tol 5-5kb), the nucleotide sequence of which had been determined in advance, failed. The cause thereof could not be identified at this time. However, the present inventors considered that the problem existed due to the following point. That is, they had unwillingly selected a signal from among a plurality of signals detected via southern hybridization with the use of chromosomal DNA of the Tol 5 strain as a template, wherefrom the fragment containing such signal was excised, and then designed the probe based on the fragments. Thus, these clones were temporarily set aside, and acquisition of a fragment that would follow Tol 5-5kb was attempted.

pUC118::Tol 5-5kb plasmid DNA was used as a template instead of the Tol 5 chromosomal DNA to prepare a novel DIG-labeled probe via PCR. Since this plasmid contains only one probe region, it was considered that only one amplification product labeled with DIG is produced. PCR conditions used are shown below.

PCR conditions

PCR DIG Probe Synthesis Kit

Template DNA: pUC118::Tol 5-5kb plasmid DNA

Primer F: Tol 5-ad-probe2F

5 Primer R: Tol 5-ad-probe2R

Annealing: 68°C

Extension: 2 minutes

As expected above, only one amplified fragment having the target size (i.e., 160
10 bp) was found. New probes with high purity were successfully prepared. With the
use of this probe, the plates obtained after the transformation with the use of the
aforementioned vector comprising Tol 5-3kb and Tol 5-2.3kb were subjected to colony
hybridization. Colonies that were deduced to have emitted signals were picked up,
cultured on the plate, and then subjected to colony hybridization again for verification.
15 Colonies in which signals were detected (i.e., clones No. 3 (3 kb) and Nos. 6, 12, 13, 21,
and 22 (2.3 kb)) were picked up and cultured in LB/Amp liquid medium, and small-scale
extraction of plasmid DNA was performed. The resultant plasmid DNA was treated
with the *Pst* I restriction enzyme. As a result, plasmids were found to comprise target
fragments of 3 kb (No. 3) or 2.3 kb (Nos. 6, 12, 13, 21, and 22), and all such insert
20 fragments were found to have hybridized with the 160-bp probe. If these fragments
comprise downstream regions that follow Tol 5-5kb as expected, such fragments should
comprise a nucleotide sequence of about 1.5 kb sandwiched between the *Pst* I site and
the *Hinc* II site, as a region overlapping with Tol 5-5kb. If the aforementioned plasmid,
in which such fragment has been inserted into the *Pst* I site of the multicloning site, is
25 digested with the restriction enzyme *Hinc* II, accordingly, a DNA fragment of about 1.5
kb resulting from the addition of a very small part of the multicloning site to the 1.5-kb
overlapped fragment should be obtained. Plasmids Nos. 3, 6, and 22 were digested
with *Hinc* II and roughly-1.5-kb DNA fragments were obtained therefrom. However,
the sizes thereof were somewhat different from each other. Among them, Plasmid No.
30 6 were subjected to sequencing at first. Plasmid No. 6 was cut into a 3.3-kb fragment,
which is considered to comprise a 3.1-kb vector fragment, and a 600-bp fragment, in

addition to the 1.5-kb fragment. Based on the result of sequencing or southern hybridization that had been carried out previously, it was considered that sequencing of the plasmid may not be accurately conducted via primer walking, which is a well-established high-throughput technique at present, since the plasmid comprises many repeat sequences and thus may cause annealing to a plurality of sites. In addition, it was deduced to have problems in the computational sequence assembly. That is, difficulty, which was experienced in the case of Tol 5-5kb, might recur. Thus, production of a series of deletion mutants was to be carried out instead of primer walking, although such procedure is laborious and time-consuming. In order to prepare mutants by successively deleting the 2.3-kb Tol 5 DNA fragment inserted in No. 6 from the aforementioned 1.5-kb overlapping fragment, the plasmid was treated with *Sac* I for a 3' protruding end and *Xba* I for a 5' protruding end, followed by digestion with exonuclease III. By preparing a series of samples by differentiating the duration during which exonuclease III would be allowed to react, deletion products with different lengths of deletion were obtained. Thereafter, blunt ending with the use of Mung Bean Nuclease and end repair with the use of the Klenow fragment were further carried out, and the samples were subjected to blunt end ligation in the end to obtain a series of deletion plasmids. After purification via ethanol precipitation, the samples were treated with *Xba* I, which should have disappeared due to digestion with exonuclease III, to eliminate the influence by unreacted plasmids, and *E. coli* DH5a was transformed with this DNA to obtain a series of deletion mutants. Many transformed colonies were picked up and cultured, and the extracted plasmids were digested with the restriction enzyme *Hinc* II. By taking the size of a sequencing path (800 bp or longer) into consideration, deletion mutants were selected at adequate intervals, and they were sequenced. The 2,280-bp nucleotide sequence was determined by assembling the sequencing results. The determined sequence is shown in SEQ ID NO: 8 (Tol 5-No.6). Tol 5-No.6 comprises a sequence of about 1.5 kb overlapping with Tol 5-5kb, and thus a nucleotide sequence of about 750 bp following Tol 5-5kb was determined based thereon. Thus, ORF encoding to the aforementioned protein exhibiting low homology to the autotransporter adhesin was extended to as long as 4,707 bp, and the amino acid sequence of the protein encoded thereby contained 1,569 residues. However, the ORF

had not yet been terminated, and the sequence was discontinued at the 3' end. This necessitated further cloning of a downstream region following Tol 5-No.6. In addition, the sequence exhibited the highest homology to EmaA of *Actinobacillus actinomycetemcomitans*, but it was as low as 26% homology at the amino acid level.

5 Further, it had not yet reached the coding region of the membrane anchor domain. Thus, the corresponding protein was not concluded to be a protein of the TAA family. In any case, it was deduced that a protein encoded by the adhesion-associated gene, which had been destroyed via transposon insertion, was a novel adhesive protein which was very large and in which long-repeat sequences were arranged in a complex manner.

10 In order to obtain sequential information on the downstream following Tol 5-No.6, a 158-bp probe was produced via PCR using the plasmid containing Tol 5-No.6 as a template, which had been designed based on the sequence newly determined with avoiding a repeat sequence. PCR conditions used are shown below.

15 PCR conditions

PCR DIG Probe Synthesis Kit

Template DNA: pUC118::Tol 5-No.6

Primer F: Tol 5-ad-probe5F

Primer R: Tol 5-ad-probe6R

20 Annealing: 65°C

Extension: 2 minutes

The restriction enzyme *Hind* III site existed in a region immediately upstream of the synthesized probe region. Thus, chromosomal DNA of Tol 5 was digested with
25 *Hind* III, subjected to E.P., and then subjected to southern hybridization with the use of a 158-bp probe. As a result, three signals were observed again, all the signal portions were excised from agarose gel, and DNAs were extracted (using DEAE papers). The resultants were designated as Tol 5-B (2,500 bp), Tol 5-M (1,200 bp), and Tol 5-S (600 bp) in descending order of molecular sizes. The excised DNA fragments were ligated
30 to *Hind* III-treated pUC118. After the ligation, DNAs were purified and then transformed (electroporation; blue/white colony selection). The plates on which

colonies had been formed were subjected to colony hybridization with the use of the 158-bp probe mentioned above. Colonies in which signals were detected were picked up and cultured in LB/Amp liquid medium. Small-scale extraction of plasmid DNA was performed and it was treated with the restriction enzyme *Hind* III to verify the length of the insert fragment. As a result, insertion of DNA fragments of the strain Tol 5 of 2,500 bp, 1,200 bp, and 600 bp in length was confirmed, and these plasmids were subjected to sequencing. Since the M fragment and the S fragment were short, the nucleotide sequences of 1,185 bp and 603 bp were determined via single-path sequencing. Deletion mutants were prepared for sequencing of the B fragment. A plasmid containing the B fragment was treated with *Sac*I for a 3' protruding end and *Xba*I for a 5' protruding end and then digested with exonuclease III. By preparing a series of samples by differentiating the duration during which exonuclease III would be allowed to react, deletion products with different length of deletion were obtained. Thereafter, blunt ending with the use of Mung Bean Nuclease and end repair with the use of the Klenow fragment were carried out, and the samples were subjected to blunt end ligation in the end, to obtain a series of deletion plasmids. After purification via ethanol precipitation, the samples were digested with *Xba*I, which should have disappeared due to digestion with exonuclease III, to eliminate the influence by unreacted plasmids, and *E. coli* DH5 α was transformed with the resultant DNA to obtain a series of deletion mutants. Many transformed colonies were picked up and cultured, and the extracted plasmids therefrom were subjected to dual digestion with the restriction enzymes *Hind*III and *Eco*RI. By taking the size of a sequencing path (800 bp or longer) into consideration, deletion mutants were selected at adequate intervals and they were sequenced. The 2,540-bp nucleotide sequence of the B fragment was determined by assembling the sequencing results. The Tol 5-B, Tol 5-M, and Tol 5-S sequences determined are shown in SEQ ID NOs: 9 to 11. The Tol 5-M fragment comprised a 533-bp nucleotide sequence as an overlapping region that is 100% consistent with Tol 5-No.6. The *Hind*III fragments, Tol 5-B, -M, and -S fragments, did not comprise overlapping regions from each other, the -M fragment was deduced to follow Tol 5-No.6, but the positional relationship between the other 2 fragments was not found. These fragments comprised almost the same nucleotide sequence regions, and

the 158-bp probe region was located therein. That is, these fragments were also deduced to be parts of a gigantic fragment in which repeat sequences are formed. ORF was extended to as long as 5,358 bp because of the overlapping of the Tol 5-M fragment, and the amino acid sequence of the protein encoded thereby comprised 1,785 residues. However, ORF had not yet been terminated, and the sequence was discontinued at the 3' end. This necessitated further elucidation of the nucleotide sequence of a downstream region following the Tol 5-M fragment. Although the sequence exhibited the highest homology to EmaA of *Actinobacillus actinomycetemcomitans* again, which was as low as 25% homology at the amino acid level, it has not yet reached the coding region of the membrane anchor domain. Thus, whether or not the protein of interest belongs to the autotransporter adhesin family is not yet known.

Sequencing of the Tol 5 DNA fragments, i.e., Tol 5-CC and Tol 5-C2, which were obtained via southern hybridization in an attempt to clone the downstream region subsequent to Tol 5-5kb but were not found to follow Tol 5-5kb, was attempted again. Since these fragments were deduced to comprise repeat sequences, independent deletion mutants thereof were produced. After the plasmids were treated with *SacI* for a 3' protruding end and *XbaI* for a 5' protruding end, a series of deletion mutants were prepared in the same manner as described above, they were sequenced and assembled into a single sequence, and the nucleotide sequences of Tol 5-CC and Tol 5-C2 were determined. The determined Tol 5-C2 and Tol 5-CC nucleotide sequences are shown in SEQ ID NOs: 12 and 13, respectively.

The positional relationship was determined by analyzing sequence overlapping of the Tol 5-B, M, and S fragments and the Tol 5-CC and C2 fragments. Only the sequences exhibiting 100% identity were determined as overlapping sequences. Although three signals were detected via southern hybridization with the use of a 158-bp probe, the results of overlapping analysis and Tol 5-C2 analysis demonstrated that there were in fact four fragments. Since the fourth fragment had almost the same size and sequence as those of Tol 5-S, it existed while overlapping with a signal at around 600 bp. This region was designated as Tol 5-S'. Based on the above, it was found that Tol 5-M, Tol 5-S', Tol 5-S, Tol 5-C2, Tol 5-B, and Tol 5-CC fragments were located in that order while overlapping with each other. As a result, ORF was extended to as long as 10,798

bp, and the amino acid sequence of the protein encoded thereby comprised 3,598 residues. However, ORF was not terminated and discontinued at the 3' end yet. This necessitated further cloning of a downstream region following Tol 5-CC. The sequence exhibited the highest homology to TAA of *Burkholderia phymatum*, but it was as low as 25% homology at the amino acid level. It seemed to contain part of the membrane anchor domain sequence, but it did not comprise the entire sequence. Further, a higher-order structure of such portion could not be predicted. Thus, it could not be concluded that it belonged to the TAA family. Also, the size thereof was found to be much greater than that of BadA of *Bartonella henselae* (3,036 amino acids), which was the largest among members of the TAA family that had been previously reported.

As a result of sequencing that had been conducted as described above, no repeat sequences were observed in the C terminal region of Tol 5-CC. Thus, a 507-bp probe was produced in the C terminal region, and southern hybridization was carried out with the use of DNA of the Tol 5 strain treated with the restriction enzyme *Hind* III as a template. As a result, a signal was detected at 5 kb and this fragment was designated as Tol 5-C5kb. This DNA was excised from agarose gel and extracted (using the DEAE paper). The extracted DNA fragment was ligated to the *Hind* III-digested pUC118 vector and subjected to transformation (electroporation; blue/white colony selection). White colonies were picked up and cultured in LB/Amp liquid medium. Plasmid DNA was extracted from bacteria in liquid medium and treated with the restriction enzyme *Hind* III to verify the insert. It was deduced that Tol 5-C5kb did not contain a repeat structure, and the sequence was determined via primer walking. The determined Tol 5-C5kb sequence is shown in SEQ ID NO: 14. Tol 5-C5kb was found to overlap with the Tol 5-CC sequence and to be a downstream region following Tol 5-CC. As a result of connection of these fragments, a gigantic ORF as large as 10,893 bp (SEQ ID NO: 1) was completed, and a gigantic protein comprising 3,630 amino acids (SEQ ID NO: 2) encoded thereby was elucidated. This is the identity of the gene associated with adhesion which was destroyed via transposon insertion. The positional relationship of the sequence fragments described above is shown in Fig. 1. Thus, the 15,011-bp nucleotide sequence of the gene fragment of the Tol 5 strain comprising a gigantic ORF encoding the protein associated with adhesion was determined.

domain, a stalk domain, and a membrane anchor domain. Thus, multiple alignments were prepared for each domain with ClustalW, and the sequence comparisons were performed. The results are shown in Fig. 4. Regarding the membrane anchor domain, the results of prediction of the secondary structure with PredictProtein are also shown.

5 The members of the TAA family were compared. As a result, the sequences of the signal peptide, the head domain, and the neck domain were found to exhibit the highest homology to EmaA of *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, which were 34%, 44%, and 52%, respectively. In addition, such sequences exhibited 11%, 23%, and 40% homology, respectively, to YadA of *Yersinia enterocolitica*, which has

10 been the most studied protein in the TAA family and can be said to be a prototype of the TAA family. The membrane anchor domain exhibited 48% homology to autotransporter adhesin of *Haemophilus somnus* and 25% homology to YadA. The stalk domain composed of 2,591 amino acids was found to exhibit partial homology to a member of the TAA family via BLAST analysis, although the homology was low. For

15 example, the stalk domain exhibited the highest homology to the YadA-like protein of *Actinobacillus succinogenes*; however, a region composed of only 800 out of 2,591 amino acids exhibited 33% homology to the protein of interest. Thus, a protein of the TAA family was demonstrated to have homology to the domains of the gigantic protein via BLAST analysis, although such homology was not sufficiently high. The most

20 characteristic feature of the TAA family is a conformation of the membrane anchor domain. TAA is a homotrimer, a polypeptide strand has an α helix and 4 β strands, 3 sets of such strands collectively form 12 β strands, the β barrel structure is formed in the outer membrane of Gram-negative bacteria, and it functions as a tunnel for protein secretion. The secondary structure was deduced based on the amino acid sequence of

25 the membrane anchor-equivalent region of the adhesion-associated protein of Tol 5, and it was deduced to have 1 α helix and 4 β strands. Based on such deduction and the domain alignment, the adhesion-associated protein of the Tol 5 strain was deduced to be a novel protein of the TAA family, and it was designated as AadA, representing an *Acinetobacter* adhesin. The nucleotide sequence of the *aadA* gene is shown in SEQ ID

30 NO: 1 and the amino acid sequence of AadA is shown in Fig. 3 and SEQ ID NO: 2. AadA is composed of 3,630 amino acids encoded by a 10,893-bp gene. AadA is a

unique member of the TAA family, which have been reported in the past, due to the points below. That is, it is much greater than proteins of the TAA family that have been already reported, the stalk domain comprising a plurality of long-repeat structures that are arranged in a complex manner has low homology to other proteins, and the head domain and the neck domain exist at sites located more closely to the carboxyl terminus at which the membrane anchor domain exists in addition to the amino terminus. Whether or not such specific properties yield high adhesive property or nonspecific adhesive property of the Tol 5 strain is not yet known at present. However, TAA proteins that have been reported in the past function for specifically adhering to host cells or extracellular matrices when pathogenic bacteria infect, and such proteins do not exhibit high adhesive property.

As a result of a homology search of ORF, an ORF of a protein exhibiting homology to the outer membrane protein OmpA of other bacteria of the genus *Acinetobacter* was found immediately downstream of the *aadA* gene. It was designated as Tol 5-OmpA. The nucleotide sequence of Tol 5-OmpA is shown in SEQ ID NO: 3, and the amino acid sequence thereof is shown in SEQ ID NO: 4. Tol 5-OmpA consists of 264 amino acids encoded by the 795-bp gene. A multiple alignment was prepared using ClustalW, and it was compared with the sequences of the outer membrane proteins exhibiting homology to the Tol 5-OmpA sequence. The results are shown in Fig. 5. In comparison with OmpA of *E. coli*, it exhibits high homology in the C terminal region but exhibits low homology in the N terminal region. In contrast, it exhibits high homology to the entire outer membrane protein of bacteria of the genus *Acinetobacter*. However, functions thereof have not been reported. Since it is adjacent to ORF of AadA, *aadA* and Tol 5-*ompA* are highly likely to exist on the same operon. Since it is an outer membrane protein, Tol 5-*ompA* may also be associated with adhesion or production of adhesive appendages.

An ORF at the 5' end of the 15,011-bp gene fragment of the Tol 5 strain, the nucleotide sequence of which has been determined, exhibited high homology to methionyl-tRNA formyltransferase of, for example, *Acinetobacter* sp. ADP1. An ORF at the 3' end exhibited high homology to dihydroxyacid dehydratase of, for example, *Acinetobacter* sp. ADP1. Since there is no report that such proteins have functions

associated with adhesion, these proteins were deduced to have no association with adhesion in *Acinetobacter* sp. Tol 5.

1-5: Cloning of full-length AadA gene

In order to verify that sequencing had been carried out accurately, full-length *aadA* comprising a putative promoter site was amplified via PCR, and the length thereof was confirmed. The Tol 5-TKD-F and Tol 5-TKD-R primer regions are shown below.

Primer Tol 5-TKD-F:

5'-AATATAGCTTAAATTTCAAAAAAATTAACCAATTGGTTTAAAAGTTAAAAAA
AGTGAAATATATCTCATTTTTTTTGATTGCTTTAATTGTATGTAAATTGTTAAATAA
10 AAAAAATTGTACATTTTATATGCATTGCTAAAGCAGAACCTACTGCCCAAATG
CATCTCCTAAGGAAAAGCGATATGAATAAAATCTACAAAGTGATTTGGAATGCG
ACTTTGTTGGCATGGG-3'

Primer Tol 5-TKD-R (complement):

5'--GGCGGTGTAGCTGCAGCGTCTCAAGGCGATGCAAGTGTTTCGTATCGGTATC
15 AGCGGTGTGATTGACTAATTCACCTCGACAGGGAAGATCTTCGGGTCTTCCTTTT
TCTTCGAAAATTTTTTAAGAGAGAAAAAATGAAAGCATTTAACAAAAAAATTA
TGTTTGGTGTATTCAGCGGTCTTGTGATGTCATTGAGCCATGCTGCTGAAGTCG
AAAGTGCAAATACGCAAGAA--3'

PCR conditions used are as follows.

PCR conditions

Polymerase: Easy-A High-Fidelity PCR Cloning Enzyme

Template DNA: Tol 5 genome

Primer F: Tol 5-TKD-F

25 Primer R: Tol 5-TKD-R

Annealing: 55°C to 64.5°C

Extension: 11 minutes

As a result of PCR, a band corresponding to a 11,145-bp sequence (about 11 kb) obtained via cloning and sequencing was obtained, and it was designated as Tol 5-11kb. Subsequently, TA cloning was carried out using the product. Transformation was

carried out via electroporation, and the resultants were spread on LB/Km agar medium. The formed colonies were cultured in LB/Km liquid medium, and after culturing plasmid DNA was extracted. DNA was treated with the *EcoR* I restriction enzyme in order to verify the insert. It was further confirmed via PCR. The conditions used are shown below.

PCR conditions

Polymerase: Blend Taq

Template DNA: pCR-XL-TOPO::Tol 5-11kb plasmid

Primer F: pUC118F

Primer R: pUC118R

Annealing: 56°C to 68°C

Extension: 11 minutes

As a result of restriction enzyme treatment, a vector and a band ideal for *aadA* were clearly observed. Also, a band having the size of *aadA* was observed as a result of PCR. As a result of sequencing of plasmid DNA, further, the sequences at the both ends of the inserted fragment were completely consistent with the nucleotide sequence, which had been determined in advance. Thus, sequencing accuracy was verified, and the *aadA* gene (about 11 kb) comprising a putative promoter sequence was satisfactorily cloned.

Further, cloning of a genetic region of about 12,505 bp comprising a putative promoter, *aadA*, and Tol 5-*ompA* was performed. The fragment was amplified via PCR. The conditions used are as follows.

PCR conditions

Polymerase: Easy-A High-Fidelity PCR Cloning Enzyme

Template DNA: Tol 5 genome

Primer F: Tol 5-TKD-F

Primer R: Tol 5-ad-op-R3

Annealing: 50°C to 60°C

Extension: 14 minutes

The primer Tol 5-ad-op-R3 region used is shown below.

5'--TATGATGTGTACATATTTCTGACTGATTTATTGCTATATCAGTTTTATTAGCCA
5 GAGTGAATCTGATTCATTTCAAGCTCAAACAATGTNGGAAATACAAATGCCNG
ACTATCGTTCAAAAACATCGACACATGGAAGAAATATGGCTGGTGCACGTGGC
TTATGGCGTGCAAC--3'

The obtained fragment of about 13 kb was designated as Tol 5-13kb and it was
10 subjected to TA cloning. Transformation was carried out via electroporation, and the
resultants were spread on LB/Km agar medium. The colonies were cultured in LB/Km
liquid medium and after culturing plasmid DNA was extracted. DNA was treated with
the restriction enzyme *EcoRI* in order to verify the insert. It was further confirmed via
PCR. The conditions used are shown below.

15 PCR conditions

Polymerase: Blend Taq

Template DNA: pCR-XL-TOPO::Tol 5-13kb plasmid

Primer F: pUC118F

20 Primer R: pUC118R

Annealing: 50°C to 60°C

Extension: 14 minutes

As a result of restriction enzyme treatment and PCR, a band of the insert having
25 a theoretical length was observed. This plasmid DNA was subjected to sequencing and,
consequently, the sequences of the both ends of the inserted fragment were found to be
completely consistent with known sequences. Thus, a genetic region comprising a
putative promoter, *aadA*, and Tol 5-*ompA*; i.e., the genetic region of about 13 kb
comprising the 11,858 bp *aadA-ompA* operon (SEQ ID NO: 5), was successfully cloned.
30 In the nucleotide sequence as shown in SEQ ID NO: 5, a region comprising nucleotides
1 to 106 is a promoter-ribosome binding region (SEQ ID NO: 29), a region comprising

nucleotides 107 to 10,999 is the *aadA* gene, and a region comprising nucleotides 11,064 to 11,858 is the Tol 5-*ompA* gene.

Example 2: Property evaluation of *E. coli* into which the adhesion-associated gene of the

5 *Acinetobacter* sp. Tol 5 strain has been introduced

Properties of the *E. coli* DH5 α strains transformed with vectors into which the *aadA* gene and the *aadA-ompA* operon have been inserted (such strains being designated as DH5 α ::*aadA* and DH5 α ::*aadA-ompA*) were compared with those of the wild-type *E. coli* DH5 α strain (WT) as a control. The *E. coli* DH5 α strain transformed with a vector
10 into which the *aadA-ompA* operon had been inserted was accepted by the Patent Microorganisms Depositary, the Department of Biotechnology, National Institute of Technology and Evaluation, Incorporated Administrative Agency (2-5-8 Kazusakamatari, Kisarazu-shi, Chiba, Japan) as "DH5 α -XLTOPO::*aadA-ompA*" under the provisional accession number: NITE ABP-490 (date of accession: February 19, 2008).

15 2-1: Morphological observation using optical microscope

In order to observe morphological changes resulting from introduction of the *aadA* gene and the *aadA-ompA* operon, 3 types of strains (i.e., DH5 α ::*aadA*, DH5 α ::*aadA-ompA*, and DH5 α WT strains) were subjected to Gram staining, and the stained strains were observed under an optical microscope. The results are shown in Fig. 6.

20 In the DH5 α ::*aadA-ompA* strain into which the *aadA-ompA* operon had been introduced, filamentous structures that link a bacterial cell to other cells were observed. The filamentous substances that look like extracellular polymers (EPS) are considered to be associated with the addition of adhesion. In the DH5 α ::*aadA* strain into which *aadA* had been introduced, bacterial cells were long and thin, and the cells were linked to each
25 other at their poles. Both transgenic strains were apparently different from the wild-type strain in terms of morphology, which indicates morphological changes resulting from introduction of the adhesion-associated gene.

2-2: Morphological observation using electron microscope

In order to observe morphological changes resulting from gene introduction in
30 greater detail, 3 types of strains (i.e., DH5 α ::*aadA*, DH5 α ::*aadA-ompA*, and DH5 α WT strains) were subjected to morphological observation under a scanning electron

microscope (FE-SEM) and under a transmission electron microscope (TEM). The results of observation via FE-SEM are shown in Figs. 7 to 9.

In the DH5 α ::*aadA* strain, polyurethane carrier surfaces to which bacterial cells had adhered were clearly distinguishable from polyurethane carrier surfaces to which the cells had not adhered. The cells autoagglutinated and seemed to adhere to the surfaces in the aggregated forms. In the aggregates, large quantities of EPS-like structures were produced and bacterial cells were stacked up and had adhered to each other throughout multiple layers. In addition, relatively large quantities of DH5 α ::*aadA* cells elongated into long and thin shapes, which is consistent with the image, obtained as a result of optical microscopic observation. In the enlarged image, portions that look like joints were observed among the long and thin shapes, and the bacterial cells were linked each other at their poles. Further, structures that look like appendages were observed in the bacterial cells that had elongated into the long and thin shapes (Fig. 7).

In the DH5 α ::*aadA-ompA* strain, numerous bacterial cells appeared to have adhered to polyurethane carriers. It seems that each of the bacterial cells adheres to a carrier in a manner such that a uniform monolayer film is formed on the carrier surface. In the DH5 α ::*aadA-ompA* strain, production of EPS-like structures was also observed. This is consistent with the results of optical microscopic observation. In the DH5 α ::*aadA-ompA* strain, many substances that look like appendages linking bacterial cells to the carrier were observed (Fig. 8).

Unlike the two recombinant strains, there were substantially no *E. coli* DH5 α WT cells that had adhered to polyurethane carriers, and the cells appeared to have "ridden by accident" rather than "adhered" to the polyurethane carriers. Also, only a small quantity of the cells produced a structure such as an appendage (Fig. 9).

Thus, introduction of the *aadA* gene and the *aadA-ompA* operon of the *Acinetobacter* sp. Tol 5 strain into the *E. coli* DH5 α strain resulted in changes in the form of adhesion.

Further, differences were observed between adhesion forms of DH5 α ::*aadA-ompA* and DH5 α ::*aadA*. In the DH5 α ::*aadA-ompA* strain, while each bacterial cell independently adhered to a carrier and formed a uniform layer, a multilayer was not formed via agglutination of the cells. In contrast, in the DH5 α ::*aadA* strain, bacterial

cells formed gigantic aggregates and adhered to the carrier in the form of a multilayer, but each cell did not independently adhere to the carrier.

2-3: Adhesion test and agglutination test

In order to examine changes in adhesion resulting from introduction of the *aadA* gene and the *aadA-ompA* operon, an adhesion test via crystal violet staining (the CV adhesion test) was carried out. The adhesion test was evaluated in the following manner.

<Adhesion test>

1. A bacterial liquid culture is centrifuged at 3,400 rpm and room temperature for 10 minutes.

2. A medium is removed via decantation, and an adequate amount of BS medium for the MATS test is added.

3. Ultrasonication is carried out with the use of UD-200 (TOMY) under the conditions of OUTPUT= 5 and TIME= 20 seconds to suspend bacterial cells, followed by adjustment of OD₆₆₀ to approximately 0.2.

4. The suspension is added to each well of a 48-well microplate (Iwaki) in amounts of 1 ml each, and incubated at 37°C for 2 hours.

5. The suspension in the wells is completely pipetted out. In this case, care should be taken so as not to scrape the plate with the end of a chip.

6. Wells are washed with 1 ml of BS medium for the MATS test, and the plate is then air dried.

7. 1% Crystal violet is added to the wells, and incubated at room temperature for 15 minutes.

8. 1% Crystal violet is completely pipetted out, the wells are washed twice with 1 ml of BS medium for the MATS test, and the plate is then air dried.

9. 1 ml of BS medium for the MATS test is added, and ultrasonication is carried out under the conditions of OUTPUT= 4 and TIME= 20 seconds to disperse stained bacteria in BS medium.

10. A₅₉₀ is measured.

The results of the adhesion test of bacteria cultured in M9 medium are shown below.

Samples	A ₅₉₀
13kb	0.226
11kb	0.173
WT	0.081

5 *E. coli* into which the adhesion-associated gene had been introduced exhibited improved adhesiveness to a solid surface compared with a wild-type strain. The results demonstrate that introduction of the adhesion-associated gene confers the adhesive property to bacteria with low adhesiveness. In addition, the results demonstrate that the *aadA* gene and the *aadA-ompA* operon obtained herein are associated with the
10 adhesion of microorganisms.

Differences were observed among culture broth of the strains (Fig. 10). While the liquid culture of the wild-type strain was homogeneously clouded, the DH5 α ::*aadA-ompA* cells and the DH5 α ::*aadA* cells aggregated to the extent that such agglutination could be visually observed, and the culture solutions were clear.

15 Agglutination tests were performed in order to quantitate autoagglutination. The agglutinating property was evaluated in the following manner.

<Autoagglutination test>

1. The bacterial culture broth was centrifuged and the culture supernatant was
20 removed.

2. An inorganic salt medium was added to the bacterial pellet and cell suspension was obtained via ultrasonication.

3. The turbidity (OD₆₆₀) of the bacterial suspension was adjusted to a constant level with a medium. The OD₆₆₀ value at this time was defined as the initial OD₆₆₀
25 value.

4. The cell suspension in a glass centrifuge tube was allowed to stand at room temperature for sedimentation of the formed aggregates of cells, and changes over time

in the OD₆₆₀ value of the supernatant were measured.

5. The percentage of autoagglutination (aggregation) was determined by the following equation.

$$5 \quad \text{Aggregation (\%)} = \frac{\text{initial OD660} - \text{OD660 after sampling}}{\text{initial OD660}} \times 100$$

The results are shown in Fig. 11.

As shown in the chart, autoagglutinating property of DH5 α ::*aadA* was improved compared with that of DH5 α WT. The results are consistent with the clarity of the liquid culture and the results of FE-SEM observation. It was thus demonstrated that introduction of the *aadA* gene would also provide autoagglutinating property to bacterial cells.

All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

CLAIMS

1. A method for providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a target microorganism, comprising introducing into the target microorganism a DNA encoding autotransporter adhesin from a microorganism having nonspecific adhesive property.

2. The method according to claim 1, wherein the microorganism having nonspecific adhesive property is a bacterium of the genus *Acinetobacter*.

3. The method according to claim 1 or 2, wherein the DNA encoding autotransporter adhesin is the following DNA (a), (b), or (c):

(a) a DNA comprising the nucleotide sequence as shown in SEQ ID NO: 1;

(b) a DNA comprising a nucleotide sequence having 70% or more homology to the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism; or

(c) a DNA consisting of a part of the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism.

4. The method according to any one of claims 1 to 3, wherein the target microorganism is a bacterium of the genus *Escherichia*.

5. The method according to claim 4, wherein the bacterium of the genus *Escherichia* is *Escherichia coli*.

6. The method according to any one of claims 1 to 5, wherein the DNA encoding autotransporter adhesin is introduced with the following DNA (a) or (b):

(a) a DNA comprising the nucleotide sequence as shown in SEQ ID NO: 3; or

(b) a DNA comprising a nucleotide sequence having 90% or more homology to

the nucleotide sequence as shown in SEQ ID NO: 3.

7. The method according to claim 6, wherein the following DNA (a) or (b) is introduced into the target microorganism:

- 5 (a) a DNA comprising the nucleotide sequence as shown in SEQ ID NO: 5; or
 (b) a DNA comprising a nucleotide sequence having 70% or more homology to the nucleotide sequence as shown in SEQ ID NO: 5 and having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a host microorganism.

10

8. A DNA of the following (a), (b), or (c):

- (a) a DNA comprising the nucleotide sequence as shown in SEQ ID NO: 1;
 (b) a DNA comprising a nucleotide sequence having 70% or more homology to the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having
15 activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism; or
 (c) a DNA consisting of a part of the nucleotide sequence as shown in SEQ ID NO: 1 and encoding a protein having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism.

20

9. A microorganism into which the DNA according to claim 8 has been introduced, for which nonspecific adhesive property and/or autoagglutinating property have been provided or enhanced.

- 25 10. The microorganism according to claim 9, which is a bacterium of the genus *Escherichia*.

11. The microorganism according to claim 10, which is *Escherichia coli*.

12. A protein of the following (a), (b), or (c):

5 (a) a protein comprising the amino acid sequence as shown in SEQ ID NO: 2;

(b) a protein comprising an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 2 by deletion, substitution, insertion, or addition of one or several amino acids and having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism; or

10 (c) a protein consisting of a part of the amino acid sequence as shown in SEQ ID NO: 2 and having activity of providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a microorganism.

13. A method for culturing a microorganism, comprising causing the
15 microorganism according to claim 9, 10 or 11 to adhere to a carrier and/or aggregate to recover the microorganism.

14. A method for production of chemical products using a microorganism wherein the microorganism according to claim 9, 10 or 11 is used.

20

15. Use of the microorganism according to claim 9, 10 or 11 in the production of chemical products using a microorganism.

METHOD AND GENE FOR PROVIDING OR ENHANCING NONSPECIFIC
ADHESIVE PROPERTY AND/OR AUTOAGGLUTINATING PROPERTY
FOR MICROORGANISMS

ABSTRACT

This invention provides a means for providing or enhancing nonspecific adhesive property and/or autoagglutinating property for microorganisms with weak or
5 without nonspecific adhesive property or autoagglutinating property. This invention relates to a method for providing or enhancing nonspecific adhesive property and/or autoagglutinating property for a target microorganism, comprising introducing into the target microorganism a DNA encoding autotransporter adhesin from a microorganism having nonspecific adhesive property.

(FIG. 1)

SEQUENCE LISTING

<110> National University Corporation Nagoya Institute of Technology

<120> Method and Gene Providing or Increasing Nonspecific Adhesive
Property and/or Agglomerating Property for Microorganisms

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<170> PatentIn version 3.4

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 <211> 3630
 <212> PRT
 <213> Acinetobacter sp.

<400> 2

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Val Ala Val Ser Glu Leu Ala Lys Gly Lys Thr Lys Ser Thr Thr Ser
 20 25 30

Lys Ser Lys Ala Lys Ser Leu Ser Ser Ser Val Ile Val Gly Gly Ile
 35 40 45

Ile Leu Thr Thr Pro Leu Ser Leu Ile Ala Ala Thr Val Gln Val Gly
 50 55 60

Gly Gly Thr Asn Ser Gly Thr Thr Ala Thr Ala Ser Thr Asn Cys Ala
 65 70 75 80

Asp Leu Tyr Asn Tyr Gln Asn Pro Glu Asn Ser Gly Ser Gly Ala Ala
 85 90 95

Gly Asn Tyr Asn Ala Gly Asn Pro Ser Val Cys Ser Ile Ala Ile Gly
 100 105 110

Glu Asn Ala Gln Gly Gly Thr Ser Gly Thr Gly Gly Ser Pro Gly Ile
 115 120 125

Ala Ile Gly Gly Asn Ser Lys Ala Thr Gly Gly Leu Ser Val Ala Ile
 130 135 140

Gly Gly Tyr Ala Gln Ala Thr Asn Val Gly Ser Ile Ala Leu Gly Thr
 145 150 155 160

Ala Ala Leu Ser Ser Gly Phe Asn Ser Leu Ala Ile Ser Arg Gln Ala
 165 170 175

Ala Ala Thr Asn Asn Tyr Ser Ile Ala Ile Gly Thr Thr Ser Val Ser
 180 185 190

Lys Gly Val Gly Ser Ile Ala Met Gly His Ser Thr Asn Ala Ser Gly
 195 200 205

Asp Gln Ser Ile Ala Ile Gly Ser Ser Asp Ala Val Asn Ser Ala Thr
 210 215 220

Ala Thr Thr Thr Tyr Asp Gly Thr Thr Asn Thr Gln Ala Ser Gly Ser
 225 230 235 240

Lys Ser Ile Ala Ile Gly Ala Ser Ala Lys Ala Ser Thr Asn Asn Ser
 245 250 255

Ile Ala Leu Gly Ala Gly Ser Val Thr Ser Ala Gln Ser Gly Asn Ser
 260 265 270

Tyr Leu Thr Gly Val Gly Ala Ser Ala Thr Asn Gly Val Val Ser Val
 275 280 285

Gly Thr Ser Thr Ala Thr Arg Arg Ile Gln Asn Val Ala Asp Gly Ser
 290 295 300

Ala Ala Ser Asp Ala Val Thr Val Ala Gln Leu Asp Lys Ala Tyr Asp
 305 310 315 320

Asp Thr Asn Gly Arg Leu Ala Ala Ala Leu Gly Thr Gly Ser Gly Ala
 325 330 335

Ala Tyr Asn Ala Ala Asn Asn Thr Tyr Thr Ala Pro Thr Asn Ile Gly
 340 345 350

Gly Thr Gly Lys Asn Thr Ile Asp Asp Ala Ile Lys Ala Thr Gln Arg
 355 360 365

Ser Val Val Ala Gly Ser Asn Ile Val Val Thr Pro Thr Thr Ala Ser
 370 375 380

Asp Gly Ser Ile Ser Tyr Ser Val Ala Thr Ser Ala Thr Pro Thr Phe
 385 390 395 400

Thr Ser Ile Thr Val Asn Asn Ala Pro Thr Ala Gly Thr Asp Ala Thr
 405 410 415

Asn Lys Thr Tyr Val Asp Ser Lys Ala Ala Ala Ser Arg Thr Glu Val
420 425 430

Ala Ala Gly Ser Asn Val Ser Gly Val Val Lys Thr Thr Gly Ala Asn
435 440 445

Gly Gln Asp Val Tyr Thr Val Asn Ala Asn Gly Thr Thr Ala Ser Ala
450 455 460

Gly Ser Ser Ala Val Thr Val Thr Pro Gly Thr Lys Asp Ala Asn Asn
465 470 475 480

Val Thr Asp Tyr Lys Val Asp Leu Ser Ala Thr Thr Lys Thr Asp Ile
485 490 495

Gln Lys Gly Val Asp Ala Lys Asn Ala Val Asp Thr Ala Gly Leu Lys
500 505 510

Phe Lys Gly Asp Thr Ala Thr Thr Ser Asn Thr Lys Lys Leu Gly Asp
515 520 525

Thr Val Ser Ile Thr Gly Asp Thr Asn Ile Ser Thr Val Ala Thr Thr
530 535 540

Asp Gly Val Gln Val Lys Leu Asn Pro Asn Leu Asp Leu Gly Ala Thr
545 550 555 560

Gly Ser Val Lys Thr Gly Asn Thr Thr Ile Asn Asn Ala Gly Val Thr
565 570 575

Ala Asp Gln Val Thr Val Gly Gly Val Val Ile Asn Asn Thr Ser Gly
580 585 590

Ile Asn Ala Gly Gly Lys Ala Ile Thr Asn Val Ala Ala Pro Thr Asn
12/76

595	600	605
Asn Thr Asp Ala Ala Asn Lys Lys Tyr Val Asp Asp Ala Gly Thr Ala		
610	615	620
Leu Thr Asn Leu Gly Phe Gly Leu Lys Ala Gln Asp Gly Thr Thr Val		
625	630	635 640
Asn Lys Lys Leu Gly Glu Ala Val Asp Ile Val Gly Ser Asn Ser Asn		
645	650	655
Ile Ser Thr Lys Val Asn Ala Gly Lys Val Glu Val Ala Leu Ser Asn		
660	665	670
Thr Leu Asp Leu Gly Thr Thr Gly Ser Val Thr Thr Gly Ser Thr Val		
675	680	685
Ile Asn Asn Thr Gly Val Thr Ala Thr Gln Val Thr Ala Asn Lys Val		
690	695	700
Thr Ile Asn Asn Ala Pro Thr Ala Gly Thr Asp Ala Thr Asn Lys Thr		
705	710	715 720
Tyr Val Asp Ser Lys Ala Ala Ala Ser Arg Thr Glu Val Ala Ala Gly		
725	730	735
Ser Asn Val Ser Gly Val Val Lys Thr Thr Gly Ala Asn Gly Gln Asp		
740	745	750
Ile Tyr Ala Val Asn Ala Asn Gly Thr Thr Ala Ser Ala Gly Ser Ser		
755	760	765
Ala Val Thr Val Thr Pro Gly Thr Lys Asp Ala Asn Asn Val Thr Asp		
770	775	780

Tyr Lys Val Asp Leu Ser Ala Thr Thr Lys Thr Asp Ile Gln Lys Gly
785 790 795 800

Val Asp Ala Lys Asn Ala Val Asp Thr Ala Gly Leu Lys Phe Lys Gly
805 810 815

Asp Thr Ala Thr Thr Ser Asn Thr Lys Lys Leu Gly Asp Thr Val Ser
820 825 830

Ile Thr Gly Asp Thr Asn Ile Ser Thr Val Ala Thr Thr Asp Gly Val
835 840 845

Gln Val Lys Leu Asn Pro Asn Leu Asp Leu Gly Ala Thr Gly Ser Val
850 855 860

Lys Thr Gly Asn Thr Thr Ile Asn Asn Ala Gly Val Thr Ala Asp Gln
865 870 875 880

Val Thr Val Gly Gly Val Val Ile Asn Asn Thr Ser Gly Ile Asn Ala
885 890 895

Gly Gly Lys Ala Ile Thr Asn Val Ala Ala Pro Thr Asn Asn Thr Asp
900 905 910

Ala Ala Asn Lys Lys Tyr Val Asp Asp Ala Gly Thr Ala Leu Thr Asn
915 920 925

Leu Gly Phe Gly Leu Lys Ala Gln Asp Gly Thr Thr Val Asn Lys Lys
930 935 940

Leu Gly Glu Ala Val Asp Ile Val Gly Ser Asn Ser Asn Ile Ser Thr
945 950 955 960

Lys Val Asn Ala Gly Lys Val Glu Val Ala Leu Ser Asn Thr Leu Asp
 965 970 975

Leu Gly Thr Thr Gly Ser Val Thr Thr Gly Ser Thr Val Ile Asn Asn
 980 985 990

Ala Gly Val Thr Ala Thr Gln Val Thr Ala Asn Lys Val Thr Val Asn
 995 1000 1005

Asn Ala Pro Thr Ala Gly Thr Asp Ala Thr Asn Lys Thr Tyr Val
 1010 1015 1020

Asp Ser Lys Ala Ala Ala Ser Arg Thr Glu Val Ala Ala Gly Ser
 1025 1030 1035

Asn Val Ser Gly Val Val Lys Thr Thr Gly Ala Asn Gly Gln Asp
 1040 1045 1050

Val Tyr Thr Val Asn Ala Asn Gly Thr Thr Ala Ser Ala Gly Ser
 1055 1060 1065

Ser Ala Val Thr Val Thr Pro Gly Thr Lys Asp Ala Asn Asn Val
 1070 1075 1080

Thr Asp Tyr Lys Val Asp Leu Ser Ala Thr Thr Lys Thr Asp Ile
 1085 1090 1095

Gln Lys Gly Val Asp Ala Lys Asn Ala Val Asp Thr Ala Gly Leu
 1100 1105 1110

Lys Phe Lys Gly Asp Thr Ala Thr Thr Ser Asn Thr Lys Lys Leu
 1115 1120 1125

Gly Asp Thr Val Ser Ile Thr Gly Asp Thr Asn Ile Ser Thr Val
 1130 1135 1140

Ala Thr Thr Asp Gly Val Gln Val Lys Leu Asn Pro Asn Leu Asp
 1145 1150 1155

Leu Gly Ala Thr Gly Ser Val Lys Thr Gly Asn Thr Thr Ile Asn
 1160 1165 1170

Asn Ala Gly Val Thr Ala Asp Gln Val Thr Val Gly Gly Val Val
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Ile Asn Asn Thr Ser Gly Ile Asn Ala Gly Gly Lys Ala Ile Thr
 1190 1195 1200

Asn Val Ala Ala Pro Thr Asn Asn Thr Asp Ala Ala Asn Lys Lys
 1205 1210 1215

Tyr Val Asp Asp Ala Gly Thr Ala Leu Thr Asn Leu Gly Phe Gly
 1220 1225 1230

Leu Lys Ala Gln Asp Gly Thr Thr Val Asn Lys Lys Leu Gly Glu
 1235 1240 1245

Ala Val Glu Val Val Gly Ala Asp Ser Asn Ile Thr Thr Lys Val
 1250 1255 1260

Ala Gly Gly Gln Val Ala Ile Glu Leu Asn Lys Asn Leu Asn Asn
 1265 1270 1275

Leu Thr Gly Ile Thr Val Asn Asp Gly Thr Asn Gly Thr Asn Gly
 1280 1285 1290

Ser Thr Val Ile Gly Lys Asp Gly Ile Ser Val Lys Asp Gly Ser
 16/76

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Gly Asn Thr Ile Ala Gly Val Asp Asn Thr Ala Leu Thr Val Lys		
1310	1315	1320
Asp Gly Ser Gly Asn Thr Glu Thr Ser Ile Asn Gln Ala Ile Asn		
1325	1330	1335
Thr Leu Asn Ala Ala Gln Gly Glu Thr Asp Lys Phe Ala Val Lys		
1340	1345	1350
Tyr Asp Lys Asn Ala Asp Gly Ser Val Asn Tyr Asn Asn Ile Thr		
1355	1360	1365
Leu Ala Gly Thr Thr Ala Ser Ser Thr Gln Asp Ala Thr Thr Gly		
1370	1375	1380
Lys Ile Thr Thr Thr Gly Gly Thr Ser Leu Asn Asn Val Ala Ser		
1385	1390	1395
Ala Gly Asp Tyr Lys Asp Val Ala Asn Ala Ser Lys Gly Val Asn		
1400	1405	1410
Ala Gly Asp Leu Asn Asn Ala Val Val Asp Ala Thr Asn Ala Ala		
1415	1420	1425
Thr Ser Lys Gly Phe Ala Leu Gln Ala Ala Asp Gly Ala Lys Val		
1430	1435	1440
Gln Lys Asn Leu Gly Glu Ala Val Glu Val Val Gly Ala Asp Ser		
1445	1450	1455
Asn Ile Thr Thr Lys Val Ala Gly Gly Gln Val Ala Ile Glu Leu		
1460	1465	1470

Asn Lys Asn Leu Asn Asn Leu Thr Gly Ile Thr Val Asn Asp Gly
1475 1480 1485

Thr Asn Gly Thr Asn Gly Ser Thr Val Ile Gly Lys Asp Gly Ile
1490 1495 1500

Ser Val Lys Asp Gly Ser Gly Asn Thr Ile Ala Gly Val Asp Asn
1505 1510 1515

Thr Ala Leu Thr Val Lys Asp Gly Ser Gly Asn Thr Glu Thr Ser
1520 1525 1530

Ile Asn Gln Ala Ile Asn Thr Leu Asn Ala Ala Gln Gly Glu Thr
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Asp Lys Phe Ala Val Lys Tyr Asp Lys Asn Thr Asp Gly Ser Thr
1550 1555 1560

Asn Tyr Asn Ser Ile Thr Ala Gly Asn Gly Asn Gly Thr Ala Ala
1565 1570 1575

Thr Ile Gly Thr Asp Thr Ala Gly Asn Ser Val Val Thr Ser Gly
1580 1585 1590

Gly Thr Lys Ile Ser Asn Val Ala Asn Gly Val Asn Ala Ser Asp
1595 1600 1605

Ala Val Asn Lys Gly Gln Leu Asp Ser Leu Ser Thr Gly Leu Thr
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Asn Thr Gly Phe Gly Leu Lys Ala Ala Asp Gly Asn Thr Val Asn
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Lys Lys Leu Gly Glu Ala Val Asp Val Val Gly Ala Asp Ser Asn
 1640 1645 1650

Ile Thr Thr Lys Val Ala Gly Gly Gln Val Ala Ile Glu Leu Asn
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Lys Asn Leu Asn Asn Leu Thr Gly Ile Thr Val Asn Asp Gly Thr
 1670 1675 1680

Asn Gly Thr Asn Gly Ser Thr Val Ile Gly Lys Asp Gly Ile Ser
 1685 1690 1695

Ile Lys Asp Gly Ser Gly Asn Thr Ile Ala Gly Val Asp Asn Thr
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Ala Leu Thr Val Lys Asp Gly Ser Gly Asn Thr Glu Thr Ser Ile
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 1730 1735 1740

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 1745 1750 1755

Tyr Asn Asn Ile Thr Leu Ala Gly Thr Thr Ala Ser Ser Thr Gln
 1760 1765 1770

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 1775 1780 1785

Asn Asn Val Ala Ser Ala Gly Asp Tyr Lys Asp Val Ala Asn Ala
 1790 1795 1800

Ser Lys Gly Val Asn Ala Gly Asp Leu Asn Asn Ala Val Val Asp
 1805 1810 1815

Ala Thr Asn Ala Ala Thr Ser Lys Gly Phe Ala Leu Gln Ala Ala
 1820 1825 1830

Asp Gly Ala Lys Val Gln Lys Asn Leu Gly Glu Ala Val Glu Val
 1835 1840 1845

Val Gly Ala Asp Ser Asn Ile Thr Thr Lys Val Val Gly Gly Gln
 1850 1855 1860

Val Ala Ile Glu Leu Asn Lys Asn Leu Asn Asn Leu Thr Gly Ile
 1865 1870 1875

Thr Val Asn Asp Gly Thr Asn Gly Thr Asn Gly Ser Thr Val Ile
 1880 1885 1890

Gly Lys Asp Gly Ile Ser Val Lys Asp Gly Ser Gly Asn Thr Ile
 1895 1900 1905

Ala Gly Val Asp Asn Thr Ala Leu Thr Val Lys Asp Gly Ser Gly
 1910 1915 1920

Asn Thr Glu Thr Ser Ile Asn Gln Ala Ile Asn Thr Leu Asn Ala
 1925 1930 1935

Ala Gln Gly Glu Thr Asp Lys Phe Ala Val Lys Tyr Asp Lys Asn
 1940 1945 1950

Ala Asp Gly Ser Val Asn Tyr Asn Asn Ile Thr Leu Ala Gly Thr
 1955 1960 1965

Thr Ala Ser Ser Thr Gln Asp Ala Thr Thr Gly Lys Ile Thr Thr
 20/76

1970	1975	1980
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1985	1990	1995
Lys Asp Val Ala Asn Ala Ser	Lys Gly Val Asn Ala	Gly Asp Leu
2000	2005	2010
Asn Asn Ala Val Val Asp Ala	Thr Asn Ala Ala Thr	Ser Lys Gly
2015	2020	2025
Phe Ala Leu Gln Ala Ala Asp	Gly Ala Lys Val Gln	Lys Asn Leu
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Gly Glu Ala Val Glu Val Val	Gly Ala Asp Ser Asn	Ile Thr Thr
2045	2050	2055
Lys Val Ala Gly Gly Gln Val	Ala Ile Glu Leu Asn	Lys Asn Leu
2060	2065	2070
Asn Asn Leu Thr Gly Ile Thr	Val Asn Asp Gly Thr	Asn Gly Thr
2075	2080	2085
Asn Gly Ser Thr Val Ile Gly	Lys Asp Gly Ile Ser	Val Lys Asp
2090	2095	2100
Gly Ser Gly Asn Thr Ile Ala	Gly Val Asp Asn Thr	Ala Leu Thr
2105	2110	2115
Val Lys Asp Gly Ser Gly Asn	Thr Glu Thr Ser Ile	Asn Gln Ala
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Ile Asn Thr Leu Asn Ala Ala	Gln Gly Glu Thr Asp	Lys Phe Ala
2135	2140	2145

Val Lys Tyr Asp Lys Asn Ala Asp Gly Ser Val Asn Tyr Asn Asn
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Ile Thr Leu Ala Gly Thr Thr Ala Ser Ser Thr Gln Asp Ala Thr
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Thr Gly Lys Ile Thr Thr Thr Gly Gly Thr Ser Leu Asn Asn Val
 2180 2185 2190

Ala Ser Ala Gly Asp Tyr Lys Asp Val Ala Asn Ala Ser Lys Gly
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Val Asn Ala Gly Asp Leu Asn Asn Ala Val Val Asp Ala Thr Asn
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Ala Ala Thr Ser Lys Gly Phe Ala Leu Gln Ala Ala Asp Gly Ala
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Lys Val Gln Lys Asn Leu Gly Glu Ala Val Glu Val Val Gly Ala
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Asp Ser Asn Ile Thr Thr Lys Val Ala Gly Gly Gln Val Ala Ile
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Glu Leu Asn Lys Asn Leu Asn Asn Leu Thr Gly Ile Thr Val Asn
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Asp Gly Thr Asn Gly Thr Asn Gly Ser Thr Val Ile Gly Lys Asp
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Gly Ile Ser Val Lys Asp Gly Ser Gly Asn Thr Ile Ala Gly Val
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Asp Asn Thr Ala Leu Thr Val Lys Asp Gly Ser Gly Asn Thr Glu
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Thr Ser Ile Asn Gln Ala Ile Asn Thr Leu Asn Ala Ala Gln Gly
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Glu Thr Asp Lys Phe Ala Val Lys Tyr Asp Lys Asn Ala Asp Gly
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Ser Ala Asn Tyr Asn Asn Val Thr Leu Ala Gly Thr Asn Gly Thr
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Ile Ile Ser Asn Val Lys Ala Gly Ala Val Thr Ser Thr Ser Thr
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Asp Ala Ile Asn Gly Ser Gln Leu Tyr Gly Val Ala Asn Ser Val
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Lys Asn Ala Ile Gly Gly Ser Thr Thr Ile Asp Ala Thr Thr Gly
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Ala Ile Thr Thr Thr Asn Ile Gly Gly Thr Gly Ser Asn Thr Ile
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Asp Gly Ala Ile Ser Ser Ile Lys Asp Ser Ala Thr Lys Ala Lys
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Ser Ser Asn Thr Ile Lys Gly Leu Ser Asn Thr Thr Trp Asn Gly
2495 2500 2505

Thr Ala Val Ser Gly Gln Ala Ala Thr Glu Asp Gln Leu Lys Thr
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Asp Val Val Gly Ala Asp Ser Asn Ile Thr Thr Lys Val Ala Gly
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Gly Gln Val Ala Ile Glu Leu Asn Lys Asn Leu Asn Asn Leu Thr
24/76

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Thr Ile Ala Gly Val Asp Asn Thr Ala Leu Thr Val Lys Asp Ser		
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Ser Gly Asn Thr Glu Thr Ser Ile Asn Gln Ala Ile Asn Thr Leu		
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Asn Ala Ala Gln Gly Glu Thr Asp Lys Phe Ala Val Lys Tyr Asp		
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Lys Asn Ala Asp Gly Ser Val Asn Tyr Asn Asn Val Thr Leu Ala		
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Gly Thr Asn Gly Thr Ile Ile Arg Asn Val Lys Ala Gly Ala Val		
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Thr Ser Thr Ser Thr Asp Ala Ile Asn Gly Ser Gln Leu Tyr Asp		
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Ala Thr Ala Lys Asp Val Asn Phe Asp Lys Val Thr Val Gly Asn
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Val Val Val Asp Lys Ala Asn Asp Thr Ile Gln Gly Leu Ser Asn
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Lys Asp Leu Asn Ser Thr Asp Phe Ala Thr Lys Gly Arg Ala Ala
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Gly Gln Thr Val Gln Lys Met Asn Thr Asp Gly Ile Lys Phe Phe
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Ala Asn Ala Val Asn Ala Thr Asp Ala Val Asn Lys Gly Gln Leu

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Leu Gly Gly Gly Ala Thr Gly	Thr Thr Ile Thr Asn	Val Lys Asp
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3395	3400	3405
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Asp Gln Ile Arg His Ile Leu Gly Asn Pro Gln Phe Ser Glu Gly Leu
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Ser Asn Gln Tyr Lys Arg Cys Gln Leu Arg Ile Asp Phe Asp Lys Gln
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Tyr Arg Ser Asp Asn Leu Tyr Trp Lys Gly Glu Gln Cys Gln Gly Leu
115 120 125

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Gln Ile Lys Gln Ser Asp Ala Asn Ser Pro Val Phe Val Ala Gly Tyr
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195 200 205

Arg Ala Asn Thr Val Val Glu Leu Leu Lys Gln Gln Gly Ile Arg Gly
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Glu Gln Ile Gln Tyr Ser Ala Glu Asn Lys Thr Asp Val Tyr Gln Lys
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