

[DESCRIPTION]

[Invention Title]

NOVEL POLYPEPTIDE AND METHOD OF PRODUCING IMP USING THE SAME

[Technical Field]

The present disclosure relates to a novel protein variant having an activity of exporting 5'-inosine monophosphate (IMP), a microorganism comprising the same, and a method for preparing IMP and a method for increasing export of IMP using the same.

[Background Art]

5'-Inosine monophosphate (hereinafter, IMP), a nucleic acid material, is an intermediate of the nucleic acid metabolism pathway and is used in many fields such as foods, medicines, various medical applications, *etc.* In particular, IMP is widely used as an additive for food seasonings or foods, along with 5'-guanine monophosphate (hereinafter, GMP). Although IMP itself is known to provide a beef taste, it is known to enhance the flavor of monosodium glutamic acid (MSG) and is thus attracting attention as a taste-enhancing nucleic acid-based seasoning.

Examples of methods for producing IMP include a method of enzymatically degrading ribonucleic acid extracted from yeast cells (Japanese Patent Publication No. 1614/1957), a method for chemically phosphorylating inosine produced by fermentation (*Agri. Biol. Chem.*, 36, 1511, *etc.*), a method for culturing microorganisms which can directly produce IMP and recovering IMP in the culture broth, *etc.* Among these, the method most frequently used at present is a method using microorganisms capable of directly producing IMP.

Meanwhile, since enzymes do not always exhibit optimal properties in nature with respect to activity, stability, substrate specificity for optical isomers, *etc.* required in industrial applications, various attempts have been made to improve enzymes to suit the intended use by modification of their amino acid sequences, *etc.* Among these, although rational design and site-directed mutagenesis of enzymes have been applied to improve enzyme function, in many cases, these attempts were shown to be disadvantageous in that information on the structure of target enzymes is not sufficient or the structure-function correlation is not clear, thus preventing their effective application. Additionally, a method of improving enzyme activity by attempting the enhancement of enzymes through directed evolution, which is for screening enzymes of desired traits from a library of modified enzymes constructed through random mutagenesis of

enzyme genes, was previously reported.

[Disclosure]

[Technical Problem]

In order to produce IMP in high yield using the method of directly producing IMP through microbial fermentation, the IMP should be smoothly exported. To accomplish such object, the inventors of the present disclosure have discovered the protein involved in the activity of exporting IMP, and also have made many efforts to increase IMP production. As a result, they have discovered protein variants having the activity of exporting IMP, thereby completing the present disclosure.

[Technical Solution]

An object of the present disclosure is to provide a protein variant having the activity of exporting IMP.

Another object of the present disclosure is to provide a polynucleotide encoding the protein variant of the present disclosure.

Still another object of the present disclosure is to provide a vector including the polynucleotide of the present disclosure.

Still another object of the present disclosure is to provide a microorganism producing IMP, including the protein variant and vector of the present disclosure.

Still another object of the present disclosure is to provide a method for preparing IMP, including culturing the microorganism of the present disclosure in a medium.

Still another object of the present disclosure is to provide a method for increasing the export of IMP, including enhancing activity of the protein variant of the present disclosure, which has the activity of exporting IMP.

[Advantageous Effects of the Invention]

IMP can be produced in high yield by culturing a microorganism of the genus *Corynebacterium* producing IMP using the protein variant of the present disclosure, which is capable of exporting IMP.

[Best Mode for Carrying out the Invention]

The present disclosure will be described in detail as follows. Meanwhile, each of the explanations and exemplary embodiments disclosed herein can be applied to other respective explanations and exemplary embodiments. That is, all of the combinations of various factors disclosed herein belong to the scope of the present disclosure. Additionally, the scope of the present disclosure should not be limited by the specific disclosure provided hereinbelow.

To achieve the above objects, an aspect of the present disclosure provides a protein variant having an activity of exporting IMP.

As used herein, the term “a protein that exports 5'-inosine monophosphate (IMP)” refers to a protein involved in the extracellular export of IMP. For the purpose of the present disclosure, the term may be used interchangeably with a protein having an activity of exporting IMP, an IMP export protein, a protein having an activity of exporting 5'-inosine monophosphate, a 5'-inosine monophosphate-exporting protein, *etc.*; specifically, the protein may be expressed as ImpE, and more specifically, may be expressed as ImpE1 or ImpE2, but is not limited thereto. Additionally, the protein may be derived from a microorganism of the genus *Corynebacterium*, and specifically from *Corynebacterium stationis*, but the microorganism is not limited thereto.

The protein, for example, may consist of the amino acid sequence represented by SEQ ID NO: 1 or SEQ ID NO: 2, but any sequence having the same activity as the protein can be included without limitation, and one of ordinary skill in the art can obtain sequence information from GenBank of NCBI, a well-known database. Additionally, the protein may include the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2, or an amino acid sequence having a homology or identity to the sequence of SEQ ID NO: 1 or SEQ ID NO: 2 of at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%. Additionally, it is obvious that any protein having an amino acid sequence with deletion, modification, substitution, or addition in part of the sequence can also be included in the scope of the present disclosure, as long as the amino acid sequence has a homology or identity described above and has an effect corresponding to that of the protein.

That is, although described as “a protein having an amino acid sequence of a particular SEQ ID NO” or “a protein consisting of an amino acid sequence of a particular SEQ ID NO” in the present disclosure, the protein may have an activity that is identical or corresponding to that

of a protein consisting of an amino acid sequence of the corresponding SEQ ID NO. In such a case, it is obvious that any proteins having an amino acid sequence with deletion, modification, substitution, conservative substitution, or addition in part of the sequence also can be used in the present disclosure. For example, in the case of having the activity that is the same as or corresponding to that of the modified protein, it does not exclude an addition of a sequence upstream or downstream of the amino acid sequence, which does not alter the function of the protein, a mutation that may occur naturally, a silent mutation thereof, or a conservative constitution, and even when the sequence addition or mutation is present, it obviously belongs to the scope of the present disclosure.

As used herein, the term “homology” or “identity” refers to a degree of matching with two given amino acid sequences or nucleotide sequences, and may be expressed as a percentage.

The terms “homology” and “identity” may often be used interchangeably with each other.

The sequence homology or identity of conserved polynucleotide or polypeptide sequences may be determined by standard alignment algorithms and can be used with a default gap penalty established by the program being used. Substantially homologous or identical sequences are generally expected to hybridize under moderate or high stringency, along the entire length or at least about 50%, about 60%, about 70%, about 80%, or about 90% of the entire length of the sequences. Polynucleotides that contain degenerate codons instead of codons in the hybridizing polypeptides are also considered.

Whether any two polynucleotide or polypeptide sequences have a homology, similarity, or identity may be determined using a known computer algorithm such as the “FASTA” program (Pearson *et al.*, (1988) [Proc. Natl. Acad. Sci. USA 85]: 2444: using default parameters in 2444). Alternately, it may be determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453), which is performed in the Needleman program of the EMBOSS package ((EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, Trends Genet. 16: 276-277) (version 5.0.0 or versions thereafter) (GCG program package (Devereux, J., *et al.*, Nucleic Acids Research 12: 387 (1984)), BLASTP, BLASTN, FASTA (Atschul, [S.] [F.,] [ET AL, J MOLEC BIOL 215]: 403 (1990); Guide to Huge Computers, Martin J. Bishop, [ED.,] Academic Press, San Diego, 1994, and [CARILLO ETA/.] (1988) SIAM J Applied Math 48: 1073). For example, the homology, similarity, or identity

may be determined using BLAST or ClustalW of the National Center for Biotechnology Information (NCBI).

The homology, similarity, or identity of polynucleotide or polypeptide sequences may be determined by comparing sequence information using, for example, the GAP computer program (*e.g.*, Needleman *et al.*, (1970), J Mol Biol.48 : 443) as published (*e.g.*, Smith and Waterman, Adv. Appl. Math (1981) 2:482). In summary, the GAP program defines the homology, similarity, or identity as the value obtained by dividing the number of similarly aligned symbols (*i.e.*, nucleotides or amino acids) into the total number of the symbols in the shorter of the two sequences. Default parameters for the GAP program may include (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov *et al.* (1986), *Nucl. Acids Res.* 14:6745, as disclosed in Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap (or a gap opening penalty of 10 and a gap extension penalty of 0.5); and (3) no penalty for end gaps. Accordingly, as used herein, the term “homology” or “identity” refers to relevance between sequences. Specifically, the protein variant of the present disclosure having the activity of exporting IMP may be one in which at least one amino acid selected from the group consisting of the 164th amino acid in the amino acid sequence of SEQ ID NO: 1, the 222nd amino acid in the amino acid sequence of SEQ ID NO: 1, the 2nd amino acid in the amino acid sequence of SEQ ID NO: 2, and the 64th amino acid in the amino acid sequence of SEQ ID NO: 2 is substituted with another amino acid, but is not limited thereto.

For example, in the protein variant having the activity of exporting IMP, the 164th amino acid in the amino acid sequence of SEQ ID NO: 1 is substituted with lysine, arginine, asparagine, glycine, threonine, or proline; the 2nd amino acid in the amino acid sequence of SEQ ID NO: 2 is substituted with isoleucine, phenylalanine, methionine, glutamic acid, histidine, or asparagine; or the 64th amino acid in the amino acid sequence of SEQ ID NO: 2 is substituted with aspartic acid, glutamic acid, asparagine, cysteine, isoleucine, or phenylalanine, but is not limited thereto.

As a specific example, the protein variant having the activity of exporting IMP may be a protein having the amino acid sequence consisting of SEQ ID NO: 141, 142, 145, 147, 149, or 151, a protein having an amino acid sequence encoded by the polynucleotide of SEQ ID NO: 153 or 154, or a protein having an amino acid sequence having a homology thereto of at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

In addition, it is apparent that a protein having a deletion, modification, substitution, or addition of some sequence may be used as the protein of the present disclosure as long as it is a protein having the amino acid sequence with the homology above and exhibiting an effect corresponding to that of the protein.

Another aspect of the present disclosure provides a polynucleotide encoding the protein variant, or a vector including the polynucleotide.

As used herein, the term “polynucleotide” refers to a polymer of nucleotides which is extended in a long chain by covalent bonds and has a DNA strand or an RNA strand longer than a certain length, and more specifically, refers to a polynucleotide fragment encoding the protein variant.

It is apparent that a polynucleotide, which can be translated by codon degeneracy into a protein consisting of the amino acid sequence of SEQ ID NO: 141, 142, 145, 147, 149, or 151, a protein consisting of an amino acid sequence encoded by the polynucleotide of SEQ ID NO: 153 or 154, or into a protein having a homology thereto, also can be included as the polynucleotide of the present disclosure. For example, the polynucleotide of the present disclosure may be a polynucleotide having a nucleotide sequence of SEQ ID NO: 143, 144, 146, 148, 150, 152, 153, or 154, and more specifically, may be a polynucleotide composed of a nucleotide sequence of SEQ ID NO: 143, 144, 146, 148, 150, 152, 153, or 154. In addition, a polynucleotide sequence, which encodes a protein having the activity of the protein consisting of an amino acid sequence of SEQ ID NO: 141, 142, 145, 147, 149, or 151 or an amino acid sequence encoded by a polynucleotide of SEQ ID NO: 153 or 154 by hybridization under stringent conditions with a probe which can be prepared from known gene sequences, *e.g.*, a complementary sequence to all or part of the nucleotide sequence, may be included without limitation.

The term “stringent conditions” refers to conditions under which specific hybridization between polynucleotides is made possible. Such conditions are specifically described in references (*e.g.*, J. Sambrook *et al.*, *supra*). For example, the conditions may include performing hybridization between genes having a high homology, a homology of 40% or higher, specifically 90% or higher, more specifically 95% or higher, even more specifically 97% or higher, and most specifically 99% or higher, while not performing hybridization between genes having a homology of lower than the above homologies; or to perform hybridization once, specifically two or three times, under conventional washing conditions for southern hybridization

of 60°C, 1× SSC, and 0.1% SDS, specifically at a salt concentration and temperature corresponding to 60°C, 0.1× SSC, and 0.1% SDS, and more specifically 68°C, 0.1× SSC, and 0.1% SDS.

Hybridization requires that two nucleic acids have a complementary sequence, although mismatches between bases may be possible depending on the stringency of the hybridization. The term “complementary” is used to describe the relationship between mutually hybridizable nucleotide bases. For example, with respect to DNA, adenosine is complementary to thymine, and cytosine is complementary to guanine. Accordingly, the present disclosure may also include isolated nucleic acid fragments complementary to the entire sequence as well as substantially similar nucleic acid sequences.

Specifically, polynucleotides having a homology can be detected at a T_m value of 55°C using hybridization conditions that include a hybridization step and using the conditions described above. Additionally, the T_m value may be 60°C, 63°C, or 65°C, but is not limited thereto and may be appropriately adjusted by an ordinary person skilled in the art according to the intended purpose.

The stringency suitable for the hybridization of polynucleotides depends on the length and complementarity of the polynucleotides and the related variables are well known in the art (see Sambrook *et al.*, *supra*, 9.50 to 9.51 and 11.7 to 11.8).

As used herein, the term “vector” refers to a DNA construct including the nucleotide sequence of the polynucleotide encoding a target protein, in which the target protein is operably linked to a suitable control sequence so that the target protein can be expressed in an appropriate host. The control sequence may include a promoter capable of initiating transcription, any operator sequence for controlling the transcription, a sequence encoding an appropriate mRNA ribosome-binding domain, and a sequence controlling the termination of transcription and translation. The vector, after being transformed into a suitable host cell, may be replicated or function irrespective of the host genome, or may be integrated into the host genome itself.

The vector used in the present disclosure may not be particularly limited as long as the vector is replicable in the host cell, and it may be constructed using any vector known in the art. Examples of the vector may include natural or recombinant plasmids, cosmids, viruses, and bacteriophages. For example, as a phage vector or cosmid vector, pWE15, M13, MBL3, MBL4, IXII, ASHII, APII, t10, t11, Charon4A, Charon21A, *etc.* may be used; and as a plasmid vector,

those based on pBR, pUC, pBluescriptII, pGEM, pTZ, pCL, pET, *etc.* may be used. Specifically, pDZ, pACYC177, pACYC184, pCL, pECCG117, pUC19, pBR322, pMW118, pCC1BAC vectors, *etc.* may be used.

In an embodiment, the polynucleotide encoding the target protein may be replaced with a modified polynucleotide within the chromosome using a vector for the insertion into the chromosome in a cell. The insertion of the polynucleotide into the chromosome may be performed using a known method in the art, for example, by homologous recombination, but is not limited thereto. In particular, a selection marker for confirming the insertion into the chromosome may be further included. The selection marker is used for selection of a transformed cell, *i.e.*, in order to confirm whether the target nucleic acid has been inserted, and markers capable of providing selectable phenotypes such as drug resistance, nutrient requirement, resistance to cytotoxic agents, and expression of surface proteins may be used. Under the circumstances where selective agents are treated, only the cells capable of expressing the selection markers can survive or express other phenotypic traits, and thus the transformed cells can be easily selected.

Still another aspect of the present disclosure provides a microorganism producing IMP, including the protein variant of the present disclosure, the polynucleotide of the present disclosure encoding the protein variant, or the vector of the present disclosure. Specifically, the microorganism including the protein variant and/or a polynucleotide encoding the protein variant may be a microorganism prepared by transformation using a vector containing the polynucleotide encoding the protein variant, but the microorganism is not limited thereto.

As used herein, the term “transformation” refers to a process of introducing a vector including a polynucleotide encoding a target protein into a host cell, thereby enabling the expression of the protein encoded by the polynucleotide in the host cell. For the transformed polynucleotide, it does not matter whether it is inserted into the chromosome of the host cell and located therein or located outside the chromosome, as long as the transformed polynucleotide can be expressed in the host cell. Additionally, the polynucleotide includes DNA and RNA which encode the target protein. The polynucleotide may be inserted in any form as long as it can be introduced into a host cell and expressed therein. For example, the polynucleotide may be introduced into a host cell in the form of an expression cassette, which is a gene construct

including all of the essential elements required for self-expression. The expression cassette may conventionally include a promoter operably linked to the polynucleotide, a transcription termination signal, a ribosome-binding domain, and a translation termination signal. The expression cassette may be in the form of a self-replicable expression vector. Additionally, the polynucleotide may be introduced into a host cell as is and operably linked to a sequence essential for its expression in the host cell, but is not limited thereto.

Additionally, as used herein, the term “operably linked” refers to a functional linkage between a promoter sequence, which initiates and mediates the transcription of the polynucleotide encoding the target protein, *i.e.*, a conjugate of the present disclosure, and the above gene sequence.

As used herein, the term “IMP-producing microorganism” refers to a microorganism which is naturally capable of producing IMP; or a microorganism introduced an ability to produce or export IMP to whose parent strain is not naturally capable of producing and/or exporting IMP which is In the present disclosure, the microorganism producing IMP can be used interchangeably with a microorganism having an activity of exporting IMP.

The IMP-producing microorganism is a cell or microorganism which includes a protein variant having an activity of exporting IMP or a polynucleotide encoding the protein variant, or which is transformed with a vector containing the polynucleotide encoding the protein variant, and is thereby capable of expressing the protein variant. For the purposes of the present disclosure, the host cell of the IMP-producing microorganism or microorganism may be any microorganism including the protein variant thus capable of producing IMP. For example, the microorganism may be a microorganism of the genus *Escherichia*, a microorganism of the genus *Serratia*, a microorganism of the genus *Erwinia*, a microorganism of the genus *Enterobacteria*, a microorganism of the genus *Salmonella*, a microorganism of the genus *Streptomyces*, a microorganism of the genus *Pseudomonas*, a microorganism of the genus *Brevibacterium*, a microorganism of the genus *Corynebacterium*, *etc.*, and specifically, a microorganism of the genus *Corynebacterium*.

As used herein, the term “IMP-producing microorganism of the genus *Corynebacterium*” refers to a microorganism of the genus *Corynebacterium* which is naturally capable of producing IMP or capable of producing IMP by modification. Specifically, as used herein, the microorganism of the genus *Corynebacterium* capable of producing IMP refers to a native strain

of the microorganism of the genus *Corynebacterium* capable of producing IMP; or a microorganism of the genus *Corynebacterium* with enhanced abilities to produce IMP prepared by inserting a gene associated with IMP production or by enhancing or attenuating the endogenous gene associated with IMP production. More specifically, in the present disclosure, the microorganism of the genus *Corynebacterium* capable of producing IMP refers to a microorganism of the genus *Corynebacterium* which has improved abilities to produce IMP by including a protein variant having an activity of exporting IMP or a polynucleotide encoding the protein variant, or by being transformed with a vector containing the polynucleotide encoding the protein variant. The “microorganism of the genus *Corynebacterium* with enhanced abilities to produce IMP” refers to a microorganism of the genus *Corynebacterium* with improved abilities to produce IMP compared to that of its parent strain before transformation or that of an unmodified microorganism of the genus *Corynebacterium*. The “unmodified microorganism of the genus *Corynebacterium*” refers to a native type of the microorganism of the genus *Corynebacterium*, a microorganism of the genus *Corynebacterium* which does not contain a protein variant capable of exporting IMP, or a microorganism of the genus *Corynebacterium* which is not transformed with a vector containing a polynucleotide encoding the protein variant capable of exporting IMP.

In an embodiment of the present disclosure, the microorganism of the present disclosure may be a microorganism of the genus *Corynebacterium*, in which the activity of adenylosuccinate synthetase and/or IMP dehydrogenase is further attenuated.

In the present disclosure, “a microorganism of the genus *Corynebacterium*” specifically refers to *Corynebacterium glutamicum*, *Corynebacterium ammoniagenes*, *Brevibacterium lactofermentum*, *Brevibacterium flavum*, *Corynebacterium thermoaminogenes*, *Corynebacterium efficiens*, *Corynebacterium stationis*, etc., but the microorganism is not necessarily limited thereto.

Still another aspect of the present disclosure provides a method for preparing IMP, including culturing the microorganism of the genus *Corynebacterium* in a medium.

Specifically, the method of the present disclosure may additionally include a step of recovering IMP from the microorganism or medium.

In the above method, the cultivation of the microorganism may be performed in a batch process, continuous process, fed-batch process, etc. known in the art, but the cultivation process

is not particularly limited thereto. In particular, with respect to the cultivation conditions, the pH of the culture may be adjusted to a suitable pH (*e.g.*, pH 5 to 9, specifically pH 6 to 8, and most specifically with an appropriate basic compound (*e.g.*, sodium hydroxide, potassium hydroxide, or ammonia) or acidic compound (*e.g.*, phosphoric acid or sulfuric acid), and the aerobic condition of the culture may be maintained by introducing oxygen or an oxygen-containing gas mixture to the culture. The cultivation temperature may generally be in the range of 20°C to 45°C, and specifically 25°C to 40°C for about 10 to 160 hours, but the cultivation conditions are not limited thereto. The IMP produced by the above cultivation may be secreted into the culture or may be retained in the cells.

Additionally, examples of the carbon sources to be used in the culture medium may include sugars and carbohydrates (*e.g.*, glucose, sucrose, lactose, fructose, maltose, molasses, starch, and cellulose); oils and fats (*e.g.*, soybean oil, sunflower oil, peanut oil, and coconut oil); fatty acids (*e.g.*, palmitic acid, stearic acid, and linoleic acid); alcohols (*e.g.*, glycerol and ethanol); and organic acids (*e.g.*, acetic acid), but are not limited thereto. These carbon sources may be used alone or in combination, but are not limited thereto. Examples of the nitrogen sources to be used in the culture medium may include nitrogen-containing organic compounds (*e.g.*, peptone, yeast extract, meat gravy, malt extract, corn steep liquor, soybean flour, and urea) or inorganic compounds (*e.g.*, ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate, and ammonium nitrate), *etc.* These nitrogen sources may be used alone or in combination, but are not limited thereto. Examples of the phosphorus sources to be used in the culture medium may include potassium dihydrogen phosphate, dipotassium hydrogen phosphate, corresponding sodium-containing salts, *etc.*, but are not limited thereto. Additionally, metal salts (*e.g.*, magnesium sulfate or iron sulfate), amino acids, vitamins, *etc.*, which are essential growth-promoting materials, may be contained in the medium.

In the present disclosure, the method for recovering the IMP produced in the step of cultivation may be performed by collecting the IMP from the culture broth using an appropriate method known in the art. For example, methods such as centrifugation, filtration, anion exchange chromatography, crystallization, HPLC, *etc.* may be used, and the desired IMP can be recovered from a culture or cultured microorganism using an appropriate method known in the art.

Further, the recovery may include a purification process and may be performed using an appropriate method known in the art. Thus, the IMP to be recovered may be in a purified form

or a microorganism fermentation broth containing IMP.

Still another aspect of the present disclosure provides a composition for producing IMP, including the protein variant of the present disclosure, which has the activity of exporting IMP, or a polynucleotide encoding the same.

The composition of the present disclosure may further include, without limitation, a constitution capable of operating the polynucleotide. In the composition of the present disclosure, the polynucleotide may be in a form included within a vector to express an operably linked gene in the introduced host cell.

Additionally, the composition may further include any suitable excipients conventionally used in the composition for producing IMP. Such excipients may be, for example, preservatives, humectants, suspending agents, buffers, stabilizing agents, or isotonic agents, but are not limited thereto.

Still another aspect of the present disclosure provides use of the protein of the present disclosure for increasing the production of IMP in the microorganism of the genus *Corynebacterium*.

Still another aspect of the present disclosure provides a method for increasing the export of IMP, including enhancing the activity of the protein variant, which has the activity of exporting IMP, in the microorganism of the genus *Corynebacterium*.

The terms “protein having the activity of exporting IMP”, “enhancement”, and “microorganism of the genus *Corynebacterium*” are as described above.

Still another aspect of the present disclosure provides use of the protein of the present disclosure for increasing the export of IMP in the microorganism of the genus *Corynebacterium*.

[Detailed Description of the Embodiment]

Hereinafter, the present disclosure will be described in detail through exemplary embodiments. However, it should be obvious to one of ordinary skill in the art that these exemplary embodiments are provided for the purpose of illustration only and are not intended to limit the scope of the present disclosure.

Example 1: Discovery of IMP export proteins

A genomic DNA library of *Corynebacterium stationis* ATCC6872 was prepared for the identification of membrane proteins of *Corynebacterium* involved in the export of IMP. Then, since the wild-type strain of *Corynebacterium* cannot produce IMP, or even if it does produce IMP, it produces only a small amount thereof, a strain called *CJI0323*, which is capable of producing IMP, derived from the ATCC6872 strain was prepared for the identification of the ability to produce IMP. The *CJI0323* strain prepared was subjected to screening of membrane proteins involved in IMP export using the genomic DNA library of the ATCC6872 strain. The specific details of the experiment are as follows.

Example 1-1: Selection of IMP-producing strain, *CJI0323*

The ATCC6872 cells were suspended in a phosphate buffer (pH 7.0) or citrate buffer (pH 5.5) at a concentration of 10^7 cells/mL to 10^8 cells/mL to prepare an ATCC6872-derived IMP-producing strain, and the cells were subjected to UV treatment to induce mutation. The resulting cells were washed twice with a 0.85% saline solution, and then diluted and plated on a medium, which was prepared by adding a resistance-providing material at an appropriate concentration to a minimal medium containing 1.7% agar, and colonies were obtained thereafter. Each colony was cultured in a nutrient medium and cultured in a seed medium for 24 hours. After culturing the colonies for 3 to 4 days in a fermentation medium, the colony with the highest abilities to produce IMP accumulated in the culture medium was selected. In the course of preparing a strain capable of producing IMP at high concentration, in order to provide adenine auxotrophy, guanine leakage, lysozyme susceptibility, 3,4-dihydroproline resistance, streptomycin resistance, azetidine carboxylic acid resistance, thiaproline resistance, azaserine resistance, sulfaguanidine resistance, norvaline resistance, and trimethoprim resistance, the procedures above were performed sequentially for each material. As a result, *CJI0323*, which showed resistance to the above materials and excellent abilities to produce IMP, was finally selected. The degree of resistance between ATCC6872 and *CJI0323* was compared and the results are shown in Table 1 below.

[Table 1]

Characteristics	ATCC6872	<i>CJI0323</i>
Adenine auxotrophy	Non-auxotrophy	Auxotrophy
Guanine leakage	Non-auxotrophy	Leaky auxotrophy

Lysozyme susceptibility	80 µg/mL	8 µg/mL
3,4-Dihydroproline resistance	1000 µg/mL	3500 µg/mL
Streptomycin resistance	500 µg/mL	2000 µg/mL
Azetidine carboxylic acid resistance	5 mg/mL	30 mg/mL
Thiaproline resistance	10 µg/mL	100 µg/mL
Azaserine resistance	25 µg/mL	100 µg/mL
Sulfaguanidine resistance	50 µg/mL	200 µg/mL
Norvaline resistance	0.2 mg/mL	2 mg/mL
Trimethoprim resistance	20 µg/mL	100 µg/mL

- Minimal medium: 2% glucose, 0.3% sodium sulfate, 0.1% KH₂SO₄, 0.3% K₂HPO₄, 0.3% magnesium sulfate, calcium chloride (10 mg/L), iron sulfate (10 mg/L), zinc sulfate (1 mg/L), manganese chloride (3.6 mg/L), L-cysteine (20 mg/L), calcium pantothenate (10 mg/L), thiamine hydrochloride (5 mg/L), biotin (30 µg/L), adenine (20 mg/L), guanine (20 mg/L), pH 7.3

- Nutrient medium: 1% peptone, 1% meat juice, 0.25% sodium chloride, 1% yeast extract, 2% agar, pH 7.2

- Seed medium: 1% glucose, 1% peptone, 1% meat juice, 1% yeast extract, 0.25% sodium chloride, adenine (100 mg/L), guanine (100 mg/L), pH 7.5

-Fermentation medium: 0.1% sodium glutamate, 1% ammonium chloride, 1.2% magnesium sulfate, 0.01% calcium chloride, iron sulfate (20 mg/L), manganese sulfate (20 mg/L), zinc sulfate (20 mg/L), copper sulfate (5 mg/L), L-cysteine (23 mg/L), alanine (24 mg/L), nicotinic acid (8 mg/L), biotin (45 µg/L), thiamine hydrochloride (5 mg/L), adenine (30 mg/L), 1.9% phosphoric acid (85%), 2.55% glucose, 1.45% fructose

Example 1-2: Experiments on fermentation titer of *CJI0323*

The seed medium (2 mL) was dispensed into test tubes (diameter: 18 mm), which were then autoclaved and each inoculated with *ATCC6872* and *CJI0323*. Thereafter, the resultants were shake-cultured at 30°C for 24 hours and then used as a seed culture solution. The

fermentation medium (29 mL) was dispensed into Erlenmeyer flasks (250 mL) for shaking, autoclaved at 121°C for 15 minutes, and the seed culture solution (2 mL) was inoculated thereto and cultured for 3 days. The culture conditions were set to 170 rpm, 30°C, and a pH of 7.5.

Upon completion of the culture, the amount of IMP produced was measured by HPLC (SHIMAZDU LC20A) and the results of the culture are shown in Table 2 below.

[Table 2]

Strain	IMP (g/L)
<i>ATCC6872</i>	0
<i>CJI0323</i>	9.52

The *CJI0323* strain was named as *Corynebacterium stationis* CN01-0323. The strain was deposited under the Budapest Treaty to the Korean Culture Center of Microorganisms (KCCM) on November 7, 2017. In addition, the strain was designated as Accession No. KCCM12151P.

Example 1-3: Discovery of exporting proteins

Screening conditions showing growth inhibition of the *CJI0323* strain were established by additionally adding IMP to the minimal medium containing 1.7% agar. The plasmids of the genomic library of the *ATCC6872* strain were transformed into the *CJI0323* strain by electroporation (van der Rest *et al.* 1999), and those colonies in which the growth inhibition was released under the medium conditions supplemented with an excess amount of IMP were selected. Plasmids were obtained from the selected colonies and analyzed by a sequencing technique. As a result, one kind of membrane protein involved in the release of the growth inhibition was identified under the condition where an excess amount of IMP was added.

The one kind of membrane protein from *Corynebacterium* was identified based on the amino acid sequence of SEQ ID NO: 2 and the nucleotide sequence of SEQ ID NO: 4 (NCBI GenBank: NZ_CP014279, WP_066795121, MFS transporter). The membrane protein is known as the MFS transporter, but its specific function has not been confirmed, and further, its function regarding the IMP export is still unknown. In the present disclosure, the membrane protein was named ImpE2(WT).

Example 2: Identification of ImpE1 and ImpE2

Example 2-1: Confirmation of *impE1* and *impE2*

In order to examine the functions of the membrane protein, ImpE2, the gene structure of SEQ ID NO: 4 was confirmed in the NCBI (NCBI GenBank: NZ_CP014279, WP_066795121, MFS transporter). As a result, it was confirmed that the 7 bp starting portion of the ORF of SEQ ID NO: 4 (*impE2*) overlaps in 7 bp with a different gene (NCBI GenBank: NZ_CP014279, WP_066795119, transcriptional regulator), which is located upstream of *impE2*. Since the functions of the gene located upstream of *impE2* and the protein encoded by the gene have not been confirmed, in the present disclosure, the protein was named ImpE1(WT) (the amino acid sequence of SEQ ID NO: 1 and the nucleotide sequence of SEQ ID NO: 3).

Example 2-2: Preparation of *impE1*- or *impE2*-deficient vector

In order to confirm whether the deletion of *ImpE1* or *ImpE2*, which are involved in releasing the growth inhibition caused by IMP as identified in Examples 1 and 2-1, in an IMP-producing strain can reduce its IMP-exporting ability, attempts were made to prepare vectors deficient in each of the genes.

The gene fragments for preparing the vectors were obtained by PCR using the genomic DNA of the ATCC6872 strain as a template.

Specifically, the PCR for *impE1* was performed using primers of SEQ ID NOS: 5 and 6 and primers of SEQ ID NOS: 7 and 8; and the PCR for *impE2* was performed using the primers of SEQ ID NOS: 9 and 10 and primers of SEQ ID NOS: 11 and 12 (Table 3).

[Table 3]

SEQ ID NO	Primer	Sequence (5' to 3')
5	<i>impE1</i> kop-1	GCTCTAGACGAGAAAGCTAAAGCCGGTGA
6	<i>impE1</i> kop-2	GTTTTTAGCTACCATTGTTACACCCCGTGCAAGTTT
7	<i>impE1</i> kop-3	GCACGGGGTGTAACAATGGTAGCTAAAAACTCCACC
8	<i>impE1</i> kop-4	GCTCTAGAAATAGTTGGGGAAGTCCACTC
9	<i>impE2</i> kop-1	GCTCTAGACTTGGATGACCTGGTGGAAAA
10	<i>impE2</i> kop-2	CTTGGAGAAAATTCCTACCATTCCAGTCCTTTCGT
11	<i>impE2</i> kop-3	GGACTGGAATGGTAGGAAATTTTCTCCAAGGGAAAT

12	impE2 kop-4	GGACTAGTGGATTGTGTTGACGCACGATG
13	impE1E2kop-2	CTTGGAGAAAATTTCTGTTACACCCCGTGCAAGTTT
14	impE1E2kop-3	GCACGGGGTGTAACAGAAATTTTCTCCAAGGGAAAT

In particular, the primers used were prepared based on information on a gene of *Corynebacterium stationis* (ATCC6872) (NCBI Genbank: NZ_CP014279) registered in NIH GenBank and the nucleotide sequences adjacent thereto.

PCR was performed by initial denaturation at 94°C for 5 minutes; 25 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 minutes, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes.

Overlapping PCR was performed using two fragments of the *impE1* gene, which were amplified using the primers of SEQ ID NOS: 5 and 6 and the primers of SEQ ID NOS: 7 and 8, as templates, and as a result, a polynucleotide template (1.8 kbp) was obtained. The obtained gene fragment was cloned into a linearized *pDZ* vector (Korean Patent No. 10-0924065 and International Patent Publication No. 2008-033001), which was digested with the restriction enzyme (*XbaI*), and ligated using T4 ligase, and thereby the *pDZ-ΔimpE1* vector was prepared. Additionally, overlapping polymerase chain reaction was performed using a fragment of the *impE2* gene, amplified using the primers of SEQ ID NOS: 9 and 10, and two fragments of the *impE2* gene, amplified using the primers of SEQ ID NOS: 11 and 12, as templates, and as a result, a polynucleotide template (1.7 kbp) was obtained. The obtained gene fragment was digested with restriction enzymes, *XbaI* and *SpeI*. The gene fragment was cloned using T4 ligase into a linearized *pDZ* vector, which had already been digested with the restriction enzyme (*XbaI*), and thereby the *pDZ-ΔimpE2* vector was prepared.

Example 2-3: Preparation of *impE1*- and *impE2*-integration-deficient vectors

Since the *impE1* and *impE2* genes, which encode proteins involved in releasing the growth inhibition caused by IMP, are overlapped, there is a need to regulate both genes simultaneously. Therefore, attempts were made to prepare a vector in which both *impE1* and *impE2* are deficient.

For the PCR of *impE1* and *impE2* genes, primers of SEQ ID NOS: 5 and 13 and primers of SEQ ID NOS: 14 and 12 were used. The primers used were prepared based on information on a gene of *Corynebacterium stationis* (ATCC6872) (NCBI Genbank: NZ_CP014279) registered in NIH GenBank and the nucleotide sequences adjacent thereto. Overlapping PCR was performed

using a fragment of the *impE1* gene, amplified using the primers of SEQ ID NOS: 5 and 13, and two fragments of the *impE2* gene, amplified using the primers of SEQ ID NOS: 14 and 12, as templates, and as a result, a polynucleotide template (2.0 kbp) was obtained. The obtained gene fragments were digested with *Xba*I and *Spe*I, respectively. The gene fragments were cloned using T4 ligase into a linearized *pDZ* vector, which had already been digested with the restriction enzyme (*Xba*I), and thereby the *pDZ-ΔimpE1E2* vector was prepared.

Example 2-4: Preparation of *impE1*- and *impE2*-deficient strains

The two kinds of plasmids prepared in Example 2-2 and one kind of plasmid prepared in Example 2-3 were each transformed into the *CJI0323* strain by electroporation (using the transformation method disclosed in *Appl. Microbiol. Biotechnol.* (1999) 52: 541 to 545). The strains in which the vector was inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over. The genetic deficiency in the finally transformed strains was confirmed by performing PCR using the primer pairs of SEQ ID NOS: 5 and 8, SEQ ID NOS: 9 and 12, and SEQ ID NOS: 5 and 12.

The selected strains were named *CJI0323_ΔimpE1*, *CJI0323_ΔimpE2*, and *CJI0323_ΔimpE1E2*. Additionally, the abilities to produce IMP of these strains was evaluated.

The seed medium (2 mL) was dispensed into test tubes (diameter: 18 mm), which were then autoclaved, each inoculated with *CJI0323*, *CJI0323_ΔimpE1*, *CJI0323_ΔimpE2*, and *CJI0323_ΔimpE1E2*, shake-cultured at 30°C for 24 hours, and used as seed culture solutions. The fermentation medium (29 mL) was dispensed into Erlenmeyer flasks (250 mL) for shaking and autoclaved at 121°C for 15 minutes. Then, the seed culture solution (2 mL) was inoculated thereto and the resultant was cultured for 3 days. The culture conditions were set to 170 rpm, 30°C, and a pH of 7.5.

Upon completion of the culture, the amount of IMP produced was measured by HPLC, and the results of the culture are shown in Table 4 below.

[Table 4]

Strain	IMP (g/L)
<i>CJI0323</i>	9.52

<i>CJI0323_ΔimpE1</i>	1.92
<i>CJI0323_ΔimpE2</i>	1.88
<i>CJI0323_ΔimpE1E2</i>	1.80

The IMP amount accumulated in each strain was compared with that of the parent strain, *Corynebacterium stationis* *CJI0323*. As a result, it was found that, as shown in Table 4 above, the IMP concentrations of the strains *CJI0323_ΔimpE1*, *CJI0323_ΔimpE2*, and *CJI0323_ΔimpE1E2* were reduced by about 8 g/L under the same conditions compared to the parent strain, confirming that ImpE1 and ImpE2 are proteins involved in the IMP export.

Example 3: Confirmation of nucleotide sequences of *impE1* and *impE2* of IMP-producing strain, *CJI0323*

In the case of the *CJI0323* strain producing IMP at high concentration in Example 1, it is possible that the strain has an improved IMP-exporting ability so as to produce IMP at high concentration. Accordingly, an attempt was made to confirm the presence of any mutation in *impE1* and *impE2* of the *CJI0323* strain.

The chromosomal DNA of the *CJI0323* strain was amplified by polymerase chain reaction (hereinafter, “PCR”). Specifically, first, PCR was performed by repeating 28 cycles consisting of denaturation at 94°C for 1 minute, annealing at 58°C for 30 seconds, and polymerization at 72°C for 2 minutes using the chromosomal DNA of the *CJI0323* strain as a template along with the primers of SEQ ID NOS: 15 and 16 (Table 5), and thereby a fragment of about 2.8 kbp was amplified.

[Table 5]

SEQ ID NO	Primer	Sequence (5' to 3')
15	impE1E2 seqF	GAACGGAGTCATCTCCTTTGC
16	impE1E2 seqR	CCAAACGCTCTGCAAGAAACTG

Upon analysis of the nucleotide sequence using the same primers, it was confirmed that the 490th nucleotide of the *impE1* gene (*i.e.*, g) was substituted with ‘a’, compared to the nucleotide sequence of the wild-type strain, *ATCC6872*. This substitution indicates that there was a modification in which the 164th amino acid of the ImpE1 protein (*i.e.*, glutamic acid) was

substituted with lysine.

Additionally, it was confirmed that the 4th nucleotide of the *impE2* gene (i.e., g) was substituted with 'a' (this means that the 666th nucleotide of the *impE1* gene (i.e., g) was substituted with 'a') and the 191st nucleotide of the *impE1* gene (i.e., g) was substituted with 'a'. These substitutions indicate that there were modifications in which the 2nd amino acid of the ImpE2 protein (i.e., valine), which corresponds to the 222nd amino acid of the ImpE1 protein, was substituted with isoleucine; and the 64th amino acid of the ImpE2 protein (i.e., glycine) was substituted with glutamic acid.

The *impE1* nucleotide of the *CJI0323* strain was named *impE1_CJI0323* (SEQ ID NO: 143) and the protein thereof was named ImpE1_CJI0323 (SEQ ID NO: 141), whereas the *impE2* nucleotide of the *CJI0323* strain was named *impE2_CJI0323* (SEQ ID NO: 144) and the protein thereof was named ImpE2_CJI0323 (SEQ ID NO: 142).

Example 4: Recovery of modifications in *impE1* and *impE2*

Example 4-1: Preparation of vectors for recovering modifications in *impE1* or *impE2*

In Example 3, the presence of any modification in *impE1* and *impE2* of the IMP-producing strain *CJI0323* was examined. As a result, it was confirmed that *impE1* had one modification and *impE2* had two modifications. Since the *CJI0323* strain produces IMP at a high concentration, it is highly likely that the modification is one that can improve the ability to export IMP. Accordingly, after recovering the mutated *impE1* and *impE2* to the native wild-type *ImpE* without modification, the following experiment was performed to confirm whether each modification actually imparted the IMP-exporting ability.

To prepare a recovery vector, PCR was performed using *Corynebacterium stationis* ATCC6872 as a template.

The *impE1impE2* gene fragment amplified using the primers of SEQ ID NOS: 17 and 18 was treated with a restriction enzyme, *Xba*I, and cloned into the *Xba*I restriction site on the *pDZ* vector, and thereby the *pDZ-impE1E2(WT)* was prepared.

Example 4-2: Preparation of vectors with single modification in *impE1* or *impE2*

A vector with a single E164K modification in the *ImpE1* gene was prepared using the native wild-type strain, *Corynebacterium stationis* ATCC6872, as a template along with the

primers of SEQ ID NOS: 19 and 20 and primers of SEQ ID NOS: 21 and 22. Overlapping PCR was performed using an E164K-1 gene fragment amplified using the primers of SEQ ID NOS: 19 and 20 and two E164K-2 gene fragments amplified using the primers of SEQ ID NOS: 21 and 22, and thereby a template with a 1.8 kbp polynucleotide was obtained. The obtained gene fragments were digested with *Xba*I and cloned into a linearized *pDZ* vector, which had already been digested with *Xba*I, using T4 ligase, and thereby the *pDZ-impE1(E164K)* vector was prepared.

A vector with a single V2I modification in the *ImpE2* gene was prepared using the *ATCC6872* strain as a template along with the primers of SEQ ID NOS: 19 and 23 and primers of SEQ ID NOS: 24 and 22. Overlapping PCR was performed using a V2I-1 gene fragment amplified using the primers of SEQ ID NOS: 19 and 23 and two V2I-2 gene fragments amplified using the primers of SEQ ID NOS: 24 and 22, and thereby a template with a 1.8 kbp polynucleotide was obtained. The obtained gene fragments were digested with *Xba*I and cloned into a linearized *pDZ* vector, which had already been digested with *Xba*I, using T4 ligase, and thereby the *pDZ-impE2(V2I)* vector was prepared.

A vector with a single G64E modification in the *ImpE2* gene was prepared using the *ATCC6872* strain as a template along with the primers of SEQ ID NOS: 19 and 25 and primers of SEQ ID NOS: 26 and 22. Overlapping PCR was performed using a G64E-1 gene fragment amplified using the primers of SEQ ID NOS: 19 and 25 and two G64E-2 gene fragments amplified using the primers of SEQ ID NOS: 26 and 22, and thereby a template with a 1.8 kbp polynucleotide was obtained. The obtained gene fragments were digested with *Xba*I and cloned into a linearized *pDZ* vector, which had already been digested with *Xba*I, using T4 ligase, and thereby the *pDZ-impE2(G64E)* vector was prepared.

[Table 6]

SEQ ID NO	Primer	Sequence (5' to 3')
17	impE1E2 WT F	GCTCTAGAGAACGGAGTCATCTCCTTTGC
18	impE1E2 WT R	GCTCTAGACCAAACGCTCTGCAAGAAACTG
19	impE1 164K-1	GCTCTAGACTTGGATGACCTGGTGGAAAA
20	impE1 164K-2	CTGGGGCGCGTTGTTTTTCAGGATGCTCCCGAAGACG
21	impE1 164K-3	AACAACGCGCCCCAGAATTGG
22	impE1 164K-4	GCTCTAGAAATAGTTGGGGAAGTCCACTC

23	impE2 V2I-2	TGGAGTTTTTTAGCTATCATTCAGTCCTTTTCGTGTAA
24	impE2 V2I-3	TAGCTAAAAAACTCCACCCCAA
25	impE2 G64E-2	CCGAAAATCATCTGCTCCAAAGAGCTCATCAGCATGG
26	impE2 G64E-3	GCAGATGATTTTTCGGTTCCGC

Example 4-3: Recovery of *impE1*, *impE2* modifications and preparation of strains with single modification

The plasmid prepared in Example 4-1 was transformed into the *CJI0323* strain by electroporation (using the transformation method disclosed in *Appl. Microbiol. Biotechnol.* (1999) 52: 541 to 545). The strains in which the vector was inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over. The recovery of the modification in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 15 and 16, followed by nucleotide sequencing analysis. The prepared strain was named *CJI0323_impE1E2(WT)*.

The three kinds of plasmids prepared in Example 4-2 were each transformed into the *CJI0323_impE1E2(WT)* strain by electroporation (using the transformation method disclosed in *Appl. Microbiol. Biotechnol.* (1999) 52: 541 to 545). The strains in which the vector was inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over,. The introduction of the modification in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 15 and 16, followed by nucleotide sequencing analysis. The selected strains were named *CJI0323_impE1(E164K)*, *CJI0323_impE2(V2I)*, and *CJI0323_impE2(G64E)*.

The *Corynebacterium stationis* *CJI0323_impE1(E164K)*, *Corynebacterium stationis* *CJI0323_impE2(V2I)*, and *Corynebacterium stationis* *CJI0323_impE2(G64E)* strains were deposited under the Budapest Treaty to the Korean Culture Center of Microorganisms (KCCM) on November 2, 2018. In addition, the strains were designated with Accession Nos. KCCM12359P, KCCM12360P, and KCCM12361P, respectively.

Example 4-4: Preparation of *impE1*- and *impE2*-integration-modified strains

The *pDZ-impE2(V2I)* and *pDZ-impE2(G64E)* plasmids prepared in Example 4-2 were

transformed into the *CJI0323_impE1(E164K)* strain by electroporation (using the transformation method disclosed in *Appl. Microbiol. Biotechnol.* (1999) 52: 541 to 545). The strains in which the vectors were inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over,. The introduction of the modification in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 15 and 16, followed by nucleotide sequencing analysis. The prepared strains were named *CJI0323_impE1(E164K)_impE2(V2I)* and *CJI0323_impE1(164K)_impE2(G64E)*.

The *pDZ-impE2(G64E)* plasmid was transformed into the *CJI0323_impE2(V2I)* strain by electroporation (using the transformation method disclosed in *Appl. Microbiol. Biotechnol.* (1999) 52: 541 to 545). The strains in which the vector was inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over,. The introduction of the modification in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 15 and 16, followed by nucleotide sequencing analysis. The selected strain was named *CJI0323_impE2(V2I)(G64E)*.

Example 4-5: Evaluation of strains with *impE1*, *impE2* modifications

The seed medium (2 mL) was dispensed into test tubes (diameter: 18 mm), which were then autoclaved, each inoculated with *CJI0323_impE1E2(WT)*, *CJI0323_impE1(E164K)*, *CJI0323_impE2(V2I)*, *CJI0323_impE2(G64E)*, *CJI0323_impE1(E164K)_impE2(V2I)*, *CJI0323_impE1(E164K)_impE2(G64E)*, and *CJI0323_impE2(V2I)(G64E)*, shake-cultured at 30°C for 24 hours, and used as seed culture solutions. The fermentation medium (29 mL) was dispensed into Erlenmeyer flasks (250 mL) for shaking and autoclaved at 121°C for 15 minutes. Then, the seed culture solutions (2 mL) were inoculated thereto and the resultants were cultured for 3 days. The culture conditions were set to 170 rpm, 30°C, and a pH of 7.5.

Upon completion of the culture, the amount of IMP produced was measured by HPLC, and the results of the culture are shown in Table 7 below.

[Table 7]

Strain	IMP (g/L)
<i>CJI0323</i>	9.52

<i>CJI0323_impE1E2(WT)</i>	2.32
<i>CJI0323_impE1(E164K)</i>	2.57
<i>CJI0323_impE2(V2I)</i>	3.11
<i>CJI0323_impE2(G64E)</i>	3.27
<i>CJI0323_impE1(E164K)_impE2(V2I)</i>	4.24
<i>CJI0323_impE1(E164K)_impE2(G64E)</i>	6.27
<i>CJI0323_impE2(V2I)(G64E)</i>	7.35

As shown above, it was confirmed that with respect to each modification position, one kind of modification, the integration of two kinds of modifications, and the integration of three kinds of modifications were all involved in the IMP export. Accordingly, in a microorganism of the genus *Corynebacterium* which does not produce IMP or produces only a small amount thereof, the increase in the amount of IMP production due to modifications of the ImpE protein of the present disclosure can be interpreted to be very meaningful.

Example 5: Substitution of amino acids in *impE1*, *impE2* modifications with another amino acids

Example 5-1: Preparation of vectors for substitutional insertion of amino acids in *impE1*, *impE2* modifications

To confirm the positional importance of the representative three kinds of modifications (*i.e.*, *impE1*(E164K), *impE2*(V2I), and *impE2*(G64E)) with enhanced abilities to produce IMP as identified in the results above, a vector for introducing modifications (*e.g.*, a modification of substituting the 164th amino acid in the amino acid sequence of *impE1*, the 2nd amino acid in the amino acid sequence of *impE2*, and the 64th amino acid in the amino acid sequence of *impE2* with an another amino acid) was prepared.

Firstly, the procedure of preparing the vector for the introduction of the *ImpE1*(E164K) modification is as follows.

Based on the reported polynucleotide sequences, the chromosomal genes of *Corynebacterium stationis CJI0323* were isolated, and gene fragments were obtained by performing PCR using the chromosomal DNA of *Corynebacterium stationis CJI0323* as a template along with primer pairs between the primer of SEQ ID NO: 27 and each of SEQ ID

NOS: 28 to 45. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes. As a result, 18 kinds of 0.7 kbp polynucleotides were obtained. Then, the chromosomal genes of *Corynebacterium stationis* CJI0323 were isolated, and gene fragments were obtained by performing PCR using the chromosomal DNA of *Corynebacterium stationis* CJI0323 as a template along with primer pairs between the primer of SEQ ID NO: 46 and each of SEQ ID NOS: 47 to 64. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes. As a result, 18 kinds of 0.7 kbp polynucleotides were obtained.

Overlapping PCR was performed using two fragments obtained from the above results as a template, and thereby 18 kinds of 1.4 kbp polynucleotides to be used as templates were obtained. The obtained gene fragments were digested with a restriction enzyme, *Spe*I, ligated to the linearized *pDZ* vector, which had already been digested with a restriction enzyme, *Xba*I, transformed into *E. coli* *DH5 α* , and the transformants were plated on a solid LB medium containing kanamycin (25 mg/L).

The sequence information on the primers used for the preparation of the vector is shown in Table 8 below.

[Table 8]

SEQ ID NO	Primer	Sequence (5' to 3')
27	Spe1-impE1 164 1F	GGGACTAGTGATTCCGGCCAACTGTCTG
28	impE1 164-R 1R	TGGGGCGCGTTGGCGTTCAGGATGCTC
29	impE1164-H 1R	TGGGGCGCGTTGGTGTTCAGGATGCTC
30	impE1 164-D 1R	TGGGGCGCGTTGATCTTCAGGATGCTC
31	impE1 164-S 1R	TGGGGCGCGTTGGGATTCAGGATGCTC
32	impE1 164-T 1R	TGGGGCGCGTTGGGTTTCAGGATGCTC
33	impE1 164-N 1R	TGGGGCGCGTTGGTTTTTCAGGATGCTC
34	impE1 164-Q 1R	TGGGGCGCGTTGCTGTTCAGGATGCTC
35	impE1 164-C 1R	TGGGGCGCGTTGGCATTTCAGGATGCTC

36	impE1 164-G 1R	TGGGGCGCGTTGGCCTTCAGGATGCTC
37	impE1 164-P 1R	TGGGGCGCGTTGCGGTTTCAGGATGCTC
38	impE1 164-A 1R	TGGGGCGCGTTGGGCTTCAGGATGCTC
39	impE1 164-V 1R	TGGGGCGCGTTGGACTTCAGGATGCTC
40	impE1 164-I 1R	TGGGGCGCGTTGGATTTTCAGGATGCTC
41	impE1 164-L 1R	TGGGGCGCGTTGCAGTTCAGGATGCTC
42	impE1 164-M 1R	TGGGGCGCGTTGCATTTTCAGGATGCTC
43	impE1 164-F 1R	TGGGGCGCGTTGGAATTCAGGATGCTC
44	impE1 164-Y 1R	TGGGGCGCGTTGGTATTCAGGATGCTC
45	impE1 164-W 1R	TGGGGCGCGTTGCCATTCAGGATGCTC
46	Spe1-impE1 164 2R	GGGACTAGTCATGAACCTTGCCGCGCTC
47	impE1 164-R 2F	GAGCATCCTGAACGCCAACGCGCCCCA
48	impE1 164-H 2F	GAGCATCCTGAACACCAACGCGCCCCA
49	impE1 164-D 2F	GAGCATCCTGAAGATCAACGCGCCCCA
50	impE1 164-S 2F	GAGCATCCTGAATCCCAACGCGCCCCA
51	impE1 164-T 2F	GAGCATCCTGAAACCCAACGCGCCCCA
52	impE1 164-N 2F	GAGCATCCTGAAAACCAACGCGCCCCA
53	impE1 164-Q 2F	GAGCATCCTGAACAGCAACGCGCCCCA
54	impE1 164-C 2F	GAGCATCCTGAATGCCAACGCGCCCCA
55	impE1 164-G 2F	GAGCATCCTGAAGGCCAACGCGCCCCA
56	impE1 164-P 2F	GAGCATCCTGAACCGCAACGCGCCCCA
57	impE1 164-A 2F	GAGCATCCTGAAGCCCAACGCGCCCCA
58	impE1 164-V 2F	GAGCATCCTGAAGTCCAACGCGCCCCA
59	impE1 164-I 2F	GAGCATCCTGAAATCCAACGCGCCCCA
60	impE1 164-L 2F	GAGCATCCTGAACTGCAACGCGCCCCA
61	impE1 164-M 2F	GAGCATCCTGAAATGCAACGCGCCCCA
62	impE1 164-F 2F	GAGCATCCTGAATTCCAACGCGCCCCA
63	impE1 164-Y 2F	GAGCATCCTGAATACCAACGCGCCCCA
64	impE1 164-W 2F	GAGCATCCTGAATGGCAACGCGCCCCA

After selecting by PCR the colonies transformed with the vector into which the target gene

was inserted, the plasmids were obtained using a conventionally known plasmid extraction method. The information on the obtained plasmids is shown in Table 9 below.

[Table 9]

No.	Plasmid
1	<i>pDZ-impE1 164R</i>
2	<i>pDZ-impE1 164H</i>
3	<i>pDZ-impE1 164D</i>
4	<i>pDZ-impE1 164S</i>
5	<i>pDZ-impE1 164T</i>
6	<i>pDZ-impE1 164N</i>
7	<i>pDZ-impE1 164Q</i>
8	<i>pDZ-impE1 164C</i>
9	<i>pDZ-impE1 164G</i>
10	<i>pDZ-impE1 164P</i>
11	<i>pDZ-impE1 164A</i>
12	<i>pDZ-impE1 164V</i>
13	<i>pDZ-impE1 164I</i>
14	<i>pDZ-impE1 164L</i>
15	<i>pDZ-impE1 164M</i>
16	<i>pDZ-impE1 164F</i>
17	<i>pDZ-impE1 164Y</i>
18	<i>pDZ-impE1 164W</i>

Secondly, the procedure of preparing the vector for the introduction of the ImpE2(V2I) is as follows.

Based on the reported polynucleotide sequences, the chromosomal genes of *Corynebacterium stationis* CJI0323 were isolated, and gene fragments were obtained by performing PCR using the chromosomal DNA of *Corynebacterium stationis* CJI0323 as a template along with primer pairs between the primer of SEQ ID NO: 65 and each of SEQ ID NOS: 66 to 83. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles

consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes. As a result, 18 kinds of 0.7 kbp polynucleotides were obtained. Then, the chromosomal genes of *Corynebacterium stationis* CJI0323 were isolated, and gene fragments were obtained by performing PCR using the chromosomal DNA of *Corynebacterium stationis* CJI0323 as a template along with primer pairs between the primer of SEQ ID NO: 84 and each of SEQ ID NOS: 85 to 102. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes. As a result, 18 kinds of 0.7 kbp polynucleotides were obtained.

Overlapping PCR was performed using two fragments obtained from the above results as a template, and thereby 18 kinds of 1.4 kbp polynucleotides to be used as templates were obtained. The obtained gene fragments were digested with a restriction enzyme, *Xba*I, ligated to the linearized *pDZ* vector, which had already been digested with a restriction enzyme, *Xba*I, transformed into *E. coli* *DH5α*, and the transformants were plated on a solid LB medium containing kanamycin (25 mg/L).

The sequence information on the primers used for the preparation of the vector is shown in Table 10 below.

[Table 10]

SEQ ID NO	Primer	Sequence (5' to 3')
65	XbaI-impE2 2 1F	GGGTCTAGATTGCATGCTGTGCAAGA
66	impE2 2-R 1R	GGAGTTTTTTAGCGCGCATTCCAGTCCT
67	impE2 2-H 1R	GGAGTTTTTTAGCGTGCATTCCAGTCCT
68	impE2 2-K 1R	GGAGTTTTTTAGCCTTCATTCCAGTCCT
69	impE2 2-D 1R	GGAGTTTTTTAGCGTCCATTCCAGTCCT
70	impE2 2-E 1R	GGAGTTTTTTAGCTTCCATTCCAGTCCT
71	impE2 2-S 1R	GGAGTTTTTTAGCGGACATTCCAGTCCT
72	impE2 2-T 1R	GGAGTTTTTTAGCGGTCATTCCAGTCCT
73	impE2 2-N 1R	GGAGTTTTTTAGCGTTCATTCCAGTCCT
74	impE2 2-Q 1R	GGAGTTTTTTAGCCTGCATTCCAGTCCT

75	impE2 2-C 1R	GGAGTTTTTTAGCGCACATTCCAGTCCT
76	impE2 2-G 1R	GGAGTTTTTTAGCGCCCATTCAGTCCT
77	impE2 2-P 1R	GGAGTTTTTTAGCTGGCATTCAGTCCT
78	impE2 2-A 1R	GGAGTTTTTTAGCAGCCATTCCAGTCCT
79	impE2 2-L 1R	GGAGTTTTTTAGCCAGCATTCAGTCCT
80	impE2 2-M 1R	GGAGTTTTTTAGCCATCATTCAGTCCT
81	impE2 2-F 1R	GGAGTTTTTTAGCGAACATTCCAGTCCT
82	impE2 2-Y 1R	GGAGTTTTTTAGCGTACATTCCAGTCCT
83	impE2 2-W 1R	GGAGTTTTTTAGCCCACATTCCAGTCCT
84	XbaI-impE2 2 2R	GGGTCTAGATTGCTCGCCACGCGCA
85	impE2 2-R 2F	AGGACTGGAATGCGCGCTAAAAACTCC
86	impE2 2-H 2F	AGGACTGGAATGCACGCTAAAAACTCC
87	impE2 2-K 2F	AGGACTGGAATGAAGGCTAAAAACTCC
88	impE2 2-D 2F	AGGACTGGAATGGACGCTAAAAACTCC
89	impE2 2-E 2F	AGGACTGGAATGGAAGCTAAAAACTCC
90	impE2 2-S 2F	AGGACTGGAATGTCCGCTAAAAACTCC
91	impE2 2-T 2F	AGGACTGGAATGACCGCTAAAAACTCC
92	impE2 2-N 2F	AGGACTGGAATGAACGCTAAAAACTCC
93	impE2 2-Q 2F	AGGACTGGAATGCAGGCTAAAAACTCC
94	impE2 2-C 2F	AGGACTGGAATGTGCGCTAAAAACTCC
95	impE2 2-G 2F	AGGACTGGAATGGGCGCTAAAAACTCC
96	impE2 2-P 2F	AGGACTGGAATGCCAGCTAAAAACTCC
97	impE2 2-A 2F	AGGACTGGAATGGCTGCTAAAAACTCC
98	impE2 2-L 2F	AGGACTGGAATGCTGGCTAAAAACTCC
99	impE2 2-M 2F	AGGACTGGAATGATGGCTAAAAACTCC
100	impE2 2-F 2F	AGGACTGGAATGTTCGCTAAAAACTCC
101	impE2 2-Y 2F	AGGACTGGAATGTACGCTAAAAACTCC
102	impE2 2-W 2F	AGGACTGGAATGTGGGCTAAAAACTCC

After selecting by PCR the colonies transformed with the vector into which the target gene was inserted, the plasmids were obtained using a conventionally known plasmid extraction

method. The information on the obtained plasmids is shown in Table 11 below.

[Table 11]

No.	Plasmid
1	<i>pDZ-impE2 2R</i>
2	<i>pDZ-impE2 2H</i>
3	<i>pDZ-impE2 2K</i>
4	<i>pDZ-impE2 2D</i>
5	<i>pDZ-impE2 2E</i>
6	<i>pDZ-impE2 2S</i>
7	<i>pDZ-impE2 2T</i>
8	<i>pDZ-impE2 2N</i>
9	<i>pDZ-impE2 2Q</i>
10	<i>pDZ-impE2 2C</i>
11	<i>pDZ-impE2 2G</i>
12	<i>pDZ-impE2 2P</i>
13	<i>pDZ-impE2 2A</i>
14	<i>pDZ-impE2 2L</i>
15	<i>pDZ-impE2 2M</i>
16	<i>pDZ-impE2 2F</i>
17	<i>pDZ-impE2 2Y</i>
18	<i>pDZ-impE2 2W</i>

Lastly, the procedure of preparing the vector for the introduction of the ImpE2(G64E) is as follows.

Based on the reported polynucleotide sequences, the chromosomal genes of *Corynebacterium stationis* CJI0323 were isolated, and gene fragments were obtained by performing PCR using the chromosomal DNA of *Corynebacterium stationis* CJI0323 as a template denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and poly along with primer pairs between the primer of SEQ ID NO: 103 and each of SEQ ID NOS: 104 to 121. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles consisting of

denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes. As a result, 18 kinds of 1 kbp polynucleotides were obtained. Then, the chromosomal genes of *Corynebacterium stationis* CJI0323 were isolated, and gene fragments were obtained by performing PCR using the chromosomal DNA of *Corynebacterium stationis* CJI0323 as a template along with primer pairs between the primer of SEQ ID NO: 84 and each of SEQ ID NOS: 85 to 102. PCR was performed by initial denaturation at 94°C for 5 minutes; 20 cycles consisting of polymerization at 72°C for 1 minute; and final polymerization at 72°C for 5 minutes. As a result, 18 kinds of 1 kbp polynucleotides were obtained.

Overlapping PCR was performed using two fragments obtained from the above results as a template, and thereby 18 kinds of 2 kbp polynucleotides to be used as templates were obtained. The obtained gene fragments were digested with a restriction enzyme, *Xba*I, ligated to the linearized *pDZ* vector, which had already been digested with a restriction enzyme, *Xba*I, transformed into *E. coli* *DH5α*, and the transformants were plated on a solid LB medium containing kanamycin (25 mg/L).

The sequence information on the primers used for the preparation of the vector is shown in Table 12 below.

[Table 12]

SEQ ID NO	Primer	Sequence (5' to 3')
103	XbaI-impE2 64 1F	GGGTCTAGAAAAGAGCTTAAGGCAGCTGCT
104	impE2 64-R 1R	GAAAATCATCTGGCGCAAAGAGCTCAT
105	impE2 64-H 1R	GAAAATCATCTGGTGCAAAGAGCTCAT
106	impE2 64-D 1R	GAAAATCATCTGGTCCAAAGAGCTCAT
107	impE2 64-K 1R	GAAAATCATCTGCTTCAAAGAGCTCAT
108	impE2 64-S 1R	GAAAATCATCTGGGACAAAGAGCTCAT
109	impE2 64-T 1R	GAAAATCATCTGGGTCAAAGAGCTCAT
110	impE2 64-N 1R	GAAAATCATCTGGTTCAAAGAGCTCAT
111	impE2 64-Q 1R	GAAAATCATCTGCTGCAAAGAGCTCAT
112	impE2 64-C 1R	GAAAATCATCTGGCACAAAGAGCTCAT
113	impE2 64-P 1R	GAAAATCATCTGTGGCAAAGAGCTCAT

114	impE2 64-A 1R	GAAAATCATCTGAGCCAAAGAGCTCAT
115	impE2 64-V 1R	GAAAATCATCTGGACCAAAGAGCTCAT
116	impE2 64-I 1R	GAAAATCATCTGGATCAAAGAGCTCAT
117	impE2 64-L 1R	GAAAATCATCTGCAGCAAAGAGCTCAT
118	impE2 64-M 1R	GAAAATCATCTGCATCAAAGAGCTCAT
119	impE2 64-F 1R	GAAAATCATCTGGAACAAAGAGCTCAT
120	impE2 64-Y 1R	GAAAATCATCTGGTACAAAGAGCTCAT
121	impE2 64-W 1R	GAAAATCATCTGCCACAAAGAGCTCAT
122	XbaI-impE2 64 2R	GGGTCTAGACGGTCAATGAAGTCTCAACGG
123	impE2 64-R 2F	ATGAGCTCTTTGCGCCAGATGATTTTC
124	impE2 64-H 2F	ATGAGCTCTTTGCACCAGATGATTTTC
125	impE2 64-D 2F	ATGAGCTCTTTGGACCAGATGATTTTC
126	impE2 64-K 2F	ATGAGCTCTTTGAAGCAGATGATTTTC
127	impE2 64-S 2F	ATGAGCTCTTTGTCCCAGATGATTTTC
128	impE2 64-T 2F	ATGAGCTCTTTGACCCAGATGATTTTC
129	impE2 64-N 2F	ATGAGCTCTTTGAACCAGATGATTTTC
130	impE2 64-Q 2F	ATGAGCTCTTTGCAGCAGATGATTTTC
131	impE2 64-C 2F	ATGAGCTCTTTGTGCCAGATGATTTTC
132	impE2 64-P 2F	ATGAGCTCTTTGCCACAGATGATTTTC
133	impE2 64-A 2F	ATGAGCTCTTTGGCTCAGATGATTTTC
134	impE2 64-V 2F	ATGAGCTCTTTGGTCCAGATGATTTTC
135	impE2 64-I 2F	ATGAGCTCTTTGATCCAGATGATTTTC
136	impE2 64-L 2F	ATGAGCTCTTTGCTGCAGATGATTTTC
137	impE2 64-M 2F	ATGAGCTCTTTGATGCAGATGATTTTC
138	impE2 64-F 2F	ATGAGCTCTTTGTTCCAGATGATTTTC
139	impE2 64-Y 2F	ATGAGCTCTTTGTACCAGATGATTTTC
140	impE2 64-W 2F	ATGAGCTCTTTGTGGCAGATGATTTTC

After selecting by PCR the colonies transformed with the vector into which the target gene was inserted, the plasmids were obtained using a conventionally known plasmid extraction method. The information on the obtained plasmids is shown in Table 13 below.

[Table 13]

No.	Plasmid
1	<i>pDZ-impE2 64R</i>
2	<i>pDZ-impE2 64H</i>
3	<i>pDZ-impE2 64D</i>
4	<i>pDZ-impE2 64K</i>
5	<i>pDZ-impE2 64S</i>
6	<i>pDZ-impE2 64T</i>
7	<i>pDZ-impE2 64N</i>
8	<i>pDZ-impE2 64Q</i>
9	<i>pDZ-impE2 64C</i>
10	<i>pDZ-impE2 64P</i>
11	<i>pDZ-impE2 64A</i>
12	<i>pDZ-impE2 64V</i>
13	<i>pDZ-impE2 64I</i>
14	<i>pDZ-impE2 64L</i>
15	<i>pDZ-impE2 64M</i>
16	<i>pDZ-impE2 64F</i>
17	<i>pDZ-impE2 64Y</i>
18	<i>pDZ-impE2 64W</i>

Example 5-2: Preparation of strains where amino acids at positions of modified products (ImpE1, ImpE2) are substituted with another amino acids, and comparison of ability to produce IMP

The 54 kinds of plasmids prepared in Example 5-1 were transformed into the *CJI0323* strain. The strains in which the vector was inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over. The introduction of the modification in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 15 and 16, followed by nucleotide sequencing analysis. The strain

names according to the inserted modifications are shown in Table 14 below.

[Table 14]

No.	Strain
1	<i>CJI0323::impE1(E164R)</i>
2	<i>CJI0323::impE1(E164H)</i>
3	<i>CJI0323::impE1(E164D)</i>
4	<i>CJI0323::impE1(E164S)</i>
5	<i>CJI0323::impE1(E164T)</i>
6	<i>CJI0323::impE1(E164N)</i>
7	<i>CJI0323::impE1(E164Q)</i>
8	<i>CJI0323::impE1(E164C)</i>
9	<i>CJI0323::impE1(E164G)</i>
10	<i>CJI0323::impE1(E164P)</i>
11	<i>CJI0323::impE1(E164A)</i>
12	<i>CJI0323::impE1(E164V)</i>
13	<i>CJI0323::impE1(E164I)</i>
14	<i>CJI0323::impE1(E164L)</i>
15	<i>CJI0323::impE1(E164M)</i>
16	<i>CJI0323::impE1(E164F)</i>
17	<i>CJI0323::impE1(E164Y)</i>
18	<i>CJI0323::impE1(E164W)</i>
19	<i>CJI0323::impE2(V2R)</i>
20	<i>CJI0323::impE2(V2H)</i>
21	<i>CJI0323::impE2(V2K)</i>
22	<i>CJI0323::impE2(V2D)</i>
23	<i>CJI0323::impE2(V2E)</i>
24	<i>CJI0323::impE2(V2S)</i>
25	<i>CJI0323::impE2(V2T)</i>
26	<i>CJI0323::impE2(V2N)</i>
27	<i>CJI0323::impE2(V2Q)</i>

28	<i>CJI0323::impE2(V2C)</i>
29	<i>CJI0323::impE2(V2G)</i>
30	<i>CJI0323::impE2(V2P)</i>
31	<i>CJI0323::impE2(V2A)</i>
32	<i>CJI0323::impE2(V2L)</i>
33	<i>CJI0323::impE2(V2M)</i>
34	<i>CJI0323::impE2(V2F)</i>
35	<i>CJI0323::impE2(V2Y)</i>
36	<i>CJI0323::impE2(V2W)</i>
37	<i>CJI0323::impE2(G64R)</i>
38	<i>CJI0323::impE2(G64H)</i>
39	<i>CJI0323:impE2(G64D)</i>
40	<i>CJI0323::impE2(G64K)</i>
41	<i>CJI0323::impE2(G64S)</i>
42	<i>CJI0323::impE2(G64T)</i>
43	<i>CJI0323::impE2(G64N)</i>
44	<i>CJI0323::impE2(G64Q)</i>
45	<i>CJI0323::impE2(G64C)</i>
46	<i>CJI0323::impE2(G64P)</i>
47	<i>CJI0323::impE2(G64A)</i>
48	<i>CJI0323::impE2(G64V)</i>
49	<i>CJI0323::impE2(G64I)</i>
50	<i>CJI0323:impE2(G64L)</i>
51	<i>CJI0323::impE2(G64M)</i>
52	<i>CJI0323::impE2(G64F)</i>
53	<i>CJI0323:impE2(G64Y)</i>
54	<i>CJI0323::impE2(G64W)</i>

The cultivation was performed in the same manner as in Example 1 and the concentration of IMP produced thereof was analyzed (Table 15).

[Table 15]

Concentration (g/L) of IMP production in strains with combined introduction of <i>impE1</i> , <i>impE2</i> modifications		
Strain		Average IMP
Control	<i>CJI0323_impE1E2</i> (WT)	2.32
1	<i>CJI0323::impE1</i> (E164R)	9.42
2	<i>CJI0323::impE1</i> (E164H)	8.47
3	<i>CJI0323::impE1</i> (E164D)	7.37
4	<i>CJI0323::impE1</i> (E164S)	8.56
5	<i>CJI0323::impE1</i> (E164T)	8.85
6	<i>CJI0323::impE1</i> (E164N)	9.13
7	<i>CJI0323::impE1</i> (E164Q)	7.45
8	<i>CJI0323::impE1</i> (E164C)	7.37
9	<i>CJI0323::impE1</i> (E164G)	9.13
10	<i>CJI0323::impE1</i> (E164P)	9.43
11	<i>CJI0323::impE1</i> (E164A)	7.44
12	<i>CJI0323::impE1</i> (E164V)	8.18
13	<i>CJI0323::impE1</i> (E164I)	8.09
14	<i>CJI0323::impE1</i> (E164L)	7.85
15	<i>CJI0323::impE1</i> (E164M)	7.39
16	<i>CJI0323::impE1</i> (E164F)	7.56
17	<i>CJI0323::impE1</i> (E164Y)	7.60
18	<i>CJI0323::impE1</i> (E164W)	8.56
19	<i>CJI0323::impE2</i> (V2R)	7.99
20	<i>CJI0323::impE2</i> (V2H)	8.75
21	<i>CJI0323::impE2</i> (V2K)	8.66
22	<i>CJI0323::impE2</i> (V2D)	8.28
23	<i>CJI0323::impE2</i> (V2E)	9.32
24	<i>CJI0323::impE2</i> (V2S)	6.37
25	<i>CJI0323::impE2</i> (V2T)	8.37
26	<i>CJI0323::impE2</i> (V2N)	9.80

27	<i>CJI0323::impE2(V2Q)</i>	7.04
28	<i>CJI0323::impE2(V2C)</i>	7.23
29	<i>CJI0323::impE2(V2G)</i>	7.71
30	<i>CJI0323::impE2(V2P)</i>	7.80
31	<i>CJI0323::impE2(V2A)</i>	6.57
32	<i>CJI0323::impE2(V2L)</i>	6.42
33	<i>CJI0323::impE2(V2M)</i>	9.20
34	<i>CJI0323::impE2(V2F)</i>	9.43
35	<i>CJI0323::impE2(V2Y)</i>	8.37
36	<i>CJI0323::impE2(V2W)</i>	7.22
37	<i>CJI0323::impE2(G64R)</i>	4.42
38	<i>CJI0323::impE2(G64H)</i>	5.14
39	<i>CJI0323::impE2(G64D)</i>	11.53
40	<i>CJI0323::impE2(G64K)</i>	4.8
41	<i>CJI0323::impE2(G64S)</i>	5.7
42	<i>CJI0323::impE2(G64T)</i>	5.52
43	<i>CJI0323::impE2(G64N)</i>	5.9
44	<i>CJI0323::impE2(G64Q)</i>	4.8
45	<i>CJI0323::impE2(G64C)</i>	5.9
46	<i>CJI0323::impE2(G64P)</i>	4.75
47	<i>CJI0323::impE2(G64A)</i>	4.58
48	<i>CJI0323::impE2(G64V)</i>	4.56
49	<i>CJI0323::impE2(G64I)</i>	5.89
50	<i>CJI0323::impE2(G64L)</i>	5.6
51	<i>CJI0323::impE2(G64M)</i>	4.3
52	<i>CJI0323::impE2(G64F)</i>	5.89
53	<i>CJI0323::impE2(G64Y)</i>	4.6
54	<i>CJI0323::impE2(G64W)</i>	4.76

As shown above, all of the modified strains showed an increase in the ability to produce IMP compared to each of the control strains, and thus, it was confirmed that the three positions

of modification are important sites that have a significant effect on the increase of the ability of the ImpE protein with respect to IMP export.

Example 6: Introduction of *impE1*, *impE2* modifications based on IMP-producing strains

Example 6-1: Preparation of strains with *impE1*, *impE2* modifications based on IMP-producing strains

To confirm the effect of introduction of *impE1* and *impE2* modifications, An IMP-producing strain was prepared in which the activities of adenylosuccinate synthetase and IMP dehydrogenase corresponding to the degradation pathway of IMP in the *ATCC6872* strain were attenuated. The initiation codon was changed by changing the first base from ‘a’ to ‘t’ in each nucleotide sequence of the two genes *purA* and *guaB*, which encode the two enzymes. The strain in which the expression of the two genes was attenuated in the *ATCC6872* strain was named *CJI9088*. The *pDZ-impE1(E164K)*, *pDZ-impE2(V2I)*, and *pDZ-impE2(G64E)* vectors prepared in Example 4-2 were transformed into the *CJI9088* strain by electroporation, and the *pDZ-impE2(G64D)* vector prepared in Example 5-1 was transformed into the *CJI9088_impE1(E164K)_impE2(V2I)* strain by electroporation. The strains in which the vectors were inserted into the chromosome by recombination of the homologous sequences were selected on a medium containing kanamycin (25 mg/L). The selected primary strains were subjected to a second cross-over. The introduction of the modification in the finally transformed strains was confirmed by performing PCR using the primer pair of SEQ ID NOS: 15 and 16, followed by nucleotide sequencing analysis.

The ability of the prepared strains (*i.e.*, *CJI9088*, *CJI9088_impE1(E164K)*, *CJI9088_impE2(V2I)*, *CJI9088_impE2(G64E)*, and *CJI9088_impE1(E164K)_impE2(V2I)(G64D)*) to produce IMP was evaluated. Upon completion of the culture, the amount of IMP production was measured by HPLC and the results are shown in Table 16 below.

[Table 16]

Strain	IMP (g/L)
<i>CJI9088</i>	0.52

<i>CJI9088_impE1(E164K)</i>	0.84
<i>CJI9088_impE2(V2I)</i>	0.93
<i>CJI9088_impE2(G64E)</i>	1.73
<i>CJI9088_impE1(E164K)_impE2(V2I)(G64D)</i>	4.30

Upon confirming the amount of IMP accumulated in the culture medium, it was confirmed that these strains showed an increase of IMP production by at least 61%, and a maximum increase of 727%, compared to the parent strain, CJ9088. Accordingly, the increase in the amount of IMP production due to modifications of the ImpE protein of the present disclosure can be interpreted to be very meaningful.

From the foregoing, a skilled person in the art to which the present disclosure pertains will be able to understand that the present disclosure may be embodied in other specific forms without modifying the technical concepts or essential characteristics of the present disclosure. In this regard, the exemplary embodiments disclosed herein are only for illustrative purposes and should not be construed as limiting the scope of the present disclosure. On the contrary, the present disclosure is intended to cover not only the exemplary embodiments but also various alternatives, modifications, equivalents, and other embodiments that may be included within the spirit and scope of the present disclosure as defined by the appended claims.



BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. CJ CheilJedang Corporation
CJ CHEILJEDANG CENTER,
330, DONGHO-RO,
JUNG-GU, SEOUL 100-400
REPUBLIC OF KOREA

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Corynebacterium stationis</i> CN01-0323	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM12151P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on November. 07. 2017 (date of the original deposit). ¹	
IV. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjeonae-2ga-gil Seodaemun-gu SEOUL 120-861 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: November. 07. 2017.

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

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한국미생물보존센터

03641 서울시 서대문구 홍제내2가길 45 유림빌딩 Tel: 02-391-0950, 396-0950 Fax: 02-392-2859

KOREAN CULTURE CENTER OF MICROORGANISMS

Yoolim Bldg., 45, Hongjeonae 2ga-gil, Seodaemun-gu, Seoul 03641, Korea Tel: 82-2-391-0950, 396-0950 Fax: 82-2-392-2859



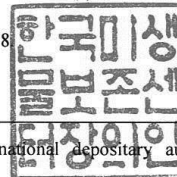
BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. CJ CheilJedang Corporation
CJ CHEILJEDANG CENTER,
330, DONGHO-RO,
JUNG-GU, SEOUL 100-400
REPUBLIC OF KOREA

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Corynebacterium stationis</i> CJI0323_impE1(E164K)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM12359P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on November. 02. 2018 (date of the original deposit). ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjenae-2ga-gil Seodaemun-gu SEOUL 03641 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: November. 02. 2018



¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

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KOREAN CULTURE CENTER OF MICROORGANISMS

KOREAN FEDERATION OF CULTURE COLLECTIONS

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FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

To. CJ CheilJedang Corporation
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330, DONGHO-RO,
JUNG-GU, SEOUL 100-400
REPUBLIC OF KOREA

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INTERNATIONAL DEPOSITARY AUTHORITY
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I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Corynebacterium stationis</i> CJ10323_impE2(V2I)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM12360P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on November. 02. 2018 (date of the original deposit). ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjeonae-2ga-gil Seodaemun-gu SEOUL 03641 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: November. 02. 2018.

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

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INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Corynebacterium stationis</i> CJ0323_impE2(G64E)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCCM12361P
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☐ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
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V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name : Korean Culture Center of Microorganisms Address : Yurim B/D 45, Hongjena-e-2ga-gil Seodaemun-gu SEOUL 03641 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: November. 02. 2018.

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[CLAIMS]

[Claim 1]

A protein variant exporting 5'-inosine monophosphate, wherein, in the amino acid sequence of SEQ ID NO: 2, (i) the 2nd amino acid, (ii) the 64th amino acid, or (iii) the 2nd amino acid and the 64th amino acid are each substituted with another amino acid.

[Claim 2]

The protein variant according to claim 1, wherein the 2nd amino acid is substituted with an amino acid selected from the group consisting of isoleucine, phenylalanine, methionine, glutamic acid, histidine, and asparagine; (ii) the 64th amino acid is substituted with an amino acid selected from the group consisting of aspartate, glutamic acid, asparagine, cysteine, isoleucine, and phenylalanine; or (iii) the 2nd amino acid and the 64th amino acid are each substituted with an amino acid selected from the group consisting of methionine, glutamic acid, histidine, asparagine, aspartate, cysteine, isoleucine, and phenylalanine.

[Claim 3]

A polynucleotide encoding the protein variant of claim 1 or 2.

[Claim 4]

A vector comprising the polynucleotide of claim 3.

[Claim 5]

A microorganism of the genus *Corynebacterium* producing 5'-inosine monophosphate, which comprises the protein variant of claim 1 or 2; or a vector comprising the polynucleotide encoding the protein variant of claim 1 or 2.

[Claim 6]

The microorganism of the genus *Corynebacterium* according to claim 5, further comprising a protein variant, in which the 164th amino acid of SEQ ID NO: 1 is substituted with another amino acid.

[Claim 7]

The microorganism of the genus *Corynebacterium* according to claim 6, wherein the

164th amino acid of SEQ ID NO: 1 is substituted with an amino acid selected from the group consisting of lysine, arginine, asparagine, glycine, threonine, and proline.

[Claim 8]

The microorganism of the genus *Corynebacterium* according to claim 5, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium stationis*.

[Claim 9]

The microorganism of the genus *Corynebacterium* according to claim 6, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium stationis*.

[Claim 10]

A method for preparing 5'-inosine monophosphate, comprising culturing the microorganism of the genus *Corynebacterium* of claim 5 in a medium; and recovering 5'-inosine monophosphate from the microorganism or medium.

[Claim 11]

A method for preparing 5'-inosine monophosphate, comprising culturing the microorganism of the genus *Corynebacterium* of claim 6 in a medium; and recovering 5'-inosine monophosphate from the microorganism or medium.

[Claim 12]

The method according to claim 10, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium stationis*.

[Claim 13]

The method according to claim 11, wherein the microorganism of the genus *Corynebacterium* is *Corynebacterium stationis*.