



US008748158B2

(12) **United States Patent**
Kameyama

(10) **Patent No.:** **US 8,748,158 B2**
(45) **Date of Patent:** **Jun. 10, 2014**

(54) **MICROORGANISM HAVING
GASTRIC-JUICE PROMOTING ACTIVITY,
AND ITS SECRETORY PRODUCT**

(76) Inventor: **Yoshichika Kameyama**, Habikino (JP)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 638 days.

(21) Appl. No.: **12/090,482**

(22) PCT Filed: **Aug. 10, 2007**

(86) PCT No.: **PCT/JP2007/065772**

§ 371 (c)(1),
(2), (4) Date: **Apr. 17, 2008**

(87) PCT Pub. No.: **WO2009/022399**

PCT Pub. Date: **Feb. 19, 2009**

(65) **Prior Publication Data**

US 2010/0291052 A1 Nov. 18, 2010

(51) **Int. Cl.**
C12N 1/20 (2006.01)

(52) **U.S. Cl.**
USPC **435/252.5; 424/93.46**

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,393,225 A * 7/1983 Hayashi et al. 549/289

OTHER PUBLICATIONS

Database GenBank [online], Accessin No. AB195283, /www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?66347569:OLD:419493> May 20, 2005 uploaded, Pham, V. T. et al., Definition: *Bacillus pumilus* gene for 16s rRNA, strain: Tbl.*

* cited by examiner

Primary Examiner — Irene Marx

(74) *Attorney, Agent, or Firm* — James W. Judge

(57) **ABSTRACT**

Designed to afford a novel microorganism promoting gastric juice secretion and having platelet increasing activity, and to afford a pharmaceutical agent composed of a product secreted by the novel microorganism. The novel microorganism, international deposit number: NITE BP-295, belongs to the species *Bacillus pumilus*, and is characterized by taking either form of coccus and *bacillus*, and makes figure-eight movement. The novel microorganism of the present invention is the novel microorganism having the gene represented by SEQ ID No: 1, and an object of the present invention is to afford the microorganism and a gastric juice secretion-promoting composition composed of the product secreted by the novel microorganism.

3 Claims, 10 Drawing Sheets

FIG. 1

【BLAST Top 20】

Sequences producing significant alignments:

	Score	E
	(bits) Value	
gi 45269058 gb AY548955.1 Bacillus pumilus strain S9 16S r...	2926	0.0
gi 45269052 gb AY548949.1 Bacillus pumilus strain 8N-4 16S...	2926	0.0
gi 45934529 gb AY505512.1 Bacillus pumilus strain GSP61 16...	2902	0.0
gi 16973340 emb AJ315067.1 BSP315067 Bacillus sp. 19499 16S...	2892	0.0
gi 34809007 gb AY373359.1 Bacillus pumilus strain c10 16S ...	2878	0.0
gi 46560693 gb AY587832.1 Bacterium Te68R 16S ribosomal RN...	2872	0.0
gi 14009321 gb AY030327.1 Bacillus pumilus strain KL-052 1...	2870	0.0
gi 45934531 gb AY505514.1 Bacillus sp. GSP46 16S ribosomal...	2859	0.0
gi 10129890 dbj AB048252.1 Bacillus pumilus gene for 16S r...	2859	0.0
gi 27530897 dbj AB098578.1 Bacillus pumilus gene for 16S r...	2839	0.0
gi 40888887 gb AY289549.1 Bacillus pumilus strain SA175001...	2833	0.0
gi 30171831 gb AY260861.1 Bacillus pumilus strain WN694 16...	2821	0.0
gi 21748162 emb AJ494730.1 BPU494730 Bacillus pumilus parti...	2821	0.0
gi 27497691 gb AY167884.1 Bacillus pumilus 16S ribosomal R...	2817	0.0
gi 27497690 gb AY167883.1 Bacillus pumilus 16S ribosomal R...	2817	0.0
gi 27497688 gb AY167881.1 Bacillus pumilus 16S ribosomal R...	2817	0.0
gi 27497687 gb AY167880.1 Bacillus pumilus 16S ribosomal R...	2817	0.0
gi 33354180 dbj AB115957.1 Bacillus pumilus gene for 16S r...	2817	0.0
gi 46560675 gb AY587814.1 Bacterium Te29R 16S ribosomal RN...	2811	0.0
gi 3970889 dbj AB020208.1 Bacillus pumilus DNA for 16S rib...	2809	0.0

FIG. 2

【Top hit】
 >gj|45269058|gb|AY548955.1| Bacillus pumilus strain S9 16S ribosomal RNA gene,
 complete sequence
 Length = 1544

Score = 2926 bits (1476), Expect = 0.0
 Identities = 1479/1480 (99%)
 Strand = Plus / Plus

```

Query: 1      tcgagcggacagaaggagcttgctcccggatgtagcgggacgggtgagtaacacgt 60
            |||
Sbjct: 55      tcgagcggacagaaggagcttgctcccggatgtagcgggacgggtgagtaacacgt 114
  
```

FIG. 3

```
Query: 61  gggtaacctgcctgtaagactgggataactccgggaaccggagctaataccggatagtt 120
          |||
Sbjct: 115  gggtaacctgcctgtaagactgggataactccgggaaccggagctaataccggatagat 174

Query: 121  ccttgaaccgcatgggtcaaggatgaaagacggtttcggctgtcacttacagatggacc 180
          |||
Sbjct: 175  ccttgaaccgcatgggtcaaggatgaaagacggtttcggctgtcacttacagatggacc 234

Query: 181  gcggcgccattagctagttgggtgggtaatggctcaccaaggcgacgatgcgtagccgacc 240
          |||
Sbjct: 235  gcggcgccattagctagttgggtgggtaatggctcaccaaggcgacgatgcgtagccgacc 294

Query: 241  tgagaggggtgatcggccacactgggactgagacacggcccagactcctacgggagggcagc 300
          |||
Sbjct: 296  tgagaggggtgatcggccacactgggactgagacacggcccagactcctacgggagggcagc 354

Query: 301  agtagggaatcttccgcaatggacgaaagtctgacggagcaacgcccgctgagtgatgaa 360
          |||
Sbjct: 355  agtagggaatcttccgcaatggacgaaagtctgacggagcaacgcccgctgagtgatgaa 414

Query: 361  ggttttcggatcgtaaaagctctgttgttagggaagaacaagtgcgagagtaactgctcgc 420
          |||
Sbjct: 415  ggttttcggatcgtaaaagctctgttgttagggaagaacaagtgcgagagtaactgctcgc 474

Query: 421  accttgaocggtacctaaccagaaagccacggctaactacgtgcccagcagccgggtaata 480
          |||
Sbjct: 475  accttgaocggtacctaaccagaaagccacggctaactacgtgcccagcagccgggtaata 534

Query: 481  cgtaggtggcaagcggttgcggaattattggcgtaaaagggctcgcagggcggtttctta 540
          |||
Sbjct: 535  cgtaggtggcaagcggttgcggaattattggcgtaaaagggctcgcagggcggtttctta 594

Query: 541  agtctgatgtgaaagccccggctcaaccggggagggtcattggaaactgggaaacttga 600
          |||
Sbjct: 595  agtctgatgtgaaagccccggctcaaccggggagggtcattggaaactgggaaacttga 654

Query: 601  gtgcagaagaggagagtggaattccacgtgtagcggtgaaatcgctagagatgtggagga 660
          |||
Sbjct: 655  gtgcagaagaggagagtggaattccacgtgtagcggtgaaatcgctagagatgtggagga 714

Query: 661  acaccagtgggcgaaggcgactctctggtctgtaactgacgctgaggagcgaagcggtggg 720
          |||
Sbjct: 715  acaccagtgggcgaaggcgactctctggtctgtaactgacgctgaggagcgaagcggtggg 774

Query: 721  gagcgaacaggattagataccctggtagtccacgccgtaaacgatgagtgctaagtgtta 780
          |||
Sbjct: 775  gagcgaacaggattagataccctggtagtccacgccgtaaacgatgagtgctaagtgtta 834

Query: 781  gggggtttccgccccttagtgctgcagctaacgcattaagcaactccgctggggagtaag 840
          |||
Sbjct: 835  gggggtttccgccccttagtgctgcagctaacgcattaagcaactccgctggggagtaag 894
```

FIG. 4

Query: 841 gtcgcaagactgaaactcaaaggaattgacgggggcccgcacaagcggaggagcatgtgg 900
|||||
Sbjct: 895 gtcgcaagactgaaactcaaaggaattgacgggggcccgcacaagcggaggagcatgtgg 954

Query: 901 ttttaattcgaagcaacgcaagaacaccttaccaggctcttgacatcctctgacaaccctaga 960
|||||
Sbjct: 955 ttttaattcgaagcaacgcaagaacaccttaccaggctcttgacatcctctgacaaccctaga 1014

Query: 961 gatagggctttcccttcggggacagagtgacaggtggtgcatggttgctcagctcgtg 1020
|||||
Sbjct: 1015 gatagggctttcccttcggggacagagtgacaggtggtgcatggttgctcagctcgtg 1074

Query: 1021 tcgtgagatggtgggtaagtcccgcacagcgcacaacccttgatcttagttgccagcat 1080
|||||
Sbjct: 1075 tcgtgagatggtgggtaagtcccgcacagcgcacaacccttgatcttagttgccagcat 1134

Query: 1081 tcagttgggcactctaaggtgactgccggtgacaaaaccggaggaaggtggggatgacgtc 1140
|||||
Sbjct: 1135 tcagttgggcactctaaggtgactgccggtgacaaaaccggaggaaggtggggatgacgtc 1194

Query: 1141 aaatcatcatgccccttatgacctgggctacacacgtgctacaatggacagaacaagg 1200
|||||
Sbjct: 1195 aaatcatcatgccccttatgacctgggctacacacgtgctacaatggacagaacaagg 1254

Query: 1201 ctgcaagaccgcaaggttagccaatcccataaatctgttctcagttcggatcgcagtct 1260
|||||
Sbjct: 1255 ctgcaagaccgcaaggttagccaatcccataaatctgttctcagttcggatcgcagtct 1314

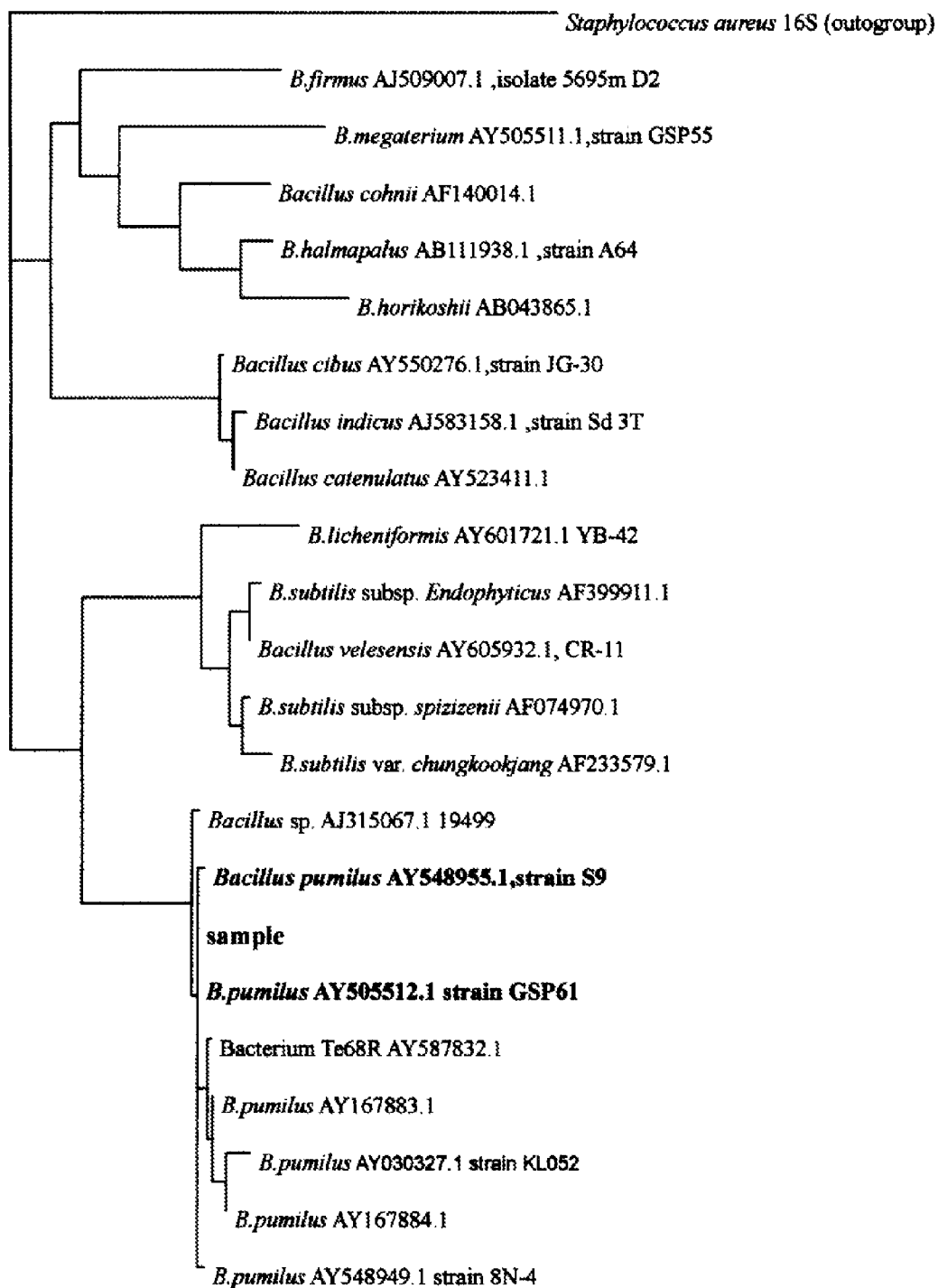
Query: 1261 gcaactcgactgcgtgaagctggaatcgctagtaatcgcggtatcagcatgccgcggtgaa 1320
|||||
Sbjct: 1315 gcaactcgactgcgtgaagctggaatcgctagtaatcgcggtatcagcatgccgcggtgaa 1374

Query: 1321 tacgttcccgggccttgtagcacaccgcccgtcacaccacgagagtttgcaacaccogaag 1380
|||||
Sbjct: 1375 tacgttcccgggccttgtagcacaccgcccgtcacaccacgagagtttgcaacaccogaag 1434

Query: 1381 tcggtgaggtaacctttatggagccagccgccgaaggtggggcagatgattggggtgaa 1440
|||||
Sbjct: 1435 tcggtgaggtaacctttatggagccagccgccgaaggtggggcagatgattggggtgaa 1494

Query: 1441 tcgtaacaaggtagccgtatcggaaggtgcccgtggatca 1480
|||||
Sbjct: 1495 tcgtaacaaggtagccgtatcggaaggtgcccgtggatca 1534

FIG. 5



0.01

FIG. 6



FIG. 7

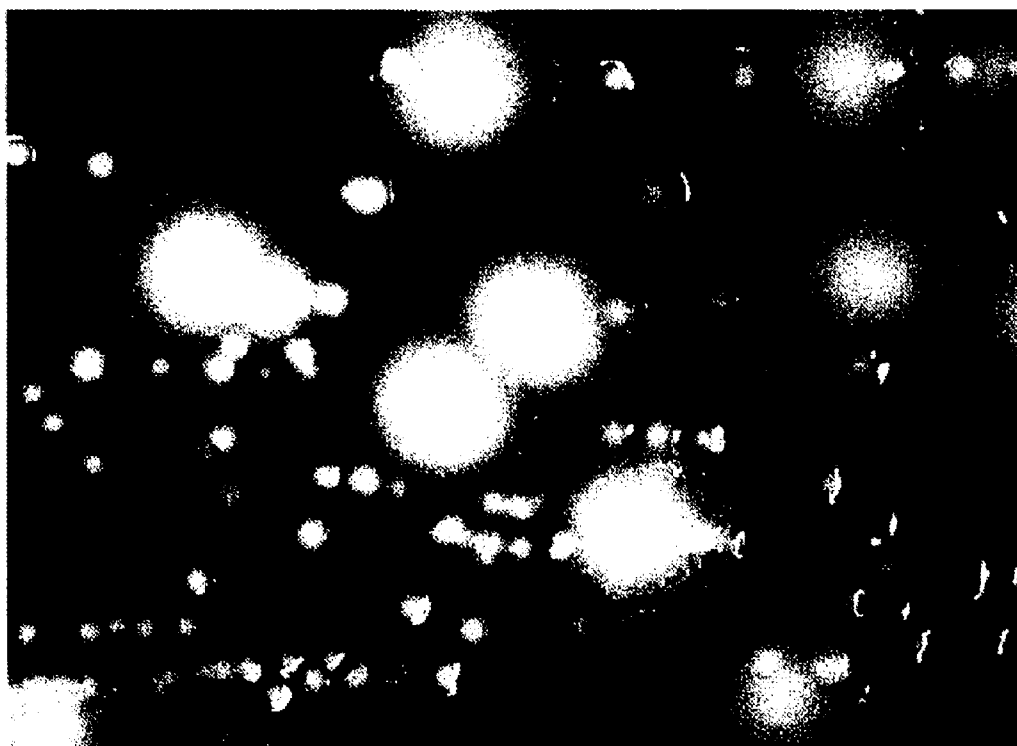


FIG. 8



FIG. 9

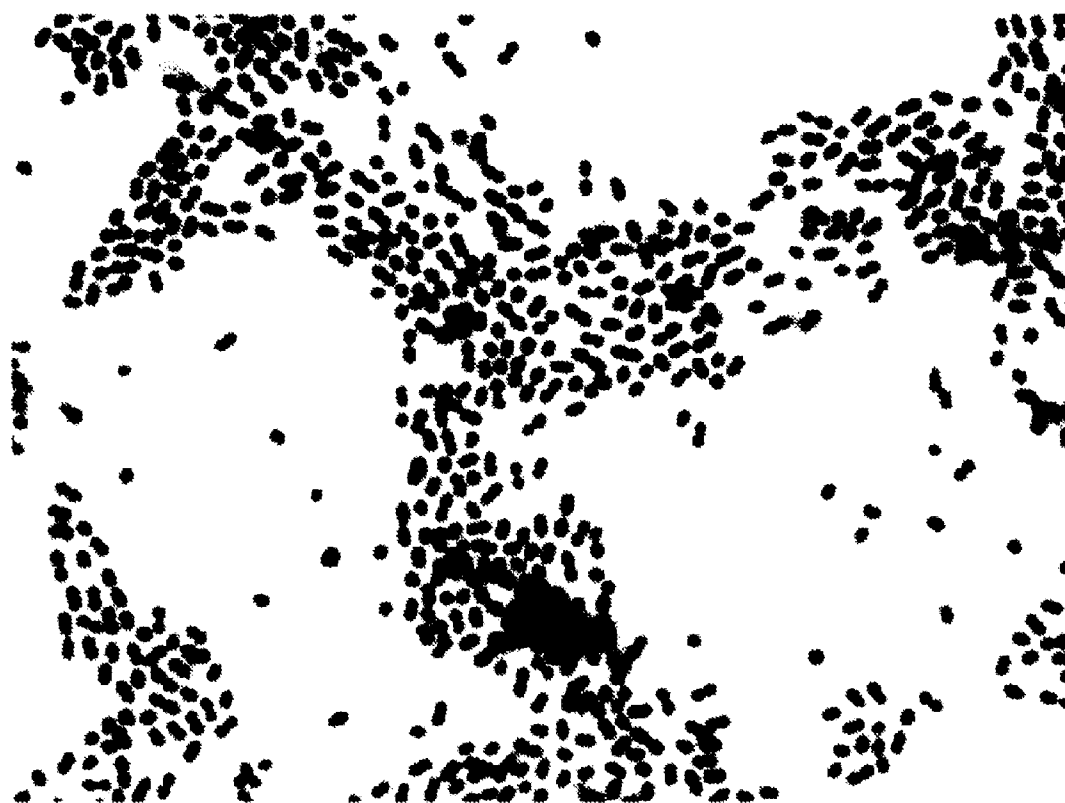
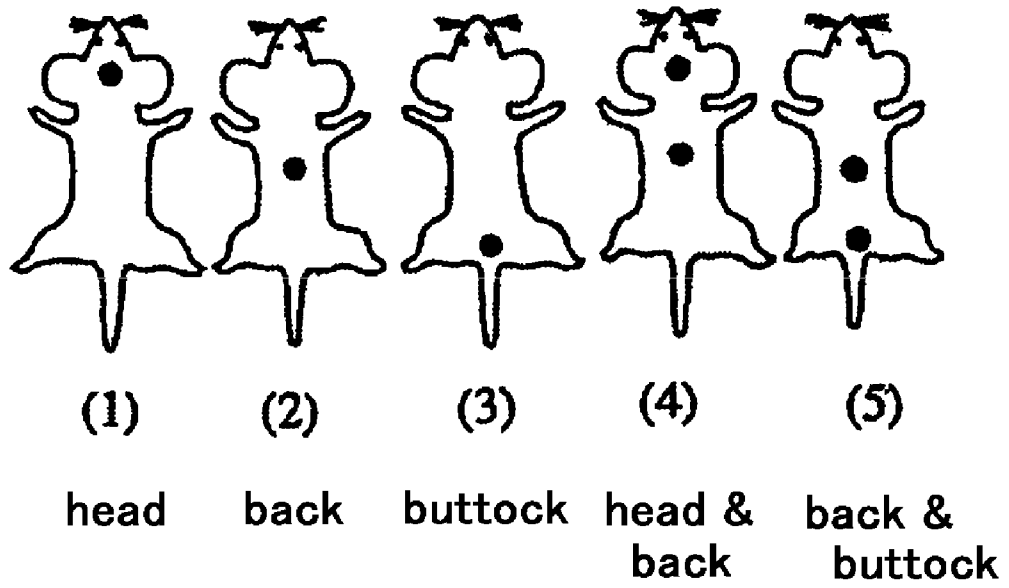


FIG. 10



**MICROORGANISM HAVING
GASTRIC-JUICE PROMOTING ACTIVITY,
AND ITS SECRETORY PRODUCT**

BACKGROUND OF THE INVENTION

1. Technical Field

The present invention, involving a novel microorganism belonging to the species *Bacillus pumilus*, relates to a novel microorganism, and to its secretory products and plasmids employing them, having an activity that prompts the secretion of gastric juice, countering gastric-acid decrease due to chronic gastritis.

2. Description of the Related Art

For gastric ulcers, duodenal ulcers, and other peptic ulcers, drugs (such as gastric-acid secretion blockers, and gastric antacids) that suppress digestive fluids and other visceral-wall invasive factors, and drugs (such as mucoprotective agents) that reinforce defense mechanisms have been used. Nevertheless, although promoting gastric-acid secretion presumably should be effective in patients with chronic gastritis, particularly atrophic gastritis, the present situation is that gastric-juice promoters that are nontoxic to living organisms have not been developed.

In cases of *Helicobacter pylori* infection, which is one of the causes of gastric ulcers, chronic gastritis is engendered, ultimately ending in inducing ulcers and cancer. That is, the bacteria is deleterious to the gastric condition; yet the reality is that a microorganism that improves the gastric condition while being a kindred microbe has yet to be discovered.

Accordingly, the discovery of a gastric-health meliorating microorganism antagonistic to *H. pylori* has been desired. Meanwhile, the manufacture of therapeutic agents utilizing discovered novel microorganisms and their secretory products has been an issue.

BRIEF SUMMARY OF THE INVENTION

According to experiments by the inventors, for an environment that is totally the opposite of *H. pylori*, that is, to counter chronic gastritis—particularly, the type of chronic gastritis in which gastric acid decreases—a novel microorganism of the present invention promotes gastric juice secretion to alleviate the chronic gastritis, and moreover is hypothesized to prevent cancer and ulcers from chronic gastritis. Thus, the novel microorganism can be detected from stomach and blood of all persons, and can also be detected from the blood of patients with chronic gastritis, ulcers, or cancer.

Not only does it have properties advantageous to promoting gastric-juice secretion, but because it also exhibits platelet-, erythrocyte-, and leukocyte-increasing action, it enables multifarious applications.

As a result of animal experiments, effectiveness in promoting gastric juice secretion was confirmed in a novel microorganism (International Deposit Number: NITE BP-295) belonging to the species *Bacillus pumilus*; moreover, it proved to be nontoxic because its LD₅₀ is 2 g or more.

This microorganism can be both punctuate and catenulate in form, is 0.5 to 1 μm×10 to 20 μm in size with a flagellum on either end, and has motility, making figure-eight movements. It has spores, and is a highly aerobic gram-positive bacillus and coccus. It takes on a catenulate form. The isolation source is the human stomach wall or blood. Alternatively, a virus giving rise to chronic gastritis may be implanted in a fertilized ovum and the antagonizing microbes that appear may be collected.

The cultivation conditions are as below. Per 1000 ml medium, (trypto-soya broth) are added the nutrients

- peptone—17 g,
 - soybean peptone—3 g,
 - sodium chloride—5 g,
 - glucose—2.5 g, and
 - potassium hydrogenphosphate—2.5 g,
- and 3 g caustic soda is added in. The pH is adjusted to 8.5. Nutrient medium sterilization conditions: 121° C., perform 15 minutes; cultivation temperature: 37° C.; cultivation period: from 2 to 7 days.

The bacteria cultivated under the above conditions are characterized by being obligatory-aerobic; viability confirmation is by unaided visual observation or by observation under a microscope.

Storage conditions: possible by generally employed methods, including freeze drying.

The novel microorganism thus collected has the following characteristics. It should be noted that they are principally by microscopic observation.

1. When it phagocytoses pathogens, antibacterial properties intensify.
2. Changes form depending on the pathogenic bacterium.
3. Secretory substances that are metabolites are brown.
4. Does not invade erythrocytes. (As to erythrocytes: When ordinary anticancer drugs are employed, the red blood cell count will not decrease—with the Hb remaining 12 to 16, the red blood cell count does not decrease; with hepatitis C, it goes to about 6.)
5. The novel microorganism cleanses dead tissue.
6. The novel microorganism is susceptible to radioactive beams, ultraviolet rays, and microacoustic waves.
7. The novel microorganism is highly heat-resistant—can withstand 100° C. for several hours, and does not lyse. Survives autoclaving.
8. Prompts increase in erythrocytes, platelets, and leukocytes; remedying of anemia is seen.

(From the fact that in a patient whose leukocyte was 6000, a white-blood-cell count of over 500 was verified even after being administered an anticancer drug, the augmenting action owing to this bacterium was verified. From the fact that in a patient whose platelet was 200,000, it fell only to the 3,000 level even after the patient was administered an anticancer drug, the augmenting action owing to this bacterium was verified.)

Its sequence: Partial sequencing was carried out; proved to have the genetic sequence represented by Seq. No. 1. Furthermore, as illustrated in FIGS. 2 through 4, the microorganism was determined to be, with 99% homology, the species *Bacillus pumilus*. The phylogenetic tree is as in FIG. 5. With the sequence having been partially specified, application to, for example, plasmid expression vectors and other practical uses are possible, and in large-scale culturing, screening, and drug manufacture numerous benefits are anticipated.

The microorganism of the invention was deposited under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure as Deposit No. NTE BP-295, with the full taxonomic description "*Bacillus pumilus*," on Dec. 25, 2006, at the Incorporated Administrative Agency, National Institute of Technology and Evaluation, Patent Microorganisms Depository (NPM), 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken 292-0818, Japan.

A method of extracting the secretory products is as follows. First, bacteria are cultivated in broth at a temperature of 33 to 37° C. for seven days. Next, pure butyl alcohol, in the same amount as, is added is stirred well into the culture filtrate. After leaving the mixture for three hours, or centrifuging it,

the clear butyl alcohol liquid is separated off. Hydrochloric acid is added to the liquid to bring the pH to 3.0, and the mixture is stirred thoroughly and left for 12 hours. The mixture is then vacuum-dried to obtain bright yellow crystals, and organic and inorganic substances.

The solution apart from the butyl alcohol liquid is mixed in with active carbon, stirred well, and left for one day.

Active carbon alone is added to butyl alcohol, and after 12 hours the butyl alcohol eluate is subjected to vacuum drying. Thereafter repeating likewise, crystal is obtained.

The present invention also relates to a method of manufacturing secretory products from the present invention, utilizing a phenotypically transformed microorganism incorporating an expression vector having a DNA sequence such as will code the amino-acid sequence of the secretory product. A phenotypically transformed microorganism according to the present invention is cultivated in the manner described above, and the secretory product is isolated from the nutrient medium.

The novel microorganism of the present invention and its secretory products have activity prompting secretion of gastric acid, and are heat-resistant. The extract is hypothesized to counter chronic gastritis of the type in which gastric acid decreases, and other chronic gastritis, by promoting gastric acid secretion to alleviate the chronic gastritis, and, by regulating the stomach condition, in turn to prevent cancer and ulcers from the chronic gastritis. The bacterium is detected from the stomach and blood of all persons, and occasionally from the blood of patients with chronic gastritis, ulcers, or cancer.

Not only does it have properties advantageous to promoting gastric-juice secretion, but because it also exhibits platelet-, erythrocyte-, and leukocyte-increasing action, and moreover is nontoxic, it enables multifarious applications.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1 is a homology search explanatory diagram.

FIG. 2 is a diagram presenting homology search results (bases 1-60).

FIG. 3 is a diagram presenting homology search results (bases 61-840).

FIG. 4 is a diagram presenting homology search results (bases 841-1480).

FIG. 5 is a diagram presenting a phylogenetic tree of a novel microorganism of the present invention.

FIG. 6 is a diagram presenting a nutrient-medium colony of the novel microorganism of the present invention.

FIG. 7 is an enlarged view of FIG. 6.

FIG. 8 is a diagram presenting a Gram-stain of a large colony.

FIG. 9 is a diagram presenting a Gram-stain of a small colony.

FIG. 10 is a diagram representing an individual identifying method in which pigment is applied to mouse fur.

DETAILED DESCRIPTION OF THE INVENTION

The present invention becomes apparent from typical embodiments cited in the following examples, but the present invention is not limited to the scope of the examples.

In the embodiments of the present invention, Embodiment 1 first represents cultivation conditions and results, and Embodiment 2 next describes how to separate secretory product off, and animal experiment employing the secretory product. Furthermore, antitumor test (Embodiment 3), toxicity

test (Embodiment 4), clinical test (Embodiment 5), staining test (Embodiment 6) are explained.

First Embodiment

This microorganism is punctuate or catenulate in shape and 0.5 to 1 $\mu\text{m} \times 10$ to 20 μm in size with flagella on its both ends, and has motility to perform figure-eight movement. Although the microorganism is aerobic gram-positive *bacillus* or coccus with spores, it also takes on catenulate shape. The isolation source is the human stomach wall or blood. The cultivation conditions are as below. Per 1000 ml medium, (tryptsoya broth) are added the nutrients

peptone—17 g,

soybean peptone—3 g,

sodium chloride—5 g,

glucose—2.5 g, and

potassium hydrogenphosphate—2.5 g,

and 3 g caustic soda is added in. The pH is adjusted to 8.5. Nutrient medium sterilization conditions: 121° C., perform 15 minutes; cultivation temperature: 37° C.; cultivation period: from 2 to 7 days. (FIGS. 6, 7, 8 and 9.)

The bacteria cultivated under the above conditions are characterized by being obligatory-aerobic; viability confirmation is by unaided visual observation or by observation under a microscope. FIG. 6 presents an image of colonies cultivated for one day in the designated medium.

Furthermore, that of the colonies which is boxed in a square on the right is the colony whose surface is greatly rough, and that of the colonies which is boxed in a rectangular on the left is the small colony whose surface is smooth. FIG. 7 is an enlarged part of the colonies. Results of Gram-staining demonstrate that the right side colony is gram-positive *bacillus* (FIG. 8), and the left side colony is gram-positive coccus (FIG. 9). As to the conditions under which the bacteria are stored, they can be stored in ways generally employed, including freeze dehydration.

Second Embodiment

As to a change in animal gastric juice, a reaction to secretion-product doses was checked.

How Secretion Product Extracted

The secretory product was extracted in the following manner: The colonies were put in an incubator with temperature of 37° C., and when the surfaces of the colonies became right gray or right yellow surfaces after two to seven days, the colonies were put in broth to cultivate bacteria for seven days at a temperature of 30° C. to 37° C.

Next, into the culture filtrate, the same amount of pure butyl alcohol as the culture filtrate was stirred.

After the culture filtrate into which the butyl alcohol had been stirred was left for three hours, or was centrifuged, clear butyl alcohol solution was separated off.

The butyl alcohol solution was added with decinomal hydrochloric acid to bring pH to 3.0, stirred thoroughly, and left for 12 hours. Subsequently, the solution was vacuum-dried to obtain right yellow crystal, organic and inorganic substances.

A solution other than butyl alcohol solution and active carbon was mixed, stirred thoroughly, and left for one day.

Only active carbon was added to butyl alcohol, and after 12 hours, butyl alcohol eluate was subjected to vacuum-dry. Afterwards, such procedures were repeated to obtain crystal. Animal Experiment

In Embodiment 2 of the present invention, as to the product secreted by the novel microorganism, a test for measuring the amount of gastric juice secretion in Heidenhain pouch dogs was carried out.

Experimental: As medium, carbohydrate solution was used in the proportion of 5% to the secretory product of 2.6 g (and was stored at room temperatures).

Experimental animal: male dogs purchased at the age of 13 months were for 13 days medically inspected, acclimated, and bread. By observing their normal conditions, and by measuring their weights, whether or not the dogs are healthy animals was checked to use 13 month-old animals whose weights on the day of surgery was from 14.2 to 14.7 kg.

Environment: The experimental environment was arranged in the range in which a temperature was from 20 to 28° C., a relative humidity was from 30 to 80%, the times of ventilation was from 12 to 18 times per hour, lighting hours were 2 hours (from 7:00 to 19:00).

Feed: Labo D Stock® (Nosan Corporation)

Drinking water: Water was taken freely from an automatic feed device in a water supply and sewerage system.

Administration Method: The secretory product of 500 mg was put in a mortar, and then the carbohydrate solution in the proportion of 5% was put in the mortar to dissolve the secretory product with a pestle. The carbohydrate solution was added to the dissolved secretory product so as to be 50 ml, and was rendered a dosing solution.

Dosing solution weight: 10 mg/kg

Dosing solution volume: 1 mL/kg, three examples

Measurement Method: The amount of secreted gastric acid was measured every 15 minutes within 2 hours after the dosing solution was administered, and changes in the secreted gastric acid amount were studied.

Heidenhain Pouch Dog Preparation and Management

The dogs were given nothing to eat for 18 hours or more as of the day before the surgery, and atropine hydrosulfate (0.1 mg/kg) was intramuscularly administered in the dogs 30 minutes before anesthesia induction. Sodium thiopental was given to the dogs from their forelimb cephalic veins to induce anesthesia, and then the dogs were laid on the back on a moisturizing pad of a body temperature controller, with the pad temperature being arranged to be 38° C. on a surgical bed. The dogs' tracheae were then cannulated and they were given

artificial respiration and an inhalant anesthetic. The artificial-respiration single inhale/exhale volume was made 20 to 25 mg/kg, at 11 to 13 cycles/min. The inhalant anesthetic was introduced with an added 1 to 4% having been nitrous-oxide gasified, and maintenance anesthesia at 0.5 to 2.0% was carried out. After the hair on and around the dogs' abdominal region was removed with an electric clipper, the entire operating area was sterilized and an incision was made through the skin and muscle layers along a medial line from slightly below the xiphisternum to above the navel region, and then the stomachs were withdrawn from the abdominal cavities to expose the stomachs on the abdominal walls. The blood vessels in the greater curvature of the stomach intersecting with an excision marking line were double-ligated, and cut. After that, the excision marking line was cut and sutured with a gastrointestinal suturing instrument and a gastrostomy tube was set alongside the pouches, and then the opened abdominal region was sutured and the pouch interior was washed several times with a warmed physiological saline solution. The dogs were given nothing to eat within two days from the day after the surgery. During the food deprivation, the dogs received fluid of 150 mL/day (Lactec D®, Otuka Pharmaceutical Co., Ltd). After the food deprivation, the dogs were bred as usual, and in order to prevent dehydration, dietary salt (approx. 0.4 g/day) was mixed into feed, and given to the dogs.

Gastric Juice Secretion Measurement Method

The Heidenhain pouch dogs were used three weeks after the surgery. The dogs were deprived of feed for 18 hours or more in the situation in which water was given, and their gastric juice was collected with the dogs being hung with an abdominal bandage in a steel pipe frame. The gastric juice was collected every 15 minutes from 30 minutes before, to two hours after, secretory product administration, and the gastric juice volume (mL) and acidity (value measured by neutralizing titration technique with 0.01 N NaOH: mEq/L) were measured to calculate from the obtained gastric juice volume and acidity the secreted gastric juice amount (mEq/15 min) at each measuring time.

The results are set forth in Tables 1 through 4.

TABLE 1

Change of secreted gastric juice amount by administrating (test substance) A											
Experimental	Secretion		secreted gastric juice amount (mEq/15 min)								
	amount		before	Inspection time after administration (min)							
Group	(mg/kg)	n = 3	administration	15	30	45	60	75	90	105	120
Secretion (testsubstance) ^d	10	Average standard deviation	0.82 0.09	0.97 0.12	0.96 0.16	1.18 0.19	1.20* 0.12	0.95 0.16	0.77 0.04	0.88* 0.08	0.72 0.05

*P < 0.05; Significant difference with respect to before administration rate (Paired t-test)

TABLE 2

Change of secreted gastric juice amount by administrating secretion (test substance) A											
Experimental	Individual	secreted gastric juice amount (mEq/15 min)									
		before	Inspection time after administration (min)								
Group	number	administration	15	30	45	60	75	90	105	120	
Secretion (testsubstance) ^d	1	0.75	0.80	0.90	1.20	1.00	0.65	0.72	0.80	0.64	
	2	1.00	1.20	1.25	1.50	1.40	1.20	0.84	1.05	0.80	
	3	0.72	0.90	0.72	0.84	1.20	1.00	0.75	0.80	0.72	

TABLE 3

Change of secreted gastric juice amount by administrating secretion (test substance) A											
Experimental Group	Secretion amount	n = 3	before administration	secreted gastric juice amount (mEq/15 min)							
				Inspection time after administration (min)							
				15	30	45	60	75	90	105	120
meat extract	25%	Average standard deviation	0.01	0.15	0.53	0.57	0.41	0.25	0.15	0.09	0.07
			0.0	0.1	0.1	0.0	0.0	0.1	0.0	0.0	0.0

TABLE 4

Change of secreted gastric juice amount by administrating meat extract											
Experimental Group	Individual number	before administration	secreted gastric juice amount (mEq/15 min)								
			Inspection time after administration (min)								
			15	30	45	60	75	90	105	120	
meat extract	1	0.02	0.27	0.55	0.52	0.42	0.31	0.19	0.12	0.12	
	2	0.02	0.16	0.61	0.57	0.32	0.31	0.20	0.11	0.07	
	3	0.00	0.02	0.42	0.61	0.48	0.14	0.07	0.03	0.02	

Results of administering the secretory product demonstrated gastric juice secretion amounts of 0.82 to 1.20 on an average before, and within two hours of, the secretory product administration, and demonstrated the largest increase in gastric acid secretion amount 60 minutes after the secretory product administration. The increment was 0.38 mEq/15 min.

Experimental results on individual dogs 1, 2, and 3 are set forth in Table 2. The results demonstrated statistically significant increases in gastric juice secretion amount 60 minutes and 105 minutes after the secretory product administration, compared with the gastric juice secretion amount before the administration. On an average, gastric juice secretion amounts were of 0.01 to 0.57 before, and within two hours of, meat extract administration (Tables 3 and 4). The amount of gastric acid secretion increased most largely 45 minutes after the meat extraction administration, and the increment was 0.56 mEq/15 min.

In the secretory product of the present invention, above examples showed statistically significant differences in gastric acid secretion 60 minutes and 105 minutes after the secretory product administration, and also showed a tendency for the gastric acid secretion to increase even 15, 30, 45 and 75 minutes after the gastric acid secretion administration, even though there was no statistically significant difference. Compared with the results of administering meat extract, the secretory product (test substance) administration results demonstrated slightly smaller increment than that in the meat extract administration results, but demonstrated at the time at which the gastric acid secretion most largely increased, the gastric acid secretion increase and decrease similar to those in the meat extract administration results. From these results, it is believed that the secretory product (test substance) is effective in increasing gastric juice secretion amount, and this effect was the same with that of the meat extract.

Third Embodiment

In embodiment 3 of the present invention, in order to search effectiveness of the secretory product to Xenograft model, study of influence of the secretory product on tumor cell line MKN-45 was attempted. The secretory product as test sub-

stance was administered subcutaneously in experimental animals for four weeks. As end points, weight measurement, tumor volume measurement, hematological test, and pathological test were carried out.

Experimental Overview

1. Experimental animal: Twenty animals 15.2 to 20.1 g in weight—BALB/C-nu-type male mice purchased at the age of four weeks—were used. Through the experimental period, the animals were bred in an animal room arranged in a region in which a temperature was 20 to 26° C., a relative humidity was 40 to 70%, the times of ventilation was 10 to 20 times per hour, lighting hours were 12 hours.

2. Breeding conditions: Water and feed are freely taken.

3. Experimental groups: When tumors developed to have an average volume of 200 to 300 mm³, the animals were divided into two groups.

4. Bulk powder of the product secreted by the novel micro-organism was precisely weighed with a top-loading balance for highly precise analysis, and was dissolved into normal saline solution of 10 mL. All dosing solutions were prepared when used.

5. Tumor cell preparation: Cell culture solution was prepared by adding to medium penicillin-streptomycin of 100 u/mL to 100 μmL.

Cells that had been frozen were thawed in warm water of approx. 37° C. to transfer the cells to a centrifuge tube containing the culture solution previously incubated at temperature of 37° C. After the culture solution in which the cells were transferred was centrifuged at 1000 rpm for five minutes, supernatant was cleared out, and the culture solution was added to the cells, and suspended thoroughly by pipetting. Subsequently, the suspension was moved into a culture flask to start culture in a CO₂ incubator (MCO-175 from Sanyo Electric Co., Ltd) in which temperature was arranged to be 37° C., and carbon dioxide concentration was arranged to be 5%. In subcultivating the cells, the culture solution was removed from the incubator before cell density increased excessively, and the surfaces of the cells were cleaned with PBS solution. After that, the cells were added with 0.25% trypsin and 1 m EDTA solution, put in the CO₂ incubator, and completely separated off. The separated cells were col-

lected with a proper amount of culture solution, and the cells and culture solution were centrifuged at 1000 rpm for 5 minutes. Then, supernatant was removed, and culture solution was added to the cells, and pipetted. After that, the cells were seeded in a different culture flask containing the culture solution. Subcultivation was carried out as frequently as two to four days, the cells subcultivated two times or more were used for transplantation.

6. Administration method: The test substances were subcutaneously administered dorsally in the mice once-daily for four weeks with a disposable syringe and a 27 G injection needle. The amount of dosing solution was calculated based on weights measured two times a week.

7. Tumor cells: Tumor cells were subcutaneously administered dorsally in the mice with the disposable syringe and 27 G injection needle on the day of transplantation.

In the foregoing experiment, normal condition of the mice and whether or not the mice were living were checked once a day, and their weights were measured twice a week. Furthermore, the tumor longest diameter (A) and shortest diameter (B) were measured twice a week with a digital caliper. From the measured longest and shortest diameters, tumor volume was calculated using the following expression.

$$\text{Tumor volume (mm}^3\text{)} = AB^2/2$$

Furthermore, hematological test was carried out. As to blood EDTA-2K-treated after taken from abdominal great veins of all the mice, the following inspection items were measured with an automatic hematology analyzer (Sysmex corporation, E-4000). The results are set forth in Table 5.

TABLE 5

Inspection Item	Measuring Method or Instrument	Unit/Sign
Red blood cell (RBC) count	Electrical-resistance detection	10 ⁴ /μL
Hematocrit (Ht)	Maximum erythroid pulse detection	%
Hemoglobin (Hb) content	SLS hemoglobin method	g/dL
Platelet (Plt) count	Electrical-resistance detection	10 ⁴ /μL
White blood cell (WBC) count	Electrical-resistance detection	10 ² /μL
Mean red blood cell index calculation method		
Mean corpuscular volume (MCV)	$\frac{\text{Ht}(\%)}{\text{RBC}(10^4/\mu\text{L})} \times 10^3$	fL
Mean corpuscular hemoglobin (MCH)	$\frac{\text{Hb}(\text{g/dL})}{\text{RBC}(10^4/\mu\text{L})} \times 10^3$	pg

TABLE 5-continued

Inspection Item	Measuring Method or Instrument	Unit/Sign
5 Mean corpuscular hemoglobin concentration (MCHC)	$\frac{\text{Hb}(\text{g/dL})}{\text{Ht}(\%)} \times 10^2$	%

10 Pathology test: After the blood drawing was completed, tumor tissues were extirpated to measure their weights, and then were fixed with a neutral buffered formalin solution. After the fixing, paraffin sections were prepared in accordance with a normal method, and then were subjected to hematoxylin-eosin staining. Subsequently, by carrying out immunohistochemical staining (TUNEL), positive cells in 15 1000 tumor cells were counted to calculate the ratio positive cells/tumor cells.

20 Twenty mice were used to subcutaneously transplant tumors dorsally in the mice. After the tumor transplantation, when the tumor developed to have an average volume of approx. 200 to 300 mm, the mice were divided into two groups so that the average tumor volume in one group equals to that in another. The test substances were administered subcutaneously in the mice continuously for four weeks. From the day of the test substance administration, normal condition of the mice, and whether or not the mice were living were observed once-daily for four weeks, and weights of the mice and sizes of the tumors were measured twice a week. 30 Four weeks after the test substance administration, blood of the mice was taken from their abdominal great veins under ether anesthesia, and tumor tissues were extirpated.

35 The foregoing test results will be described in detail.

1. Shifts in weight: Weight changes in the two groups for four weeks after the test substance administration were set forth in Tables 6 and 7. In both of the two groups: that of the groups in which normal saline solution was administered, and that of the groups in which the secretory product was administered, significant weight decreases were observed during the administration period. The peak of the weight decreases was on the twelfth day from the administration in the normal saline solution-administering group, and was on the second day from the administration in the secretory product-administering group. Compared with the weight on the first day from the administration, the normal saline solution-administering group experienced weight decrease of 1.9 g on the twelfth day, and the secretory product-administering group experienced weight decrease of 1.2 g on the second day.

TABLE 6

Influence of repeated oral dosing of secretory product (test substance) A for four weeks on weight in nude mouse antitumor test (MKN-45)											
sample		Weight (g)									
Dosage material	number	1	5	8	12	15	19	22	26	29 (day)	
Normal saline solution	10	Average	18.1	17.2*	17.3	16.2**	16.3**	16.5**	16.3**	16.7**	17.4
		standard deviation	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.5	0.5
Secretion (test substance) ⁴	10	Average	19.3	19.2	19.1	18.6**	18.2**	18.2**	18.1**	18.6*	19.4
		standard deviation	0.5	0.6	0.6	0.5	0.5	0.4	0.5	0.5	0.5

*, **p < 0.05, 0.01 Significant difference with respect to before administration rate (Paired t test)

TABLE 7

Influence on four weeks repetition oral administration of the secretion which gives it to the weight in the antitumor examination of the nude mouse (MKN-45)										
Dosage material	Animal No.	Weight (g)								
		1	5	8	12	15	19	22	26	29 (day)
Normal saline solution	1	17.2	16.8	17.6	17.0	17.0	16.9	17.3	17.4	17.7
	2	17.8	16.3	16.0	14.9	14.9	15.1	14.5	15.0	15.9
	3	18.7	18.6	18.6	17.4	18.0	18.0	17.8	18.8	19.2
	4	18.0	16.4	16.6	15.6	15.6	15.7	15.6	16.4	17.7
	5	17.6	17.0	17.5	16.6	16.9	17.1	16.6	16.2	18.0
	6	17.7	16.4	16.2	15.1	15.1	15.7	15.8	16.2	16.8
	7	19.5	18.7	19.3	17.8	18.1	18.5	18.0	18.7	19.0
	8	16.9	18.3	17.9	16.0	15.9	16.8	16.5	16.8	17.2
	9	18.7	16.5	16.3	15.6	15.6	15.9	15.3	15.6	16.0
	10	18.5	17.4	17.2	15.9	15.8	15.7	15.7	16.0	16.5
Secretion (test substance) ^d	11	20.8	19.3	19.7	19.1	19.3	18.7	18.9	19.4	20.1
	12	17.7	17.6	17.3	16.7	16.5	16.8	16.3	17.3	17.9
	13	16.5	15.9	16.0	15.9	15.4	15.8	15.5	15.5	16.7
	14	21.3	20.9	20.7	20.2	19.5	19.1	18.7	18.5	18.8
	15	19.6	19.8	19.7	19.6	19.0	18.9	18.8	19.7	20.0
	16	19.6	19.6	19.1	18.7	18.0	18.4	17.8	18.4	19.4
	17	18.9	19.3	19.2	18.3	18.1	18.3	18.3	19.1	20.6
	18	18.5	18.5	18.7	18.0	17.5	17.5	17.7	18.2	19.2
	19	21.0	22.3	22.2	20.9	20.4	20.4	20.6	21.3	22.2
	20	19.1	18.4	18.5	18.3	18.2	18.3	18.2	18.1	19.3

2. Tumor weight measurement: Tumor volume changes within four weeks of the test substance administration are set forth in Tables 8 to 9. The normal saline solution-administering group demonstrated day-by-day tumor volume increase during the solution administration period. Also in the

secretory product-administering group, the similar tumor volume increase to that in the normal saline solution-administering group was observed. Both of the two groups experienced no significant tumor volume increase on each measurement date.

TABLE 8

Influence of repeated oral dosing of secretory product (test substance) A for four weeks on tumor volume in nude mouse antitumor test (MKN-45)											
material	The number of the examples		tumor volume (mm ³)								
			1	5	8	12	15	19	22	26	29 (day)
Normal saline solution	10	Average	255.9	382.5	478.5	584.3	729.5	920.1	1094.6	1255.9	1479.9
		standard deviation	21.9	22.9	23.3	33.8	42.8	55.2	66.9	76.0	89.8
Secretion (test substance) ^d	10	Average	223.7	350.3	433.8	575.5	703.9	884.8	1014.1	1189.6	1412.2
		standard deviation	21.8	33.8	37.2	49.4	69.3	87.0	92.8	125.3	142.8

TABLE 9

Influence of repeated oral dosing of secretory product (test substance) A for four weeks on hematological inspection in nude mouse antitumor test (MKN-45)										
Dosage material	Animal No.	tumor volume (mm ³)								
		1	5	8	12	15	19	22	26	29 (day)
Normal saline solution	1	290.1	578.0	760.8	940.8	1136.2	1403.6	1811.6	1963.5	2216.8
	2	292.5	366.7	539.6	602.8	761.3	873.3	1207.1	1488.2	1851.3
	3	137.7	296.5	425.3	457.9	577.6	658.6	754.3	853.9	927.4
	4	193.9	309.0	348.0	524.3	629.3	719.3	901.6	1111.6	1432.1
	5	386.0	423.9	465.5	538.7	789.6	1074.6	1149.3	1362.9	1574.3
	6	252.4	342.4	433.4	593.0	621.9	780.4	899.2	951.7	1081.3
	7	234.5	371.2	465.7	543.3	685.6	875.5	1055.6	1204.0	1458.6
	8	215.1	282.4	377.4	408.9	533.3	792.4	957.2	1099.0	1325.4
	9	252.0	328.1	382.2	469.8	615.3	811.1	795.6	955.9	1207.3
	10	305.2	527.3	586.8	763.1	945.3	1212.3	1414.5	1568.1	1724.7
(secretion test substance) ^d	11	305.8	396.1	471.0	684.1	879.3	1050.8	1228.1	1326.9	1526.7
	12	321.4	445.5	536.2	686.7	804.8	958.2	1094.0	1617.4	1779.9
	13	256.0	379.5	405.0	450.6	540.7	821.5	915.1	990.9	1289.7
	14	169.0	306.6	366.1	539.3	652.9	767.3	843.7	941.5	1158.6

TABLE 9-continued

Influence of repeated oral dosing of secretory product (test substance) A for four weeks on hematological inspection in nude mouse antitumor test (MKN-45)										
Dosage	tumor volume (mm ³)									
material	Animal No.	1	5	8	12	15	19	22	26	29 (day)
	15	113.4	136.1	186.4	252.5	288.8	385.6	489.1	635.1	788.2
	16	245.0	436.4	564.1	766.4	993.7	1350.6	1453.8	1758.2	2002.8
	17	134.5	256.0	489.8	530.5	551.4	773.3	943.2	993.6	1146.9
	18	241.9	392.1	447.5	622.4	676.3	742.6	799.3	899.4	976.8
	19	210.8	326.1	402.7	605.0	819.3	984.6	1196.1	1495.0	1898.7
	20	239.1	428.5	469.3	617.2	831.7	1013.7	1178.6	1237.8	1553.7

3. Hematological test: Results of hematological test in the two groups within four weeks of the test substance administration are set forth in Tables 10 and 11. The secretory product-administering group experienced a more significant increase in platelet count among inspection items, compared

¹⁵ with the saline solution administering-group, but neither normal saline solution-administering group nor the secretion-administering group experienced any significant increase in other seven inspection items.

TABLE 10

Influence of repeated oral dosing of secretory product (test substance) A for four weeks on hematological inspection in nude mouse antitumor test (MKN-45)										
Administered substance	No. of Subjects		RBC (10 ⁴ /μL)	Ht (%)	Hb (g/dL)	MCV (fL)	MCH (pg)	MCHC (%)	Plt (10 ⁴ /μL)	WBC (10 ² /μL)
Normal saline solution	10	Average	1046.6	46.7	15.5	44.7	14.8	33.1	76.0	16.8
		± standard deviation	9.3	0.4	0.2	0.3	0.2	0.3	2.7	1.8
Secretion(test substance) ^d	10	Average	1040.6	45.6	14.9	43.9	14.3	32.7	94.0##	23.3
		± standard deviation	12.5	0.5	0.2	0.3	0.2	0.4	2.8	3.8

RBC: red blood cell count,

Ht: hematocrit,

Hb: hemoglobin content,

MCV: mean corpuscular volume,

MCH: mean corpuscular hemoglobin,

MCHC: mean corpuscular hemoglobin concentration,

Plt: platelet count,

WBC: white blood cell count

#, ##p < 0.05, 0.01 significant difference with respect to normal saline solution (student t test)

TABLE 11

Influence of repeated oral dosing of secretory product (test substance) A for four weeks on hematological inspection in nude mouse antitumor test (MKN-45)										
Dosage material	Animal No.	RBC (10 ⁴ /μL)	Ht (%)	Hb (g/dL)	MCV (fL)	MCH (pg)	MCHC (%)	Plt (10 ⁴ /μL)	WBC (10 ² /μL)	
Normal saline solution	1	1030	46.7	15.3	45.3	14.9	32.8	88.0	23	
	2	1051	46.1	15.6	43.9	14.8	33.8	59.5	19	
	3	1012	46.0	14.9	45.5	14.7	32.4	73.0	12	
	4	1019	44.5	14.6	43.7	14.3	32.8	77.8	10	
	5	1078	47.6	15.6	44.2	14.5	32.8	82.1	21	
	6	1030	45.4	14.7	44.1	14.3	32.4	71.3	10	
	7	1101	48.4	15.7	44.0	14.3	32.4	82.4	11	
	8	1044	48.4	17.0	46.4	16.3	35.1	69.4	15	
	9	1026	45.8	15.7	44.6	15.3	34.3	72.1	23	
	10	1075	48.3	15.5	44.9	14.4	32.1	84.0	24	
Secretion (test substance) ^d	11	1061	46.0	14.0	43.4	13.2	30.4	90.7	19	
	12	991	44.6	14.9	45.0	15.0	33.4	89.5	53	
	13	1021	46.5	14.9	45.5	14.6	32.0	94.1	23	
	14	1118	48.0	15.6	42.9	14.0	32.5	90.6	15	
	15	998	44.7	14.3	44.8	14.3	32.0	104.6	28	
	16	1053	45.3	15.1	43.0	14.3	33.3	100.0	22	
	17	1001	43.3	14.3	43.3	14.3	33.0	109.1	10	
	18	1074	47.7	15.5	44.4	14.4	32.5	94.1	28	

TABLE 11-continued

Influence of repeated oral dosing of secretory product (test substance) A for four weeks on hematological inspection in nude mouse antitumor test (MKN-45)									
Dosage material	Animal No.	RBC (10 ⁴ /μL)	Ht (%)	Hb (g/dL)	MCV (fL)	MCH (pg)	MCHC (%)	Plt (10 ⁴ /μL)	WBC (10 ² /μL)
	19	1049	45.1	15.9	43.0	15.2	35.3	89.6	12
	20	1040	45.1	14.7	43.4	14.1	32.6	77.9	23

RBC: red blood cell count,
 Ht: hematocrit,
 Hb: hemoglobin,
 MCV: mean corpuscular volume,
 MCH: mean corpuscular hemoglobin,
 MCHC: mean corpuscular hemoglobin concentration,
 Plt: platelet count,
 WBC: white blood cell count

4. Tumor weight measurement: Changes of tumor weight measured 4 weeks after the test substance administration are set forth in Tables 12 and 13. The tumor weights in the normal saline solution-administering and secretion-administering groups were respectively 0.093±0.109 g and 1.022±0.016 g, and neither of the two groups experienced significant tumor weight changes.

TABLE 12

Influence of repeated oral dosing of secretory product (test substance)A for four weeks on tumor weight in nude mouse antitumor test (MKN-45)			
Dosage material	sample No.		tumor weight (g)
Normal saline solution	10	Average ± standard deviation	1.093 ± 0.109
Secretion (test substance)A	10	Average ± standard deviation	1.022 ± 0.016

TABLE 13

Influence of repeated oral dosing of secretory product (test substance)A for four weeks on tumor weight in nude mouse antitumor test(MKN-45)			
Dosage material	Animal No.		tumor weight (g)
Normal saline solution	1		1.665
	2		1.534

TABLE 13-continued

Influence of repeated oral dosing of secretory product (test substance)A for four weeks on tumor weight in nude mouse antitumor test(MKN-45)			
Dosage material	Animal No.		tumor weight (g)
Secretion (test substance) ^d	3		0.925
	4		1.006
	5		0.605
	6		0.599
	7		1.141
	8		1.099
	9		1.220
	10		1.140
	11		1.174
	12		0.905
	13		0.937
	14		1.026
	15		0.751
	16		1.613
	17		1.047
	18		0.595
	19		1.658
	20		0.511

5. Pathological test: Average values in the two groups in pathological test within four weeks of the test substance administration are set forth in Tables 14 and 15. Results of calculating the ratio positive cells/tumor cells in the normal saline solution-administering and secretion-administering groups demonstrated no numerical significant change.

TABLE 14

Influence of repeated oral dosing of secretory product (test substance)A for four weeks on illness physical examination in nude mouse antitumor test (MKN-45)			
parameter	number of sample	Dosage material	
		Normal saline solution	Secretion (test substance) ^d
		10	10
Histological grade	Grade		
No therapeutic effects observed	0	10 (100.0) ^c	10 (100)
Degenerative changes in tumor cells, but no destruction of tumor nests	I	0	0
Destruction and disappearance of tumor nests, but viable cells remain			
viable cells occupy large areas (≥1/3)	IIa	0	0
viable cells occupy small areas (<1/3)	IIb	0	0

TABLE 14-continued

Influence of repeated oral dosing of secretory product (test substance)A for four weeks on illness physical examination in nude mouse antitumor test (MKN-45)			
parameter	number of sample	Dosage material	
		Normal saline solution 10	Secretion (test substance) ^d 10
Tumor cells remain but appear non-viable	III	0	0
No tumor cells remain	IV	0	0
TUNEL ^d index (% Average \pm standard deviation)		1.17 \pm 0.16	1.09 \pm 0.08

^cValue in parenthesis indicates the % incidence

^dTdT (terminal deoxynucleotidyl transferase) mediated dUTP-biotin Nick End Labeling

TABLE 15

Influence of repeated oral dosing of secretory product (test substance) A for four weeks on illness physical examination in nude mouse antitumor test (MKN-45)					
Dosage material	Animal No.	Organs/tissues	Findings	Histological grade	TUNEL index (%)
Normal saline solution	1	Tumor	No therapeutic effects observed	0	1.6
	2	Tumor	No therapeutic effects observed		1.2
	3	Tumor	No therapeutic effects observed	0	0.7
	4	Tumor	No therapeutic effects observed	0	1.2
	5	Tumor	No therapeutic effects observed	0	2.4
	6	Tumor	No therapeutic effects observed	0	0.9
	7	Tumor	No therapeutic effects observed	0	1.3
	8	Tumor	No therapeutic effects observed	0	0.8
	9	Tumor	No therapeutic effects observed	0	0.9
	10	Tumor	No therapeutic effects observed	0	0.7
	Average standard deviation				1.17 0.16
Secretion (test substance) ^d	11	Tumor	No therapeutic effects observed	0	0.9
	12	Tumor	No therapeutic effects observed	0	1.3
	13	Tumor	No therapeutic effects observed	0	1.0
	14	Tumor	No therapeutic effects observed	0	0.8
	15	Tumor	No therapeutic effects observed	0	0.9
	16	Tumor	No therapeutic effects observed	0	1.5
	17	Tumor	No therapeutic effects observed	0	1.2
	18	Tumor	No therapeutic effects observed	0	1.3
	19	Tumor	No therapeutic effects observed	0	1.2
	20	Tumor	No therapeutic effects observed	0	0.8
	Average standard deviation				1.09 0.08

45

In both of the normal saline solution-administering and secretory product-administering groups, as to weight during the administration period, above results demonstrated weight decrease until the second or third day from the administration, but showed a tendency of regaining the weight on the first day of the administration after the second or third day from the administration. Such a weight decrease is believed to occur under the influence of the tumor transplantation, not of the test substance, because the normal saline solution-administering group also experiences the weight decrease. As to tumor volume, in the normal saline solution-administering and secretory product-administering groups, a tumor volume increase similar to the tumor weight increase was observed during the administration period, and a tumor volume decrease caused by secretory product administration was not demonstrated. In the hematological test, the secretory product-administering group experienced a significant increase in platelet count. These results made it clear that the secretory product of the present invention dose not have activity increasing tumor cells. Accordingly, it is apparent that the secretory product of the present invention can be safely given

even to patients with stomach cancer and other tumors in digestive system in order to increase gastric juice of the patients.

In addition, although the weight decrease was demonstrated until the second or third day from the administration, no significant change was experienced. Tumor weight measurement: Tumor weight changes in the two groups within four weeks of the test substance administration are set forth in Tables 8 and 9. The normal saline solution-administering group demonstrated a day-by-day tumor volume increase during the administration period. Also in the secretory product-administering group, a similar tumor volume increase to that in the normal saline solution-administering group was observed. Neither of the two groups experienced any significant tumor volume increase.

Fourth Embodiment

Embodiment 4 (single subcutaneous dose toxicity test on mice with the product secreted by the novel microorganism) of the present invention will be described with FIG. 10. In this

test, a study was made as to lethal dose and toxicological appearance in the situation in which the product secreted by the novel microorganism was subcutaneously administered in the mice one time.

Secretory Product from Present-Invention Novel Microorganism Employed

1. Characteristics: brownish-yellow colored; acicular crystalline.
2. Solubility: Soluble in distilled water, and in 5% dextrose in water.
3. Stability: Stable because not hydrolyzable, air-oxidizing, photodegradable, or thermally degradable. At a pH of 8 or more, however, becomes clouded.
4. Storing conditions: Hermetically sealed (with a desiccant being included) at room temperature.
5. Storing location: Stored in a room-temperature cabinet.

Experimental Mice

1. Species, genealogy, sex: mouse {Slc:ICR}, SPF animal, male

2. Age in week: Mice that are four weeks old are grouped at the age of 5 weeks to start using the mice at the age of 6 weeks.

3. Weights of the mice when they are grouped: 29.4 to 33.9 g

4. Breeding environment conditions: A temperature is from 22.2 to 22.7° C.; a relative humidity is from 48.6 to 64.2%; RH•light-dark cycle is 12 hours; the times of ventilation is 10 times per hour; and feed and drinking water are taken freely.

5. Individual identification: In 5 animals in each of the administering groups, fur of the mice illustrated in FIG. 10 was applied with pigment by employing saturated picric acid solution to perform individual identification.

6. Dosing solution adjusting method: After the secretory product and solvent (distilled water) were weighed, they were gradually blended, crushed and made cloud, and then were rendered a predetermined amount of dosing solution.

7. Dosing solution amount: the dosing solution weight and grouping as shown in Table 5 with 2000 mg/kg that was the upper limit in guideline for non-clinical trials for pharmaceuticals being defined as high dose, dosing solution weight was 1,000 mg/kg half of the high dose, and a control (comparative group) was arranged.

8. Administration Method: Dosing solution was subcutaneously administered, and in both the administering groups, dosing solution of 20 mL/kg was administered. Each dosing solution calculated from weight right before the administration was administered subcutaneously in backs of necks of the mice with a syringe on which a 26 G needle was put. Administration period was determined to be only one time on the first day (Day 1), and the administration was carried out in the morning.

9. Observation period: Observation period was determined to be until the fifteenth day with the day of administration as the first day. As to when observation was carried out, whether or not the mice were living and normal condition of the mice were observed right after the administration, 1, 2, 4, and 6 hours after the administration only on the first day. On days other than the day of administration, whether or not the mice were living and normal condition of the mice were observed in the morning, and in the afternoon, only whether or not the mice were living was checked. In observing the normal condition, whether or not the mice was abnormal in appearance (fur, eyes, ears, nose, anus, and vulvar), behavior, posture, breathing, muscle stress, and enteruria, and the extent of the abnormality were observed with the naked eye, and weights of the mice were measured.

10. Pathology inspection: There is no fatal case in the test. The survived mice in the groups therefore were abdominally

operated on the fifteenth day under pentobarbital sodium salt anesthesia (100 mg/kg, intraperitoneal administration), and the abdominal great arterio-veins of the mice were cut to euthanize the mice by exsanguinations. After that, the mice were quickly dissected in accordance with pathological manner to observe organs and tissues on the surfaces of the bodies and in orifices, brainpans, chest cavities and abdominal cavities.

As a result of the experiment, the following became evident.

1. Incidence of Animal Death

Neither of the groups experienced death nor moribund condition of the mice.

2. Normal Condition

Neither of the groups experienced changes in normal condition of the mice.

3. Shifts in Weight

The group in which dosing solution of 1,000 mg/kg was administered and the group in which dosing solution of 2,000 mg/kg was administered showed a (slight) decrease in weight on the day after the administration. In the 2,000 mg/kg administering group experienced a significant decrease, compared with the comparative group. Herein, weight changes in the 1,000 mg/kg and 2,000 mg/kg administering groups from the third day to the fifteenth day were increase similar to those in the comparative group.

4. Pathology Inspection

In 2/5 cases in the 1,000 mg/kg administering group and 5/5 cases in the 2,000 mg/kg administering group, green-brown (slight) deposition that seemed to be secretory product was found in a site of the administration (subcutaneous tissue of the back of neck) depending on dosing solution weight. Furthermore, 1/5 cases in the 1000 mg/kg administering group experienced (left) testis (slight) diminishing. Abnormal findings other than the testis diminishing were not observed.

From the foregoing results, in the study of the lethal dose and toxicological appearance in the situation in which secretory product was administered one time subcutaneously in the mice to observe the mice for 15 days, although the weights of the mice on the second day (slightly) decreased depending on the dosing solution weight, weight changes similar to those in the comparative group was observed after the third day, so that it was believed that administering secretory product had a slight degree of influence on the weight changes. In the pathological inspection, although the fact that a (slight) deposition that seemed to be remaining secretory product was found on the site of administration suggested that the secretory product was not be subcutaneously absorbed well, the secretory product was presumably weak irritant because in the site of administration, changes such as inflammatory reaction was not observed. Furthermore, there were not any abnormal findings in other observation sites in the pathological inspection, so that clear toxicity changes originating in the exertion were not found.

From above results, it was apparent that the minimum lethal dose was 2,000 mg/kg or more because there were not any clear toxicity changes originating in the secretory products, and furthermore, moribund or dead animals were not found. Accordingly, the microorganism secretory product of the present invention is nontoxic, and thus can be used for treatment.

Embodiment 5 of the present invention will be explained.
Case 1

In case 1, Results of subcutaneously injecting a patient A under treatment for *Helicobacter pylori* with the novel microorganism of the present invention, together with *Helicobacter pylori* bacteria scavenging agent.

Preparation method: The secretory product was extracted in the same manner as in Embodiment 2.

Administration Method: The secretory product of 0.2 g and a normal saline solution were mixed, and administered by subcutaneously injecting the patient A with the mixture twice a day for seven days.

Results: The patient A showed gastric improvement 8 days later. The novel microorganism was viewed in any section collected from gastric wall under a microscope. The stomach felt light and free from something lying, heartburn did not occur next morning. Stomach condition was sufficiently improved.

Embodiment 6 of the present invention will be explained. When the section collected in Embodiment 5 was subjected to Giemsa staining, the novel microorganism of the present invention was confirmed to make figure-eight movement under a microscope.

INDUSTRIAL APPLICABILITY

According to the present invention, the novel microorganism was confirmed from the experiments to be able to promote gastric juice secretion, and furthermore, to be nontoxic. The present invention therefore has industrial applicability.

The material in the text file entitled "UY004_SeqList," created on Aug. 2, 2010, 3 kilobytes in size, and electronically submitted as part of the application of which this specification forms an essential part, is hereby incorporated by reference.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 1

<210> SEQ ID NO 1

<211> LENGTH: 1480

<212> TYPE: DNA

<213> ORGANISM: *Bacillus pumilus*

<400> SEQUENCE: 1

```

tcgagcggac agaagggagc ttgctcccgg atgttagcgg cggacgggtg agtaaacagt    60
gggtaacctg cctgtaagac tgggataact cgggaaacc ggagctaata ccggatagtt    120
ccttgaaccg catggttcaa ggatgaaaga cggtttcggc tgtcacttac agatggaccc    180
gcggcgcatt agctagtggg tggggtaatg gctcaccaag gcgacgatgc gtagccgacc    240
tgagaggggtg atcggccaca ctgggactga gacacggccc agactcctac gggaggcagc    300
agtagggaat cttccgcaat ggacgaaagt ctgacggagc aacgccgctg gtagtgatgaa    360
ggttttcgga tcgtaaagct ctgttgttag ggaagaacaa gtgcgagagt aactgctcgc    420
accttgacgg tacctaacca gaaagccacg gctaactacg tgccagcagc cgcgtaata    480
cgtaggtggc aagcgttctc cggaaattatt gggcgtaaag ggctcgcagg cggtttctta    540
agtctgatgt gaaagccccc ggctcaaccg gggagggtca ttggaaactg gaaacttga    600
gtgcagaaga ggagagtgga attccacgtg tagcggtgaa atgcgtagag atgtggagga    660
acaccagtgg cgaagggcag tctctgttct gtaactgacg ctgaggagcg aaagcgtggg    720
gagcgaacag gattagatac cctggtagtc cacgccgtaa acgatgagtg ctaagtgtta    780
gggggtttcc gcccttagt gctgcagcta acgcattaag cactccgect ggggagtagc    840
gtcgaagac tgaactcaa aggaattgac gggggcccgc acaagcggtg gagcatgtgg    900
ttaattcga agcaacgcga agaaccttac caggtcttga catcctctga caacctaga    960
gatagggctt tcccttcggg gacagagtga caggtggtgc atggttgctg tcagctcgtg   1020
tcgtgagatg ttgggttaag tcccgaacg agcgaaccc ttgatcttag ttgccagcat   1080
tcagttgggc actctaaggt gactgccggg gacaaaaccg aggaaggtgg ggatgacgtc   1140
aatcatcat gcccttatg acctgggcta cacacgtgct acaatggaca gaacaaaggg   1200
ctgcaagacc gcaaggttta gccaatccca taaatctgtt ctcagttcgg atcgagctct   1260

```

-continued

gcaactcgac tgcgtgaagc tggaaatcgct agtaatcgcg gatcagcatg ccgcggtgaa	1320
tacggtcccg ggccctgtac acaccgcccg tcacaccacg agagtttgca acaccggaag	1380
tcggtgaggt aacctttatg gagccagccg ccgaaggtgg ggcagatgat tgggggtgaag	1440
tcgtaacaag gtagccgtat cgggaaggtgc ggctggatca	1480

What is claimed is:

1. A biologically pure culture of the strain *Bacillus pumilus* NITE BP-295, isolated from either the human stomach wall or from human blood wherein said strain is gastric-juice promoting and nontoxic.

2. The *Bacillus pumilus* NITE BP-295 set forth in claim 1, characterized in taking the form of cocci as well as rods.

3. The *Bacillus pumilus* NITE BP-295 set forth in claim 1, further characterized in having the following bacteriological properties:

10

(1) being Gram-positive;

(2) being non-photodegradable;

(3) being motile;

(4) having a flagellum on each end;

15

(5) being aerobic;

(6) being 0.5 to 1 μm \times 10 to 20 μm in size; and

(7) being non-pyrogenic.

* * * * *