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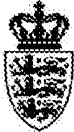

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(54) Benævnelse: **Nye genprodukter, der danner eller nedbryder polyaminosyrer, af Bacillus licheniformis og herpå byggende forbedrede bioteknologiske produktionsfremgangsmåder**

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Description

The present invention relates to improved biotechnological production methods by microorganisms which are characterized by an inactivation of a novel gene and its gene product from *Bacillus licheniformis* and sufficiently similar genes and proteins which are involved *in vivo* in the formation, the modification and/or the degradation of polyamino acids, and can be used for this purpose, and to microorganisms in which the *ywtA* gene coding for a gene product involved in the formation of poly-gamma-glutamate has been functionally inactivated.

The present invention is in the area of biotechnology, in particular the preparation of viable products by fermentation of microorganisms able to form the viable products of interest. This includes for example the preparation of low molecular weight compounds, for instance of dietary supplements or pharmaceutically relevant compounds, or of proteins for which, because of their diversity, there is in turn a large area of industrial uses. In the first case, the metabolic properties of the relevant microorganisms are utilized and/or modified to prepare the viable products; in the second case, cells which express the genes of the proteins of interest are employed. Thus in both cases, genetically modified organisms (GMO) are mostly involved.

There is an extensive prior art on the fermentation of microorganisms, especially also on the industrial scale; it extends from the optimization of the relevant strains in relation to the formation rate and the nutrient utilization via the technical design of the fermenters and up to the isolation of the valuable products from the relevant cells themselves and/or the fermentation medium. Both genetic and microbiological, and process engineering and biochemical approaches are applied thereto. The aim of the present invention is to improve this process in relation to a common property of the microorganisms employed, which impairs the

actual fermentation step, specifically at the level of the genetic properties of the strains employed.

For industrial biotechnological production, the relevant microorganisms are cultured in fermenters which are configured appropriate for their metabolic properties. During the culturing, they metabolize the substrate offered and, besides the actual product, normally form a large number of other substances in which there is ordinarily no interest and/or which – as explained hereinafter – may lead to difficulties in the fermentation or the working up.

Fermentations are normally very complicated processes in which a large number of different parameters must be adjusted and monitored. Thus, for example, aerobic processes are very often involved, meaning that the microorganisms employed must be supplied adequately with oxygen throughout the fermentation (control of the aeration rate). Further examples of such parameters are the reactor geometry, the continuously changing composition of the nutrient medium, the pH or the CO_2 formation rate. A particularly important parameter both in terms of the economics and in relation to the process management per se is the necessary energy input, for example via agitation systems which ensure that the reactor content is mixed as thoroughly as possible. In addition, besides the substrate distribution, also an adequate supply of oxygen to the organisms is ensured.

After completion of the fermentation it is normally necessary, besides the removal of the producer organisms, for the valuable product of interest to be purified and/or concentrated from the so-called fermenter slurry. The working up process can include for example various chromatographic and/or filtration steps. Thus, besides the content of valuable products, also decisive for the success of the overall working up process are the biophysical properties of the fermenter slurry, especially its viscosity immediately after completion of the fermentation.

The properties thereof are also influenced by the metabolic activities of the chosen microorganisms, it also being possible for unwanted effects to occur. These include for example a frequent increase in the viscosity of the nutrient medium during the fermentation. This impairs the mixing and thus the transport of matter and the oxygen supply inside the reactor. Additional difficulties mostly arise during the subsequent working up because increased viscosities considerably impair for example the efficiency of filtration processes.

It is known in particular that species of the genus *Bacillus* produce slime which consists essentially of poly-gamma-glutamate (PGA) and/or -aspartate, meaning polyamino acids linked via the relevant gamma peptide bonds. In scientific studies on *Bacillus subtilis* it is mainly the three genes *ywsC, ywtA* and *ywtB* and the gene products derived therefrom which are connected with the production of poly-gamma-glutamate; the gene product of *ywtD* is involved in the degradation. The general designation "*ywt*" for genes is in this connection synonymous with the abbreviations "*cap*" and "*pgs*" which are in common use for the same functions. This is explained below.

The publication "Physiological and biochemical characteristics of poly gamma-glutamate synthetase complex of *Bacillus subtilis*" (2001) by M. Ashiuchi et al., in *Eur. J. Biochem.*, volume 268, pages 5321-5328, describes the PgsBCA (poly-gamma-glutamate synthetase complex BCA) enzyme complex, which consists of the three subunits PgsB, PgsC and PgsA, from *B. subtilis*. This complex is, according to this, an atypical amide ligase which converts both the D and the L enantiomer of glutamate into the corresponding polymer. According to this publication, a gene disruption experiment described therein is to be regarded as proof that this complex is the only one catalyzing this reaction in *B. subtilis*.

Y. Urushibata et al. demonstrate in the publication

"Characterization of the *Bacillus subtilis ywsC* gene, involved in gamma-polyglutamic acid production" (2002), in *J. Bacteriol.*, volume 184, pages 337-343, inter alia via deletion mutations in the three genes *ywsC*, *ywtA* and *ywtB*, that the three gene products responsible in *B. subtilis* for the formation of PGA are encoded by these three genes. They form in this sequence and together with the subsequent gene ywtC a coherent operon in this microorganism.

The fact that a further gene relevant for the metabolism of PGA is located in the genome of *B. subtilis* downstream from *ywtC* in its own operon is shown by T. Suzuki and Y. Tahara in the publication "Characterization of the *Bacillus subtilis ywtD* gene, whose product is involved in gamma-polyglutamic acid degradation" (2003), *J. Bacteriol.*, volume 185, pages 2379-2382. This gene codes for a DL-endopeptidase which is able to hydrolyze PGA and thus can be referred to as gamma-DL-glutamyl hydrolase.

An up-to-date survey of these enzymes is additionally provided by the article "Biochemistry and molecular genetics of poly-gamma-glutamate synthesis" by M. Ashiuchi and H. Misono in *Appl. Microbiol. Biotechnol.*, volume 59, pages 9-14 of 2002. The genes homologous to *pgsB*, *pgsC* and *pgsA* and coding for the PGA synthase complex in *B. anthracis* are referred to therein as *capB*, *capC* and *capA*. The gene located downstream is referred to according to this article as *dep* (for "D-PGA depolymerase") in *B. anthracis* and as *pgdS* (for "PGA depolymerase") in *B. subtilis*.

In the current state of the art, these enzymic activities are already in positive use mainly for preparing poly-gamma-glutamate as raw material, for example for use in cosmetics, although their exact DNA sequences and amino acid sequences have not to date been known – especially from *B. licheniformis*. Thus, for example, the application JP 08308590 A discloses the preparation of PGA by fermentation of the PGA-producing strains itself, namely of *Bacillus* species such as

B. subtilis and *B. licheniformis*; the isolation of this raw material from the culture medium is also described therein. *B. subtilis* var. chunkookjang represents, according to the application WO 02/055671 A1, a microorganism which is particularly suitable therefor.

Thus, in some fermentations there is an interest in GLA as the valuable product to be produced by the fermentation.

However, the interest in all other fermentations is to prepare other valuable products; in this connection, the formation of polyamino acids means, for the reasons stated above, a negative side effect. A typical procedure for mastering the increased viscosity of the fermentation medium attributable to the formation thereof is to increase the agitator speed. However, this has an effect on the energy input. In addition, the fermented microorganisms are exposed thereby to increasing shear forces representing a considerable stress factor for them. In the end, very high viscosities cannot be overcome even thereby, so that premature termination of the fermentation may be necessary, although production could otherwise be continued.

Slime formation, as a negative side effect of numerous fermentation processes, may thus have negative effects on the overall result of fermentation for diverse reasons. Conventional methods for successfully continuing fermentations in progress despite an increasing viscosity of the nutrient medium can be designated only as inadequate, especially because they do not represent a causal control.

The more pressing problem was thus to suppress as far as possible an unwanted formation of slime, especially a slime attributable to poly-gamma-amino acids such as poly-gamma-glutamate, during the fermentation of microorganisms. It was intended in particular to find a solution representing a causal control. A further aspect of this problem is the provision of the

relevant genes for a positive utilization of the GLA-synthesizing gene products.

One solution to this problem is a method of reducing slime attributable to poly-gamma-glutamate, wherein the protein YwtA (CapC, PgsC) encoded by the *ywtA* gene and having an amino acid sequence which is at least 94% identical to the amino acid sequence set forth in SEQ ID NO. 6 is prevented from functioning as an enzyme involved in the formation of polyamino acids or as a subunit of such an enzyme.

In one embodiment of said method, the YwtA protein is prevented from functioning during fermentation of the microorganism.

In another embodiment, the method is characterized by a reduction in the slime attributable to polyamino acids to 50%.

In another embodiment of said method, the microorganism is a bacterium, and/or is a gram-negative bacterium or a gram-positive bacterium.

In another embodiment of said method, the microorganism is selected from any of the genera *Escherichia, Klebsiella, Pseudomonas, Xanthomonas, Bacillus, Staphylococcus* and *Corynebacterium.*

In another embodiment, the method is characterized by the following steps of preventing the YwtA protein encoded by the *ywtA* gene from functioning:

a) selecting two regions of the sequence SEQ ID NO. 5,
b) cloning the regions into a vector either to flank a section coding for an inactive protein or to follow directly upon each other, excluding the intermediate region,
c) deleting the *ywtA* gene using the vector produced in step b), and
d) detecting the gene deletion.

In another embodiment of said method, the YwtA (CapC, PsgC) protein encoded by the *ywtA* gene is prevented from functioning by employing a nucleic acid having a deletion or insertion mutation, preferably comprising the border sequences, each of which comprises at least 70 to 150 nucleic acid positions, of the region coding for the protein.

Another solution is a method of producing a valuable product by fermentation of a microorganism, wherein the formation of poly-gamma-glutamate by the microorganism during fermentation is reduced by preventing the YwtA (CapC, PgsC) protein encoded by the *ywtA* gene and having an amino acid sequence which is at least 94% identical to the amino acid sequence set forth in SEQ ID NO. 6 from functioning as an enzyme involved in the formation of polyamino acids or as a subunit of such an enzyme.

In one embodiment of said method, the YwtA (CapC, PgsC) protein encoded by the *ywtA* gene is prevented from functioning by a method comprising the following steps:

a) selecting two regions of the sequence SEQ ID NO. %,

b) cloning the regions into a vector either to flank a section coding for an inactive protein or to follow directly upon each other, excluding the intermediate region,

c) deleting the *ywtA* gene using the vector produced in step b), and

d) detecting the gene deletion.

In another embodiment of said method, the YwtA (CapC, PsgC) protein encoded by the *ywtA* gene is prevented from functioning by employing a nucleic acid having a deletion or insertion mutation, preferably comprising the border sequences, each of which comprises at least 70 to 150 nucleic acid positions, of the region coding for the protein.

In another embodiment of said method, the valuable product is a natural product, a food supplement or a pharmaceutically relevant compound or an enzyme.

In another embodiment of said method, the enzyme is selected from the group consisting of α-amylases, proteases, cellulases, lipases, oxidoreductases, peroxidases, laccases, oxidases, and hemicellulases.

Another solution is the use of a nucleic acid coding for a YwtA (CapC, PgsC) protein involved in the formation of poly-gamma-glutamate and having an amino acid sequence which is at least 94% identical to the amino acid sequence set forth in SEQ ID NO. 6, or in each case parts thereof for preventing the YwtA (CapC, PgsC) protein from functioning as an enzyme involved in the formation of polyamino acids or as a subunit of such an enzyme.

Another solution is the use of a nucleic acid coding for a YwtA (CapC, PgsC) protein involved in the formation of poly-gamma-glutamate and having an amino acid sequence which is at least 94% identical to the amino acid sequence set forth in SEQ ID NO. 6, or in each case parts thereof for reducing slime attributable to poly-gamma-glutamate to 50% during fermentation of a microorganism.

In one embodiment of said use, the nucleic acid has the sequence SEQ ID NO. 5.

Another solution is the use of a nucleic acid having a deletion or insertion mutation comprising the border sequences, each of which comprises at least 70 to 150 nucleic acid positions, of the region coding for a YwtA (CapC, PsgC) protein having an amino acid sequence which is at least 94% identical to the amino acid sequence set forth in SEQ ID NO. 6, for reducing slime attributable to poly-gamma-glutamate during fermentation of a microorganism.

Another solution is a microorganism in which the *ywtA* gene coding for a gene product involved in the formation of poly-gamma-glutamate has been functionally inactivated, the *ywtA*

coding nucleotide sequence having a nucleotide sequence which is at least 94% identical to the nucleotide sequence set forth in SEQ ID NO. 5.

5 In one embodiment, the microorganism is *Bacillus licheniformis*.

Further disclosed are the relevant nucleic acid *ywtA* and, based thereon, the use of relevant nucleic acids for reducing 10 the formation of slime attributable to polyamino acids during the fermentation of the microorganism, and corresponding methods for fermentation of microorganisms. In the reduction according to the invention of the formation of slime at the genetic level, the gene *ywtA* is functionally inactivated. In 15 addition, there is the positive use of this gene or of the derived gene products for the preparation of poly-gamma-glutamate.

This invention which is applicable in principle to all 20 fermentable microorganisms, especially to those of the genus *Bacillus*, leads to the microorganisms employed for the fermentative production of valuable products other than polyamino acids, in particular of pharmaceutically relevant low molecular weight compounds or of proteins, being prevented 25 at the genetic level from forming polyamino acids, especially GLA. On the one hand, this has an advantageous effect on the viscosity of the culture medium and additionally on the mixability, the oxygen input and the energy to be expended, and on the other hand the working up of the product of 30 interest is considerably facilitated. In addition, most of the raw materials employed, for instance the N source, is not converted into a product of no interest, so that overall a higher fermentation yield is to be expected.

35 The said gene can be used for a positive use of the GLA-synthesizing gene product, specifically by the derived protein YwtA being produced biotechnologically and being introduced in the cells producing it or independently thereof as catalyst

into appropriate reaction mixtures.

There is disclosed a protein YwtA (CapC, PgsC) which is involved in the formation of polyamino acids and which is encoded by a nucleotide sequence *ywtA* which shows at least 82% identity to the nucleotide sequence indicated in SEQ ID NO. 5.

This specific enzyme was obtained by analysis of the genome of *B. licheniformis* DSM 13 (see Example 1). This protein is made reproducibly available through the nucleotide and amino acid sequences indicated in SEQ ID NO. 5 and 6 of the present application (see Example 1).

This takes the form, in agreement with the literature information mentioned in the introduction, of a further subunit of the poly-gamma-glutamate synthetase complex. The protein known in the state of the art and most similar thereto has been found to be the homolog YwsA from *B. subtilis* which is noted in the GenBank database (National Center for Biotechnology Information NCBI, National Institutes of Health, Bethesda, MD, USA) under the access number AB046355.1 and has a homology of 77.8% identity at the nucleic acid level, while the agreement is 89.9% identity at the amino acid level (see Example 2). These significant agreements suggest not only the same biochemical function, but also the presence within the claimed range of a large number of related proteins having the same function.

The following variants are to be allocated to this protein YwtA:

- Any corresponding protein YwtA which is encoded by a nucleotide sequence which shows with increasing preference at least 85%, 90%, 92%, 94%, 96%, 97%, 98%, 99% and particularly preferably 100% identity to the nucleotide sequence indicated in SEQ ID NO. 5.
- Any protein YwtA (CapC, PgsC) involved in the formation of polyamino acids and having an amino acid sequence which shows at least 94% identity, with increasing preference at least

95%, 96%, 97%, 98%, 99% and particularly preferably 100% identity to the amino acid sequence indicated in SEQ ID NO. 6. The specific protein obtained from *B. licheniformis* DSM13 is most preferred in each case, because this is specifically described in the present application and is made available 100% reproducibly.

Preference is given in each case among these in each case to a previously described protein which is involved in the formation or degradation of polyamino acids and which is naturally produced by a microorganism, preferably by a bacterium, particularly preferably by a Gram-positive bacterium, preferably among these by one of the genus *Bacillus*, particularly preferably among these by one of the species *B. licheniformis* and very particularly preferably among these by *B. licheniformis* DSM13.

This is because, in accordance with the problem, there was interest in improving the fermentation of microorganisms, for which bacteria from among these particularly Gram-positive ones, are frequently used, especially those which, like *Bacillus*, are able to secrete produced valuable products and proteins. In addition, there is a wealth of clinical experience concerning this. In addition, it was possible to detect, as mentioned, the proteins indicated in the sequence listing for *B. licheniformis*, specifically *B. licheniformis* DSM13. It is to be expected that an increasing degree of relationship of the relevant organisms will be associated with an increasing extent of agreement of the nucleotide and amino acid sequences and thus their exchangeability.

The disclosure furthermore relates to nucleic acids:

- nucleic acid *ywtA (capC, pgsC)*, which codes for a gene product involved in the formation of polyamino acids and has a nucleotide sequence which shows at least 82% identity to the nucleotide sequence indicated in SEQ ID NO. 5;
- a corresponding nucleic acid *ywtA* having a nucleotide sequence which shows with increasing preference at least 85%,

90%, 92%, 94%, 96%, 97%, 98%, 99% and particularly preferably 100% identity to the nucleotide sequence indicated in SEQ ID NO. 5.

5 The nucleic acids provided herewith can be employed by methods of molecular biology known per se for inactivating or enhancing the activity of the relevant proteins. Thus, inactivations are possible for example via appropriate deletion vectors (see below); enhancement of the activity 10 advantageously takes place by an overexpression which can be achieved with the aid of an expression vector (see below).

The corresponding genes falling within the homology ranges indicated in each case can be obtained from the organisms of 15 interest for example with the aid of probes which can be prepared on the basis of sequence5. These complete genes may also serve as model for generating PCR primers via which the relevant genes can be rendered accessible from corresponding total DNA preparations; these genes in turn provide the 20 proteins described previously. The success rate in this connection usually increases with the closeness of the relationship of the relevant strain to that which has served to construct the probe or the PCR primers, and thus in the present case to *B. licheniformis*.

25

Preference is given in each case among these in each case to a nucleic acid which is naturally present in a microorganism, preferably a bacterium, particularly preferably a Gram-positive bacterium, and among these preferably one of the 30 genus *Bacillus*, particularly preferably among these one of the species *B. licheniformis* and very particularly preferably among these *B. licheniformis* DSM13.

This is because, as stated above, there is a particular 35 interest in utilizing these genes for fermentations of such microorganisms. On the other hand, the present disclosure is also linked to the possibility of adjusting, via the genes and/or proteins described herein, the metabolism of the

polyamino acids, especially gamma-glutamic acid, at least in parts when they are to be synthesized, modified and/or degraded. The success rate for this generally, especially in appropriate transgenic host cells, increases with the degree 5 of agreement of the relevant genes with those of the natural cells.

It is additionally possible to isolate alternatives of the genes and proteins easily from in principle all natural 10 organisms.

There are furthermore disclosed nucleic acids which code for a protein described above.

15 Thus, differences exist, particularly between remotely related species, in the usage of synonymous codons coding for the respective amino acids, with which the protein biosynthesis apparatus also conforms, for instance via the available number of appropriate loaded tRNAs. Transfer of one of said genes 20 into a less related species can be used particularly successfully for example for deletion mutation or for synthesis of the relevant protein if it is appropriately optimized in terms of the codons. It is possible thereby to introduce increasing percentage differences at the DNA level 25 which, however, have no consequence at the amino acid level.

There are furthermore disclosed vectors which comprise a previously designated nucleic acid region.

30 This is because in order to handle the nucleic acids, and thus in particular to prepare for the production of proteins, they are suitably ligated into vectors. Such vectors and the relevant working methods are described in detail in the prior art. Vectors are commercially available in large number and 35 range of variation, both for cloning and for expression. These include for example vectors derived from bacterial plasmids, from bacteriophages or from viruses, or predominantly synthetic vectors. They are also distinguished according to

the nature of the cell types in which they are able to establish themselves, for example into vectors for Gram-negative, for Gram-positive bacteria, for yeasts or for higher eukaryotes. They form suitable starting points for example for molecular biological and biochemical investigations and for the expression of the relevant gene or associated protein. They are as virtually indispensable - as is evident from the prior art relevant thereto - in particular for the preparation of constructs for deletion or enhancement of expression.

The vectors may be cloning vectors.

This is because cloning vectors are, besides the storage, the biological amplification or the selection of the gene of interest, suitable for its molecular biological characterization. At the same time, they represent transportable and storable forms of the claimed nucleic acids and are also starting points for molecular biological techniques which are not linked to cells, such as, for example, PCR or in vitro mutagenesis methods.

The vectors are preferably expression vectors.

This is because such expression vectors are the basis for implementing the corresponding nucleic acids in biological production systems and thus producing the relevant proteins. Preference is given to expression vectors which carry genetic elements necessary for expression, for example the natural promoter originally located in front of this gene, or a promoter from a different organism. These elements may be disposed for example in the form of a so-called expression cassette. An alternative possibility is for one or all regulatory elements also to be provided by the respective host cell. Expression vectors are particularly preferred in relation to further properties such as, for example, the optimum copy number matched to the chosen expression system, especially the host cell (see below).

There are furthermore disclosed cells which, after genetic modification, comprise one of the nucleic acids designated above.

This is because these cells comprise the genetic information for synthesizing a protein. By these are meant in particular cells which have been provided with the nucleic acids by methods known per se, or which are derived from such cells. The host cells suitably selected for this purpose are those which can be cultured relatively simply and/or provide high product yields.

It is necessary in principle in countries where human embryonic stem cells may not be placed under patent protection for such human embryonic stem cells of the invention to be excluded from the protection conferred.

Cells make it possible for example to amplify the corresponding genes, but also for them to be mutagenized or transcribed and translated and eventually for the relevant proteins to be produced biotechnologically. This genetic information may be present either extrachromosomally as separate genetic element, meaning located in plasmids in the case of bacteria, or be integrated into a chromosome. The choice of a suitable system depends on questions such as, for example, the nature and duration of the storage of the gene or of the organism or the nature of the mutagenesis or selection.

These include in particular those cells which comprise the gene *ywtA* via a vector *in trans* and can thus be used for corresponding deletions (see below).

Preference is given to the said nucleic acid being part of a vector, in particular of a previously described vector, in such a cell.

Host cells which are bacteria are preferred among these.

This is because bacteria are distinguished by short generation times and low demands on the culturing conditions. It is possible thereby to establish cost-effective methods. In addition, there is a wealth of experience in the techniques of fermentation of bacteria. Gram-negative or Gram-positive bacteria may be suitable for a specific production for a wide variety of reasons which are to be ascertained experimentally in the individual case, such as nutrient sources, product formation rate, time required etc.

Preference is given to there being involved a Gram-negative bacterium, in particular one of the genera *Escherichia coli, Klebsiella, Pseudomonas* or *Xanthomonas,* in particular strains of *E. coli* K12, *E. coli* B or *Klebsiella planticola*, and very especially derivatives of the strain *Escherichia coli* BL21 (DE3), *E. coli* RV308, *E. coli* DH5α, *E. coli* JM109, *E. coli* XL-1 or *Klebsiella planticola* (Rf).

This is because a large number of proteins are secreted into the periplasmic space with Gram-negative bacteria such as, for example, *E. coli*. This may be advantageous for specific applications. The application WO 01/81597 A1 discloses a method which achieves expulsion of the expressed proteins by Gram-negative bacteria too. The Gram-negative bacteria mentioned as preferred are usually available easily, meaning commercially or through public collections of strains, and can be optimized for specific preparation conditions in association with other components such as, for instance, vectors which are likewise available in large number.

Not less preferably there is involved a Gram-positive bacterium, in particular one of the genera *Bacillus, Staphylococcus* or *Corynebacterium,* very particularly of the species *Bacillus lentus, B. licheniformis, B. amyloliquefaciens, B. subtilis, B. globigii* or *B. alcalophilus, Staphylococcus carnosus* or *Corynebacterium glutamicum*, and among these in turn very particularly preferably a derivative of *B. licheniformis* DSM 13.

This is because Gram-positive bacteria have the fundamental difference from Gram-negative ones of immediately releasing secreted proteins into the nutrient medium which surrounds the cells and from which if desired the expressed proteins can be directly purified from the nutrient medium. In addition, they are related or identical to most of the organisms of origin of industrially important enzymes and mostly themselves produce comparable enzymes, so that they have a similar codon usage and their protein synthesis apparatus is naturally configured appropriately. Derivatives of *B. licheniformis* DSM 13 are very particularly preferred because they on the one hand are likewise widely used as biotechnological producer strains in the state of the art and because on the other hand the present application makes exactly the genes and proteins from *B. licheniformis* DSM 13 available, so that implementation of the present invention ought most likely to be successful in such strains.

There are furthermore disclosed methods for preparing a gene product YwtA described above.

This includes any method for preparing a protein described above, for example chemical synthetic methods. However, in relation thereto, all molecular biological, microbiological and biotechnological preparation methods which have been discussed above in individual aspects and are established in the state of the art are preferred. The aim thereof is primarily to obtain the proteins in order to make them available for appropriate applications, for example for the synthesis of poly-gamma-glutamate.

Methods preferred in this connection are those taking place with use of a nucleic acids designated above, preferably taking place with use of a vector designated above and particularly preferably with use of a cell designated above.

This is because said nucleic acids, especially the nucleic

acid indicated in the sequence listing under SEQ ID NO. 5, makes the correspondingly preferred genetic information available in microbiologically utilizable form, i.e. for genetic production methods. It is increasingly preferred to provide on a vector which can be utilized particularly successfully by the host cell, or such cells themselves. The relevant production methods are known per se to the skilled worker.

The basis of the relevant nucleic acid sequences may also be cell-free expression systems in which the protein biosynthesis is duplicated *in vitro*. All the elements already mentioned may also be combined to novel methods in order to prepare proteins. A large number of possible combinations of method steps is conceivable for each protein moreover, so that optimal methods need to be ascertained experimentally for each specific individual case.

Methods of such types are further preferred when the nucleotide sequence has been adapted in one or, preferably, more codons to the codon usage of the host strain.

This is because, in accordance with that stated above, transfer of one of said genes into a less related species can be used particularly successfully for synthesizing the relevant protein if it is appropriately optimized in relation to the codon usage.

There is furthermore disclosed the use of a nucleic acid *ywtA* described above or of a corresponding nucleic acid which codes for one of the proteins described above or in each case parts thereof for the functional inactivation of the respectively relevant gene *ywtA* in a microorganism.

Functional inactivation means in the context of the present application any type of modification or mutation by which the function of the relevant protein as an enzyme involved in the formation of polyamino acids, or as subunit of such an enzyme,

is suppressed. This includes the embodiment where a virtually complete but inactive protein is formed, where inactive parts of such a protein are present in the cell, up to the possibilities where the relevant gene is no longer translated or is even completely deleted. Thus, a specific "use" of these factors or genes in this embodiment consists of them no longer acting in their natural manner precisely in the relevant cell. This is achieved according to the subject matter of the invention at the genetic level by switching off the relevant gene.

In preferred embodiments, both uses are those where the functional inactivation or increase in activity takes place during the fermentation of the microorganism, preferably with a reduction of the slime attributable to polyamino acids to 50%, particularly preferably to less than 20%, very particularly preferably to less than 5%, once again all intermediate integral or fractional percentages being understood in appropriately preferred gradation.

To determine these values, cells of an untreated strain and of a treated strain are fermented under conditions which are otherwise identical and suitably the viscosity of the respective medium is determined during the fermentation. Since the strains are otherwise identical, the differences in viscosity are attributable to the different contents of polyamino acids. Every reduction in viscosity is desired according to the invention. Comparable values as percentages are obtained by taking samples from both fermentations and determining the content of polyamino acid-containing slime by methods known per se. It is increasingly preferred for the value which can be determined in the sample of the invention to be at the transition into the stationary growth phase less than 50%, 40%, 30%, 20%, 10%, 5% and very especially less than 1% of the corresponding value for the comparative fermentation.

For the use for functional inactivation of the gene *ywtA*, a

nucleic acid coding for an inactive protein and having a point mutation may be employed.

Nucleic acids of this type can be generated by methods of point mutagenesis known per se. Such methods are described for example in relevant handbooks such as that of Fritsch, Sambrook and Maniatis "Molecular cloning: a laboratory manual", Cold Spring Harbour Laboratory Press, New York, 1989. In addition, numerous commercial construction kits are now available therefor, for instance the QuickChange® kit from Stratagene, La Jolla, USA. The principle thereof is for oligonucleotides having single exchanges (mismatch primers) to be synthesized and hybridized with the gene in single-stranded form; subsequent DNA polymerization then affords corresponding point mutants. It is possible to use for this purpose the respective species-specific sequences of these genes. Owing to the high homologies, it is possible and particularly advantageous according to the invention to carry out this reaction on the basis of the sequence provided by SEQ ID NO. 5. This sequence can also serve to design appropriate mismatch primers for related species, especially on the basis of the conserved regions identifiable in the alignments of Figures 1 and 2.

In one embodiment of this use, in each case a nucleic acid with a deletion mutation or insertion mutation is employed for the functional inactivation, preferably including the border sequences, in each case comprising at least 70 to 150 nucleic acid positions, of the region coding for the protein.

These methods are also familiar per se to the skilled worker. It is thus possible to prevent the formation of a factor YwtA by the host cell by cutting out part of the relevant gene on an appropriate transformation vector via restriction endonucleases, and subsequently transforming the vector into the host of interest, where the active gene is replaced by the inactive copy via the homologous recombination which is still possible until then. In the embodiment of insertion mutation

it is possible merely to introduce the intact gene interruptingly or, instead of a gene portion, another gene, for example a selection marker. Phenotypical checking of the mutation event is possible thereby in a manner known per se.

In order to enable these recombination events which are necessary in each case between the defective gene introduced into the cell and the intact gene copy which is endogenously present for example on the chromosome, it is necessary according to the current state of knowledge that in each case there is agreement in at least 70 to 150 connected nucleic acid positions, in each case in the two border sequences to the non-agreeing part, with the part lying between being immaterial. Accordingly, preferred embodiments are those including only two flanking regions with at least one of these sizes.

For the use, nucleic acids having a total of two nucleic acid segments which in each case comprise at least 70 to 150 nucleic acid positions, and thus flank at least partly, preferably completely, the region coding for the protein, may be employed. The flanking regions can in this connection be ascertained starting from the known sequences by methods known per se, for example with the aid of outwardly directed PCR primers and a preparation of genomic DNA as template (anchored PCR). This is because it is not obligatory for the segments to be protein-encoding in order to make it possible to exchange the two gene copies by homologous recombination. According to the present invention it is possible to design the primers required for this on the basis of SEQ ID NO. 5 also for other species of Gram-positive bacteria and, among these, in particular for those of the genus *Bacillus*. As an alternative to this experimental approach it is possible to take such regions which are at least in part non-coding for many of these genes from related species, for example from *B. subtilis* database entries, for example the SubtiList database of the Institute Pasteur, Paris, France (http://genolist.pasteur.fr/SubtiList/genome.cgi).

The present invention is also implemented in the form of genetically modified microorganisms, to which that stated above applies correspondingly.

5

These are very generally microorganisms in which the gene *ywtA* is functionally inactivated.

These are preferably microorganisms in the form of bacteria.

10

The microorganisms among these which are preferred are Gram-negative bacteria, especially those of the genera *Escherichia coli, Klebsiella, Pseudomonas* or *Xanthomonas,* especially strains of *E. coli* K12, *E. coli* B or *Klebsiella planticola,*
15 and very especially derivatives of the strains *Escherichia coli* BL21 (DE3), *E. coli* RV308, *E. coli* DH5α, *E. coli* JM109, *E. coli* XL-1 or *Klebsiella planticola* (Rf).

Microorganisms which are not less preferred are Gram-positive
20 bacteria, especially those of the genus *Bacillus, Staphylococcus* or *Corynebacterium,* very particularly of the species *Bacillus lentus, B. licheniformis, B. amyloliquefaciens, B. subtilis, B. globigii* or *B. alcalophilus, Staphylococcus carnosus* or *Corynebacterium*
25 *glutamicum* and, among these, very especially *B. licheniformis* DSM 13.

According to the problem on which the present application is based, the intention was primarily to improve industrial
30 fermentation methods. Accordingly, the invention is implemented especially in corresponding fermentation methods of the invention.

These are very generally methods for the fermentation of a
35 microorganism of the invention described above.

According to statements hitherto, the methods characterized thereby are correspondingly preferred. These include in

particular the gene *ywtA* being functionally inactivated. For this purpose, recourse is particularly preferably had to the nucleic acids described above, especially that indicated under SEQ ID NO. 5. This applies correspondingly also to the species selected as suitable for the respective fermentation. According to the statements above, those among these which are increasingly preferred have an increasing extent of relationship to *B. licheniformis* DSM13, because the prospects of success on use of the stated nucleic acids increase thereby.

Among the fermentation methods of the invention, those for preparing a valuable product are preferred, especially for preparing a low molecular weight compound or a protein.

This is because this is the most important area of application of industrial fermentations.

These are preferably methods where the low molecular weight compound is a natural product, a dietary supplement or a pharmaceutically relevant compound.

In this way for example amino acids or vitamins which are used in particular as dietary supplements are produced. Pharmaceutically relevant compounds may be precursors or intermediates for medicaments or even the latter themselves. In all these cases, the term biotransformation is also used, according to which the metabolic properties of the microorganisms are utilized to replace, entirely or at least in individual steps, the otherwise elaborate chemical synthesis.

No less preferred are corresponding methods in which the protein produced in this way is an enzyme, in particular one from the group of α-amylases, proteases, cellulases, lipases, oxidoreductases, peroxidases, laccases, oxidases and hemicellulases.

Industrial enzymes prepared by such methods are used for example in the food industry. Thus, α-amylases are used for example to prevent bread becoming stale or to clarify fruit juices. Proteases are used for the lysis of proteins. All these enzymes have been described for use in detergent and cleaner compositions, a prominent place being occupied in particular by the *Subtilis*in proteases prepared naturally by Gram-positive bacteria. They are used in particular in the textile and leather industries for processing the natural raw materials. A further possibility is for all these enzymes in turn to be employed in the context of biotransformation as catalysts for chemical reactions.

Many of these enzymes are originally derived from *Bacillus* species and are therefore produced particularly successfully in Gram-positive organisms, especially those of the genus *Bacillus*, including in many cases also derivatives of *B. licheniformis* DSM13. Production methods based on these microbial systems in particular can be improved with the aid of the present invention, because the sequences indicated in particular in SEQ ID NO. 5 is derived from precisely this organism.

Finally, the factors made available with the present application can also be employed positively, meaning in the sense of their natural function, meaning in connection with a targeted preparation of poly-gamma-glutamate.

There are thus disclosed microbial methods for the preparation of poly-gamma-glutamate in which one of the nucleic acids *ywtA* described above or a corresponding nucleic acid which codes a protein described above is employed transgenically, preferably to form the corresponding protein described above.

Preferred methods among these are those in which a microorganism from the genus *Bacillus*, in particular *B. subtilis* or *B. licheniformis*, is employed.

It is thus possible, as described for example in the applications JP 08308590 A or WO 02/055671 A1, to produce GLA microbially, specifically in *B. subtilis* and *B. licheniformis*. The DNA sequences made available with the present application can be utilized for example to increase the respective gene activities in appropriate cells, and thus to increase the yield.

As alternative thereto, cell-free methods for the preparation of poly-gamma-glutamate are now also possible, involving a gene product YwtA described above, which is involved in the formation of polyamino acids, preferably with use of a corresponding nucleic acid described above.

Thus, these factors can be reacted for example in a bioreactor. The design of such enzyme bioreactors is known from the prior art.

The following examples illustrate the present invention further.

Examples

All molecular biological working steps follow standard methods as indicated for example in the handbook by Fritsch, Sambrook and Maniatis "Molecular cloning: a laboratory manual", Cold Spring Harbour Laboratory Press, New York, 1989, or comparable relevant works. Enzymes, construction kits and apparatuses were employed in accordance with the respective manufacturer's instructions.

Example 1

Identification of the genes *ywsC, ywsC', ywtA, ywtB* and *ywtD* from *B. licheniformis* DSM 13

The genomic DNA was prepared by standard methods from the strain *B. licheniformis* DSM 13, which is available to anyone

from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig (http://www.dsmz.de), mechanically fractionated and fractionated by electrophoresis in a 0.8% agarose gel. For a shotgun cloning of the smaller fragments, the fragments 2 to 2.5 kb in size were eluted from the agarose gel, dephosphorylated and ligated as blunt-ended fragments into the SmaI restriction cleavage site of the vector pTZ19R-Cm. This is a derivative which confers chloramphenicol resistance of the plasmid pTZ19R which is obtainable from Fermentas (St. Leon-Rot). A gene library of the smaller fragments was obtained thereby. As second shotgun cloning, the genomic fragments obtained by a partial restriction with the enzyme SauIIIaI were ligated into the SuperCos 1 vector system ("Cosmid Vector Kit") from Stratagene, La Jolla, USA, resulting in a gene library over the predominantly larger fragments.

The relevant recombinant plasmids were isolated and sequenced from the bacteria E. coli DH5α (D. Hannahan (1983): "Studies on transformation on Escherichia coli"; *J. Mol. Microbiol.*, volume 166, pages 557-580) obtainable by transformation with the relevant gene libraries. The dye termination method (dye terminator chemistry) was employed in this case, carried out by the automatic sequencers MegaBACE 1000/4000 (Amersham Bioscience, Piscataway, USA) and ABI Prism 377 (Applied Biosystems, Foster City, USA).

In this way, inter alia the sequences SEQ ID NO. 1, 3, 5, 7 and 9 which are indicated in the sequence listing of the present application were obtained and stand in this sequence for the genes *ywsC, ywsC'* (as truncated variant of *ywsC*), *ywtA, ywtB* and *ywtD*. The amino acid sequences derived therefrom are indicated in the corresponding sequence in SEQ ID NO. 2, 4, 6, 8 and 10, respectively. A truncated variant *ywsC'* (or YwsC') is indicated for the gene or protein *ywsC* (or YwsC) because the comparison, shown in Figure 6, of the amino acid sequences for the homologous protein in *B. subtilis* shows

a polypeptide which is N-terminally shorter by 16 amino acids with otherwise quite high homology and therefore comparable activity.

5 **Reproducibility**

These genes and gene products can now be artificially synthesized by methods known per se, and without the need to reproduce the described sequencing, in a targeted manner on
10 the basis of these sequences. It is possible, as further alternative thereto, to isolate the relevant genes from a *Bacillus* strain, in particular the strain *B. licheniformis* DSM 13 which is obtainable from the DSMZ, via PCR, it being possible to use the respective border sequences indicated in
15 the sequence listing for synthesizing primers. If further strains are used, the genes homologous thereto in each case are obtained, and the success of the PCR should increase with the closeness of the relationship of the selected strains to *B. licheniformis* DSM 13, because this is likely to be
20 associated with an increasing agreement of sequences also within the primer binding regions.

Example 2

25 **Sequence homologies**

After ascertaining the DNA and amino acid sequences as in Example 1, in each case the most similar homologs disclosed to date were ascertained by a search in the databases GenBank
30 (National Center for Biotechnology Information NCBI, National Institutes of Health, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov) and *Subtilis*t of the Institute Pasteur, Paris, France (http://genolist.pasteur.fr/*Subtilis*t/genome.cgi).

35

The ascertained DNA and amino acid sequences were compared with one another via the alignments depicted in Figures 1 to 10; the computer program used for this was Vector NTI® Suite

Version 7 which is obtainable from Informax Inc., Bethesda, USA. In this case, the standard parameters of this program were used, meaning for comparison of the DNA sequences: K-tuple size: 2; Number of best Diagonals: 4; Window size: 4; Gap penalty: 5; Gap opening penalty: 15 and Gap extension penalty: 6.66. The following standard parameters applied to the comparison of the amino acid sequences: K-tuple size: 1; Number of best Diagonals: 5; Window size: 5; Gap penalty: 3; Gap opening penalty: 10 and Gap extension penalty: 0.1. The results of these sequence comparisons are compiled in Table 1 below, the access numbers indicated being those from the NCBI database.

Table 1: Genes and proteins of greatest similarity to the genes and proteins found in Example 1.

Gene or protein found in *B. licheniformis*/SEQ ID NO.	Most closely related gene or protein	Database entry of the most closely related gene or protein	Homology in % identity
ywsC/1	*ywsC* from *B. subtilis*	AB046355.1	75.4
ywsC'/3	*ywsC* from *B. subtilis*	AB046355.1	78.5
ywtA/5	ywsA from *B. subtilis*	AB046355.1	77.8
ywtB/7	*ywsB* from *B. subtilis*	AB046355.1	67.1
ywtD/9	*ywtD* from *B. subtilis*	AB080748	62.3
YwsC/2	YwsC from *B. subtilis*	AB046355.1	86.1
YwsC'/4	YwsC from *B. subtilis*	AB046355.1	89.6
YwtA/6	YwsA from *B.*	AB046355.1	89.9

	subtilis		
YwtB/8	YwsB from *B. subtilis*	AB046355.1	65.8
YwtD/10	YwsD from *B. subtilis*	AB046355.1	57.3

Example 3

Functional inactivation of one or more of the genes *ywsC, ywsC′*, *ywtA* and *ywtB* in *B. licheniformis*

Principle of the preparation of a deletion vector

Each of these genes can be functionally inactivated for example by means of a so-called deletion vector. This procedure is described per se for example by J. Vehmaanperä et al. (1991) in the publication "Genetic manipulation of *Bacillus* amyloliquefaciens"; *J. Biotechnol.*, volume 19, pages 221-240.

A suitable vector for this is pE194 which is characterized in the publication "Replication and incompatibility properties of plasmid pE194 in *Bacillus subtilis*" by T.J. Gryczan et al. (1982), *J. Bacteriol.*, volume 152, pages 722-735. The advantage of this deletion vector is that it possesses a temperature-dependent origin of replication. pE194 is able to replicate in the transformed cell at 33°C, so that initial selection for successful transformation takes place at this temperature. Subsequently, the cells comprising the vector are incubated at 42°C. The deletion vector no longer replicates at this temperature, and a selection pressure is exerted on the integration of the plasmid via a previously selected homologous region into the chromosome. A second homologous recombination via a second homologous region then leads to excision of the vector together with the intact gene copy from the chromosome and thus to deletion of the gene which is located in the chromosome *in vivo*. Another possibility as second recombination would be the reverse reaction to

integration, meaning recombination of the vector out of the chromosome, so that the chromosomal gene would remain intact. The gene deletion must therefore be detected by methods known per se, for instance in a southern blot after restriction of the chromosomal DNA with suitable enzymes or with the aid of the PCR technique on the basis of the size of the amplified region.

It is thus necessary to select two homologous regions of the gene to be deleted, each of which should include 70 base pairs in each case, for example the 5′ region and the 3′ region of the selected gene. These are cloned into the vector in such a way that they flank a part coding for an inactive protein, or are in direct succession, omitting the region inbetween. The deletion vector is obtained thereby.

Deletion of the genes ywsC, ywsC′, ywtA and ywtB considered here

A deletion vector of the invention is constructed by PCR amplification of the 5′ and 3′ regions of one of these four or three genes. The sequences SEQ ID NO. 1, 3, 5 and 7 indicated in the sequence listing are available for designing suitable primers and originate from *B. licheniformis*, but ought also to be suitable, because of the homologies to be expected, for other species, especially of the genus *Bacillus*.

The two amplified regions suitably undergo intermediate cloning in direct succession on a vector useful for these operations, for example on the vector pUC18 which is suitable for cloning steps in E. coli.

The next step is a subcloning into the vector pE194 selected for deletion, and transformation thereof into *B. subtilis* DB104, for instance by the method of protoplast transformation according to Chang & Cohen (1979; "High Frequency Transformation of *Bacillus subtilis* Protoplasts by Plasmid DNA"; *Molec. Gen. Genet.* (1979), volume 168, pages 111-115).

All working steps must be carried out at 33°C in order to ensure replication of the vector.

In a next step, the vector which has undergone intermediate cloning is likewise transformed by the method of protoplast transformation into the desired host strain, in this case *B. licheniformis*. The transformants obtained in this way and identified as positive by conventional methods (selection via the resistance marker of the plasmid; check by plasmid preparation and PCR for the insert) are subsequently cultured at 42°C under selection pressure for presence of the plasmid through addition of erythromycin. The deletion vector is unable to replicate at this temperature, and the only cells to survive are those in which the vector is integrated into the chromosome, and this integration most probably takes place in homologous or identical regions. Excision of the deletion vector can then be induced subsequently by culturing at 33°C without erythromycin selection pressure, the chromosomally encoded gene being completely deleted from the chromosome. The success of the deletion is subsequently checked by southern blotting after restriction of the chromosomal DNA with suitable enzymes or with the aid of the PCR technique.

Such transformants in which the relevant gene is deleted are additionally distinguished by a limitation or even complete inability to form GLA.

Description of the figures

Figure 1: Alignment of the gene *ywtA* (SEQ ID NO. 5) from *B. licheniformis* DSM 13 (B.l. ywtA) with the homologous gene *ywtA* from *B. subtilis* (B.s. ywtA).

Figure 2: Alignment of the protein YwtA (SEQ ID NO. 6) from *B. licheniformis* DSM 13 (B.l. YwtA) with the homologous protein YwtA from *B. subtilis* (B.s. YwtA).

SEQUENCE LISTING

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<120>  New gene products of bacillus licheniformis forming or
decomposing polyamino acids and associated improved biotechnological
production method

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<150>  DE102004030938.8
<151>  2004-06-26

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Tyr Glu Lys Arg Arg His Gln Gln Asn Ile Asp Ala Leu Pro Val Arg
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Val Asn Ile Asn Gly Ile Arg Gly Lys Ser Thr Val Thr Arg Leu Thr
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aca ggg ata tta atc gaa gca ggc tac aaa aca gta gga aaa aca acc     240
Thr Gly Ile Leu Ile Glu Ala Gly Tyr Lys Thr Val Gly Lys Thr Thr
65                  70                  75                  80

ggg aca gac gca agg atg att tat tgg gac aca ccg gaa gag aag ccg     288
Gly Thr Asp Ala Arg Met Ile Tyr Trp Asp Thr Pro Glu Glu Lys Pro
                85                  90                  95
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atg aaa gaa acg gtg gaa aga ggg gcc aat gcg att gtc agt gag tgc      384
Met Lys Glu Thr Val Glu Arg Gly Ala Asn Ala Ile Val Ser Glu Cys
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atg gcc gtt aat cct gat tac caa atc atc ttt cag gaa gaa ttg ctt      432
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Phe Phe Lys Gln Ile Ala Lys Glu Arg Asn Thr Lys Val Ile Val Ala
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Ile Met Phe Met Leu Lys Asn Lys Leu Glu Gly Arg Val Ile Tyr Gly
    370                 375                 380

gtc gga aat atc cac gga gca gcg gag cct ctc att gaa aaa ata caa      1200
Val Gly Asn Ile His Gly Ala Ala Glu Pro Leu Ile Glu Lys Ile Gln
385                 390                 395                 400

gat tac aag att aag cag ctc gtt agc tag                              1230
Asp Tyr Lys Ile Lys Gln Leu Val Ser
                405


&lt;210&gt;  2
&lt;211&gt;  409
&lt;212&gt;  PRT
&lt;213&gt;  Bacillus licheniformis DSM 13

&lt;400&gt;  2

Met Asn Glu Phe Thr Tyr Gln Ile Pro Arg Arg Arg Cys Arg Gln Thr
1               5                   10                  15

Met Trp Val Met Leu Leu Ala Cys Val Ile Val Val Gly Ile Gly Ile
            20                  25                  30

Tyr Glu Lys Arg Arg His Gln Gln Asn Ile Asp Ala Leu Pro Val Arg
        35                  40                  45

Val Asn Ile Asn Gly Ile Arg Gly Lys Ser Thr Val Thr Arg Leu Thr
    50                  55                  60

Thr Gly Ile Leu Ile Glu Ala Gly Tyr Lys Thr Val Gly Lys Thr Thr
65                  70                  75                  80

Gly Thr Asp Ala Arg Met Ile Tyr Trp Asp Thr Pro Glu Glu Lys Pro
                85                  90                  95

Ile Lys Arg Lys Pro Gln Gly Pro Asn Ile Gly Glu Gln Lys Glu Val
            100                 105                 110

Met Lys Glu Thr Val Glu Arg Gly Ala Asn Ala Ile Val Ser Glu Cys
        115                 120                 125
```

```
Met Ala Val Asn Pro Asp Tyr Gln Ile Ile Phe Gln Glu Glu Leu Leu
    130                 135                 140

Gln Ala Asn Ile Gly Val Ile Val Asn Val Leu Glu Asp His Met Asp
145                 150                 155                 160

Val Met Gly Pro Thr Leu Asp Glu Ile Ala Glu Ala Phe Thr Ala Thr
                165                 170                 175

Ile Pro Tyr Asn Gly His Leu Val Ile Thr Asp Ser Glu Tyr Thr Asp
            180                 185                 190

Phe Phe Lys Gln Ile Ala Lys Glu Arg Asn Thr Lys Val Ile Val Ala
        195                 200                 205

Asp Asn Ser Lys Ile Thr Asp Glu Tyr Leu Arg Gln Phe Glu Tyr Met
    210                 215                 220

Val Phe Pro Asp Asn Ala Ser Leu Ala Leu Gly Val Ala Gln Ala Leu
225                 230                 235                 240

Gly Ile Asp Glu Glu Thr Ala Phe Lys Gly Met Leu Asn Ala Pro Pro
                245                 250                 255

Asp Pro Gly Ala Met Arg Ile Leu Pro Leu Met Asn Ala Lys Asn Pro
            260                 265                 270

Gly His Phe Val Asn Gly Phe Ala Ala Asn Asp Ala Ala Ser Thr Leu
        275                 280                 285

Asn Ile Trp Lys Arg Val Lys Glu Ile Gly Tyr Pro Thr Asp Gln Pro
    290                 295                 300

Ile Val Ile Met Asn Cys Arg Ala Asp Arg Val Asp Arg Thr Gln Gln
305                 310                 315                 320

Phe Ala Glu Asp Val Leu Pro Tyr Ile Glu Ala Ser Glu Leu Val Leu
                325                 330                 335

Ile Gly Glu Thr Thr Glu Pro Ile Val Lys Ala Tyr Glu Ala Gly Lys
            340                 345                 350

Ile Pro Ala Asp Lys Leu Phe Asp Phe Glu His Lys Ser Thr Glu Glu
```

```
        355                 360                 365

Ile Met Phe Met Leu Lys Asn Lys Leu Glu Gly Arg Val Ile Tyr Gly
    370                 375                 380

Val Gly Asn Ile His Gly Ala Ala Glu Pro Leu Ile Glu Lys Ile Gln
385                 390                 395                 400

Asp Tyr Lys Ile Lys Gln Leu Val Ser
                405


<210>  3
<211>  1182
<212>  DNA
<213>  Bacillus licheniformis DSM 13

<220>
<221>  gene
<222>  (1)..(1182)
<223>  ywsC'


<220>
<221>  CDS
<222>  (1)..(1182)
<223>


<400>  3
atg tgg gta atg cta tta gcc tgt gtg atc gtt gtt ggg atc ggc att        48
Met Trp Val Met Leu Leu Ala Cys Val Ile Val Val Gly Ile Gly Ile
1               5                   10                  15

tat gaa aaa agg cgc cac cag caa aat atc gat gcg ctg cct gtc cga        96
Tyr Glu Lys Arg Arg His Gln Gln Asn Ile Asp Ala Leu Pro Val Arg
            20                  25                  30

gtg aac atc aac ggt ata cgc gga aag tcc acg gtg aca aga tta aca       144
Val Asn Ile Asn Gly Ile Arg Gly Lys Ser Thr Val Thr Arg Leu Thr
        35                  40                  45

aca ggg ata tta atc gaa gca ggc tac aaa aca gta gga aaa aca acc       192
Thr Gly Ile Leu Ile Glu Ala Gly Tyr Lys Thr Val Gly Lys Thr Thr
    50                  55                  60

ggg aca gac gca agg atg att tat tgg gac aca ccg gaa gag aag ccg       240
Gly Thr Asp Ala Arg Met Ile Tyr Trp Asp Thr Pro Glu Glu Lys Pro
65                  70                  75                  80

atc aaa aga aag ccg caa ggg ccg aat atc gga gag cag aag gag gtt       288
Ile Lys Arg Lys Pro Gln Gly Pro Asn Ile Gly Glu Gln Lys Glu Val
                85                  90                  95

atg aaa gaa acg gtg gaa aga ggg gcc aat gcg att gtc agt gag tgc       336
Met Lys Glu Thr Val Glu Arg Gly Ala Asn Ala Ile Val Ser Glu Cys
```

```
            100                 105                 110

atg gcc gtt aat cct gat tac caa atc atc ttt cag gaa gaa ttg ctt       384
Met Ala Val Asn Pro Asp Tyr Gln Ile Ile Phe Gln Glu Glu Leu Leu
        115                 120                 125

cag gct aat atc ggc gtg atc gtg aac gtg ctg gag gat cac atg gat       432
Gln Ala Asn Ile Gly Val Ile Val Asn Val Leu Glu Asp His Met Asp
    130                 135                 140

gtg atg gga ccg act ttg gat gaa atc gca gaa gca ttc aca gca acc       480
Val Met Gly Pro Thr Leu Asp Glu Ile Ala Glu Ala Phe Thr Ala Thr
145                 150                 155                 160

att cct tat aat gga cat ttg gtt att act gat agt gag tat acc gat       528
Ile Pro Tyr Asn Gly His Leu Val Ile Thr Asp Ser Glu Tyr Thr Asp
                165                 170                 175

ttc ttt aag caa att gca aaa gaa agg aac aca aaa gtc atc gtc gca       576
Phe Phe Lys Gln Ile Ala Lys Glu Arg Asn Thr Lys Val Ile Val Ala
            180                 185                 190

gac aat tct aaa ata aca gat gaa tac ctc aga cag ttt gag tac atg       624
Asp Asn Ser Lys Ile Thr Asp Glu Tyr Leu Arg Gln Phe Glu Tyr Met
        195                 200                 205

gta ttc cct gat aat gcg tct ctt gcg ctc ggt gta gct caa gcg ttg       672
Val Phe Pro Asp Asn Ala Ser Leu Ala Leu Gly Val Ala Gln Ala Leu
    210                 215                 220

ggc att gac gaa gaa acc gcc ttt aaa ggc atg ctg aat gcg ccg cct       720
Gly Ile Asp Glu Glu Thr Ala Phe Lys Gly Met Leu Asn Ala Pro Pro
225                 230                 235                 240

gat ccg gga gcc atg aga att ctg ccg ctg atg aac gcc aag aat ccc       768
Asp Pro Gly Ala Met Arg Ile Leu Pro Leu Met Asn Ala Lys Asn Pro
                245                 250                 255

gga cat ttc gtc aac ggt ttt gcg gcc aat gac gca gct tcc act tta       816
Gly His Phe Val Asn Gly Phe Ala Ala Asn Asp Ala Ala Ser Thr Leu
            260                 265                 270

aac att tgg aag cgt gta aaa gaa ata ggc tat cct acg gat cag ccg       864
Asn Ile Trp Lys Arg Val Lys Glu Ile Gly Tyr Pro Thr Asp Gln Pro
        275                 280                 285

atc gtc att atg aac tgc cgc gcc gac agg gta gac aga aca cag cag       912
Ile Val Ile Met Asn Cys Arg Ala Asp Arg Val Asp Arg Thr Gln Gln
    290                 295                 300

ttt gcg gaa gat gtc ctt cct tat att gaa gca agt gaa ctt gtg ctg       960
Phe Ala Glu Asp Val Leu Pro Tyr Ile Glu Ala Ser Glu Leu Val Leu
305                 310                 315                 320

att gga gaa aca aca gag ccg atc gtc aaa gca tat gaa gca ggc aaa      1008
Ile Gly Glu Thr Thr Glu Pro Ile Val Lys Ala Tyr Glu Ala Gly Lys
                325                 330                 335

att cct gcg gac aag ctg ttt gat ttt gag cac aaa tca acg gaa gaa      1056
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```
Ile Pro Ala Asp Lys Leu Phe Asp Phe Glu His Lys Ser Thr Glu Glu
            340                 345                 350

atc atg ttc atg ctg aaa aac aag ctt gag ggc cgc gtt att tac gga      1104
Ile Met Phe Met Leu Lys Asn Lys Leu Glu Gly Arg Val Ile Tyr Gly
        355                 360                 365

gtc gga aat atc cac gga gca gcg gag cct ctc att gaa aaa ata caa      1152
Val Gly Asn Ile His Gly Ala Ala Glu Pro Leu Ile Glu Lys Ile Gln
    370                 375                 380

gat tac aag att aag cag ctc gtt agc tag                              1182
Asp Tyr Lys Ile Lys Gln Leu Val Ser
385                 390


<210>  4
<211>  393
<212>  PRT
<213>  Bacillus licheniformis DSM 13

<400>  4

Met Trp Val Met Leu Leu Ala Cys Val Ile Val Val Gly Ile Gly Ile
1               5                   10                  15


Tyr Glu Lys Arg Arg His Gln Gln Asn Ile Asp Ala Leu Pro Val Arg
            20                  25                  30


Val Asn Ile Asn Gly Ile Arg Gly Lys Ser Thr Val Thr Arg Leu Thr
        35                  40                  45


Thr Gly Ile Leu Ile Glu Ala Gly Tyr Lys Thr Val Gly Lys Thr Thr
    50                  55                  60


Gly Thr Asp Ala Arg Met Ile Tyr Trp Asp Thr Pro Glu Glu Lys Pro
65                  70                  75                  80


Ile Lys Arg Lys Pro Gln Gly Pro Asn Ile Gly Glu Gln Lys Glu Val
                85                  90                  95


Met Lys Glu Thr Val Glu Arg Gly Ala Asn Ala Ile Val Ser Glu Cys
            100                 105                 110


Met Ala Val Asn Pro Asp Tyr Gln Ile Ile Phe Gln Glu Glu Leu Leu
        115                 120                 125


Gln Ala Asn Ile Gly Val Ile Val Asn Val Leu Glu Asp His Met Asp
    130                 135                 140
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```
Val Met Gly Pro Thr Leu Asp Glu Ile Ala Glu Ala Phe Thr Ala Thr
145                 150                 155                 160

Ile Pro Tyr Asn Gly His Leu Val Ile Thr Asp Ser Glu Tyr Thr Asp
                165                 170                 175

Phe Phe Lys Gln Ile Ala Lys Glu Arg Asn Thr Lys Val Ile Val Ala
            180                 185                 190

Asp Asn Ser Lys Ile Thr Asp Glu Tyr Leu Arg Gln Phe Glu Tyr Met
        195                 200                 205

Val Phe Pro Asp Asn Ala Ser Leu Ala Leu Gly Val Ala Gln Ala Leu
    210                 215                 220

Gly Ile Asp Glu Glu Thr Ala Phe Lys Gly Met Leu Asn Ala Pro Pro
225                 230                 235                 240

Asp Pro Gly Ala Met Arg Ile Leu Pro Leu Met Asn Ala Lys Asn Pro
                245                 250                 255

Gly His Phe Val Asn Gly Phe Ala Ala Asn Asp Ala Ala Ser Thr Leu
            260                 265                 270

Asn Ile Trp Lys Arg Val Lys Glu Ile Gly Tyr Pro Thr Asp Gln Pro
        275                 280                 285

Ile Val Ile Met Asn Cys Arg Ala Asp Arg Val Asp Arg Thr Gln Gln
    290                 295                 300

Phe Ala Glu Asp Val Leu Pro Tyr Ile Glu Ala Ser Glu Leu Val Leu
305                 310                 315                 320

Ile Gly Glu Thr Thr Glu Pro Ile Val Lys Ala Tyr Glu Ala Gly Lys
                325                 330                 335

Ile Pro Ala Asp Lys Leu Phe Asp Phe Glu His Lys Ser Thr Glu Glu
            340                 345                 350

Ile Met Phe Met Leu Lys Asn Lys Leu Glu Gly Arg Val Ile Tyr Gly
        355                 360                 365

Val Gly Asn Ile His Gly Ala Ala Glu Pro Leu Ile Glu Lys Ile Gln
    370                 375                 380
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Asp Tyr Lys Ile Lys Gln Leu Val Ser
385                 390


<210>  5
<211>  450
<212>  DNA
<213>  Bacillus licheniformis DSM 13

<220>
<221>  gene
<222>  (1)..(450)
<223>  ywtA


<220>
<221>  CDS
<222>  (1)..(450)
<223>


<400>  5
atg ttt gga tca gat tta tat atc gcc ctc att tta gga gtc tta ctc       48
Met Phe Gly Ser Asp Leu Tyr Ile Ala Leu Ile Leu Gly Val Leu Leu
1               5                   10                  15

agt ttg att ttt gca gag aaa acg gga att gta cca gcc ggc ctc gtc       96
Ser Leu Ile Phe Ala Glu Lys Thr Gly Ile Val Pro Ala Gly Leu Val
            20                  25                  30

gta ccg ggt tat ttg gga ctt gtc ttc aat cag ccg att ttc atg ctg      144
Val Pro Gly Tyr Leu Gly Leu Val Phe Asn Gln Pro Ile Phe Met Leu
        35                  40                  45

ctc gtt ctt ttt gtc agt ttg ctg acg tat gtc atc gtg aaa ttc gga      192
Leu Val Leu Phe Val Ser Leu Leu Thr Tyr Val Ile Val Lys Phe Gly
    50                  55                  60

ctt tcc aaa att atg att cta tac gga cgc aga aaa ttc gca gca atg      240
Leu Ser Lys Ile Met Ile Leu Tyr Gly Arg Arg Lys Phe Ala Ala Met
65                  70                  75                  80

ctg att acg gga att ctt ttg aaa atc ggt ttt gat ttt ata tat ccg      288
Leu Ile Thr Gly Ile Leu Leu Lys Ile Gly Phe Asp Phe Ile Tyr Pro
                85                  90                  95

gtg atg ccg ttt gag att gcc gaa ttc agg gga atc gga atc atc gtg      336
Val Met Pro Phe Glu Ile Ala Glu Phe Arg Gly Ile Gly Ile Ile Val
            100                 105                 110

ccg ggg ctg atc gcc aat acc att caa aga cag gga tta acg att acg      384
Pro Gly Leu Ile Ala Asn Thr Ile Gln Arg Gln Gly Leu Thr Ile Thr
        115                 120                 125

ctt gga agt acg ctt tta ttg agc gga gca aca ttc gtc att atg tat      432
Leu Gly Ser Thr Leu Leu Leu Ser Gly Ala Thr Phe Val Ile Met Tyr
    130                 135                 140
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gct tac tat cta atc taa 450
Ala Tyr Tyr Leu Ile
145

<210> 6
<211> 149
<212> PRT
<213> Bacillus licheniformis DSM 13

<400> 6

Met Phe Gly Ser Asp Leu Tyr Ile Ala Leu Ile Leu Gly Val Leu Leu
1 5 10 15

Ser Leu Ile Phe Ala Glu Lys Thr Gly Ile Val Pro Ala Gly Leu Val
20 25 30

Val Pro Gly Tyr Leu Gly Leu Val Phe Asn Gln Pro Ile Phe Met Leu
35 40 45

Leu Val Leu Phe Val Ser Leu Leu Thr Tyr Val Ile Val Lys Phe Gly
50 55 60

Leu Ser Lys Ile Met Ile Leu Tyr Gly Arg Arg Lys Phe Ala Ala Met
65 70 75 80

Leu Ile Thr Gly Ile Leu Leu Lys Ile Gly Phe Asp Phe Ile Tyr Pro
85 90 95

Val Met Pro Phe Glu Ile Ala Glu Phe Arg Gly Ile Gly Ile Ile Val
100 105 110

Pro Gly Leu Ile Ala Asn Thr Ile Gln Arg Gln Gly Leu Thr Ile Thr
115 120 125

Leu Gly Ser Thr Leu Leu Leu Ser Gly Ala Thr Phe Val Ile Met Tyr
130 135 140

Ala Tyr Tyr Leu Ile
145

<210> 7
<211> 1170
<212> DNA
<213> Bacillus licheniformis DSM 13

<220>
<221> gene

<222> (1)..(1170)
<223> ywtB

<220>
<221> CDS
<222> (1)..(1170)
<223>

<400> 7
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atg aaa aaa caa ctg aac ttt cag gaa aaa ctg ctg aag ttg acg aag        48
Met Lys Lys Gln Leu Asn Phe Gln Glu Lys Leu Leu Lys Leu Thr Lys
1               5                   10                  15

cag gag aaa aag aaa aca aac aag cac gtc ttt atc gta ttg ccc gtt        96
Gln Glu Lys Lys Lys Thr Asn Lys His Val Phe Ile Val Leu Pro Val
            20                  25                  30

att ttc tgt tta atg ttt gtc ttt act tgg gtc gga agc gcc aaa act       144
Ile Phe Cys Leu Met Phe Val Phe Thr Trp Val Gly Ser Ala Lys Thr
        35                  40                  45

cct tcg caa atg gac aaa aaa gaa gat gcc aag ctt aca gct act ttt       192
Pro Ser Gln Met Asp Lys Lys Glu Asp Ala Lys Leu Thr Ala Thr Phe
    50                  55                  60

gtt ggc gat atc atg atg gga aga aac gta gaa aaa gtg aca aac ttg       240
Val Gly Asp Ile Met Met Gly Arg Asn Val Glu Lys Val Thr Asn Leu
65                  70                  75                  80

cac ggt tcg gaa agt gtc ttc aaa aat gtg aag ccg tac ttt aat gtg       288
His Gly Ser Glu Ser Val Phe Lys Asn Val Lys Pro Tyr Phe Asn Val
                85                  90                  95

tca gat ttt atc aca gga aac ttt gaa aac cct gta acc aat gca aag       336
Ser Asp Phe Ile Thr Gly Asn Phe Glu Asn Pro Val Thr Asn Ala Lys
            100                 105                 110

gac tat caa gag gca gaa aag aac atc cat ctg caa acg aat caa gaa       384
Asp Tyr Gln Glu Ala Glu Lys Asn Ile His Leu Gln Thr Asn Gln Glu
        115                 120                 125

tca gtc gaa aca ttg aaa aag ctg aac ttc agc gta ctg aat ttt gcc       432
Ser Val Glu Thr Leu Lys Lys Leu Asn Phe Ser Val Leu Asn Phe Ala
    130                 135                 140

aac aac cat gcg atg gac tac ggg gaa gac ggt ttg aag gat acg ctc       480
Asn Asn His Ala Met Asp Tyr Gly Glu Asp Gly Leu Lys Asp Thr Leu
145                 150                 155                 160

aat aaa ttt tca aat gag aat ctg gag ctt gtc gga gca gga aat aat       528
Asn Lys Phe Ser Asn Glu Asn Leu Glu Leu Val Gly Ala Gly Asn Asn
                165                 170                 175

ctt gaa gac gcg aaa cag cac gta tcc tat cag aat gtg aac ggc gta       576
Leu Glu Asp Ala Lys Gln His Val Ser Tyr Gln Asn Val Asn Gly Val
            180                 185                 190
```

```
aaa att gca acg ctc ggt ttt aca gac gtc tac aca aag aac ttt aca        624
Lys Ile Ala Thr Leu Gly Phe Thr Asp Val Tyr Thr Lys Asn Phe Thr
        195                 200                 205

gcc aaa aag aac aga ggc gga gtg ctg ccg ctc agt ccg aaa atc ttt        672
Ala Lys Lys Asn Arg Gly Gly Val Leu Pro Leu Ser Pro Lys Ile Phe
    210                 215                 220

att cca atg att gcg gaa gca tcg aaa aaa gcg gat ctt gtc ctt gtc        720
Ile Pro Met Ile Ala Glu Ala Ser Lys Lys Ala Asp Leu Val Leu Val
225                 230                 235                 240

cat gtg cac tgg gga caa gaa tat gac aat gaa ccg aac gac aga cag        768
His Val His Trp Gly Gln Glu Tyr Asp Asn Glu Pro Asn Asp Arg Gln
                245                 250                 255

aag gat ctg gcc aag gcg att gca gat gcc gga gca gat gtc atc atc        816
Lys Asp Leu Ala Lys Ala Ile Ala Asp Ala Gly Ala Asp Val Ile Ile
            260                 265                 270

ggc gct cat ccc cat gtt ctc gaa ccg atc gaa gtg tat aac ggt act        864
Gly Ala His Pro His Val Leu Glu Pro Ile Glu Val Tyr Asn Gly Thr
        275                 280                 285

gtg att ttc tac agc ctc ggc aac ttt gta ttt gat cag ggc tgg tca        912
Val Ile Phe Tyr Ser Leu Gly Asn Phe Val Phe Asp Gln Gly Trp Ser
    290                 295                 300

aga aca cgg gac agc gcg ctt gta caa tac cat tta atg aat gac ggc        960
Arg Thr Arg Asp Ser Ala Leu Val Gln Tyr His Leu Met Asn Asp Gly
305                 310                 315                 320

aaa ggg cgc ttt gag gta acg cct ctc aac att cgc gaa gca acg ccg       1008
Lys Gly Arg Phe Glu Val Thr Pro Leu Asn Ile Arg Glu Ala Thr Pro
                325                 330                 335

acg cct tta ggc aag agc gac ttc tta aaa cga aaa gcg atc ttc cgt       1056
Thr Pro Leu Gly Lys Ser Asp Phe Leu Lys Arg Lys Ala Ile Phe Arg
            340                 345                 350

caa ttg aca aaa gga aca aac ctc gac tgg aaa gaa gag aac gga aaa       1104
Gln Leu Thr Lys Gly Thr Asn Leu Asp Trp Lys Glu Glu Asn Gly Lys
        355                 360                 365

tta acg ttt gaa gtc gat cat gcg gac aag ctg aaa aat aat aaa aac       1152
Leu Thr Phe Glu Val Asp His Ala Asp Lys Leu Lys Asn Asn Lys Asn
    370                 375                 380

gga gtg gtg aac aaa tga                                               1170
Gly Val Val Asn Lys
385
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<210> 8
<211> 389
<212> PRT
<213> Bacillus licheniformis DSM 13

<400> 8

Met Lys Lys Gln Leu Asn Phe Gln Glu Lys Leu Leu Lys Leu Thr Lys
1 5 10 15

Gln Glu Lys Lys Lys Thr Asn Lys His Val Phe Ile Val Leu Pro Val
20 25 30

Ile Phe Cys Leu Met Phe Val Phe Thr Trp Val Gly Ser Ala Lys Thr
35 40 45

Pro Ser Gln Met Asp Lys Lys Glu Asp Ala Lys Leu Thr Ala Thr Phe
50 55 60

Val Gly Asp Ile Met Met Gly Arg Asn Val Glu Lys Val Thr Asn Leu
65 70 75 80

His Gly Ser Glu Ser Val Phe Lys Asn Val Lys Pro Tyr Phe Asn Val
85 90 95

Ser Asp Phe Ile Thr Gly Asn Phe Glu Asn Pro Val Thr Asn Ala Lys
100 105 110

Asp Tyr Gln Glu Ala Glu Lys Asn Ile His Leu Gln Thr Asn Gln Glu
115 120 125

Ser Val Glu Thr Leu Lys Lys Leu Asn Phe Ser Val Leu Asn Phe Ala
130 135 140

Asn Asn His Ala Met Asp Tyr Gly Glu Asp Gly Leu Lys Asp Thr Leu
145 150 155 160

Asn Lys Phe Ser Asn Glu Asn Leu Glu Leu Val Gly Ala Gly Asn Asn
165 170 175

Leu Glu Asp Ala Lys Gln His Val Ser Tyr Gln Asn Val Asn Gly Val
180 185 190

Lys Ile Ala Thr Leu Gly Phe Thr Asp Val Tyr Thr Lys Asn Phe Thr
195 200 205

Ala Lys Lys Asn Arg Gly Gly Val Leu Pro Leu Ser Pro Lys Ile Phe
210 215 220

Ile Pro Met Ile Ala Glu Ala Ser Lys Lys Ala Asp Leu Val Leu Val
225 230 235 240

```
His Val His Trp Gly Gln Glu Tyr Asp Asn Glu Pro Asn Asp Arg Gln
                245                     250                     255

Lys Asp Leu Ala Lys Ala Ile Ala Asp Ala Gly Ala Asp Val Ile Ile
            260                     265                     270

Gly Ala His Pro His Val Leu Glu Pro Ile Glu Val Tyr Asn Gly Thr
        275                     280                     285

Val Ile Phe Tyr Ser Leu Gly Asn Phe Val Phe Asp Gln Gly Trp Ser
    290                     295                     300

Arg Thr Arg Asp Ser Ala Leu Val Gln Tyr His Leu Met Asn Asp Gly
305                     310                     315                     320

Lys Gly Arg Phe Glu Val Thr Pro Leu Asn Ile Arg Glu Ala Thr Pro
                325                     330                     335

Thr Pro Leu Gly Lys Ser Asp Phe Leu Lys Arg Lys Ala Ile Phe Arg
            340                     345                     350

Gln Leu Thr Lys Gly Thr Asn Leu Asp Trp Lys Glu Glu Asn Gly Lys
        355                     360                     365

Leu Thr Phe Glu Val Asp His Ala Asp Lys Leu Lys Asn Asn Lys Asn
    370                     375                     380

Gly Val Val Asn Lys
385
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<210> 9
<211> 1245
<212> DNA
<213> Bacillus licheniformis DSM 13

<220>
<221> gene
<222> (1)..(1245)
<223> ywtD

<220>
<221> CDS
<222> (1)..(1245)
<223>

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<220>
<221>  misc_feature
<222>  (1)..(3)
<223>  First codon translated as Met.


<400>  9
ttg ata aaa aaa gcg gca aac aaa aag ttg gtt ttg ttt tgt gga att        48
Leu Ile Lys Lys Ala Ala Asn Lys Lys Leu Val Leu Phe Cys Gly Ile
1               5                   10                  15

gcg gtg ctt tgg atg tct tta ttt tta acg aat cat aat gat gta cgc        96
Ala Val Leu Trp Met Ser Leu Phe Leu Thr Asn His Asn Asp Val Arg
            20                  25                  30

gcc gat acg atc ggc gag aaa ata gcg gaa act gcc aga cag ctt gag       144
Ala Asp Thr Ile Gly Glu Lys Ile Ala Glu Thr Ala Arg Gln Leu Glu
        35                  40                  45

ggt gcg aaa tac agc tac ggc gga gag aag ccg aaa acg ggg ttt gac       192
Gly Ala Lys Tyr Ser Tyr Gly Gly Glu Lys Pro Lys Thr Gly Phe Asp
    50                  55                  60

tcg tca ggc ttt gtg caa tat gtg ttt caa tcg ctc gat att acg ctt       240
Ser Ser Gly Phe Val Gln Tyr Val Phe Gln Ser Leu Asp Ile Thr Leu
65                  70                  75                  80

ccg aga acg gta aag gaa caa tcg act ctt ggg agc agt gtc ggc cgt       288
Pro Arg Thr Val Lys Glu Gln Ser Thr Leu Gly Ser Ser Val Gly Arg
                85                  90                  95

cag cag ctc gaa aag ggg gac ctt gtc ttt ttc aag aat gcc gag ctg       336
Gln Gln Leu Glu Lys Gly Asp Leu Val Phe Phe Lys Asn Ala Glu Leu
            100                 105                 110

gaa tcg gac gga ccg acc cat gtc gcc atc tat ttg gga aat gat caa       384
Glu Ser Asp Gly Pro Thr His Val Ala Ile Tyr Leu Gly Asn Asp Gln
        115                 120                 125

atc atc cac agc aca aaa tca aac ggg gtt gtc gtg aca aag ctt gaa       432
Ile Ile His Ser Thr Lys Ser Asn Gly Val Val Val Thr Lys Leu Glu
    130                 135                 140

ggc agc tct tac tgg agc tcg ggg tat ttt aaa gcg aaa agg atc aca       480
Gly Ser Ser Tyr Trp Ser Ser Gly Tyr Phe Lys Ala Lys Arg Ile Thr
145                 150                 155                 160

aaa gag cct gag att tcg atg gat cct gtc gtt caa aaa gca aaa agc       528
Lys Glu Pro Glu Ile Ser Met Asp Pro Val Val Gln Lys Ala Lys Ser
                165                 170                 175

tat gtc ggt gtt cct tat gta ttt gga ggc aac tct ccg gat ctc gga       576
Tyr Val Gly Val Pro Tyr Val Phe Gly Gly Asn Ser Pro Asp Leu Gly
            180                 185                 190

ttt gac tgt tcg ggg ttg acc caa tac gtc ttc aga gag gtg ctc ggc       624
Phe Asp Cys Ser Gly Leu Thr Gln Tyr Val Phe Arg Glu Val Leu Gly
        195                 200                 205
```

```
gtt tat ttg cca agg tcg gct gaa cag caa tgg gct gtc ggt caa aag        672
Val Tyr Leu Pro Arg Ser Ala Glu Gln Gln Trp Ala Val Gly Gln Lys
    210                 215                 220

gtg aag ctt gaa gat atc cgg ccg ggt gat gtt ttg ttt ttc agc aat        720
Val Lys Leu Glu Asp Ile Arg Pro Gly Asp Val Leu Phe Phe Ser Asn
225                 230                 235                 240

acg tac aaa ccg gga ata tcc cat aac ggc atc tat gcc ggg ggc ggg        768
Thr Tyr Lys Pro Gly Ile Ser His Asn Gly Ile Tyr Ala Gly Gly Gly
                245                 250                 255

cgg ttt atc cat gcg agc cgt tca aat aaa gtg acg ata tcc tac ttg        816
Arg Phe Ile His Ala Ser Arg Ser Asn Lys Val Thr Ile Ser Tyr Leu
            260                 265                 270

tcg gct tcc tat tgg cag aag aag ttc aca gga gtc aga cgt ttt gac        864
Ser Ala Ser Tyr Trp Gln Lys Lys Phe Thr Gly Val Arg Arg Phe Asp
        275                 280                 285

aac atg tcc ctg cca aaa aat ccg att gta tcc gaa gcc atc agg cat        912
Asn Met Ser Leu Pro Lys Asn Pro Ile Val Ser Glu Ala Ile Arg His
    290                 295                 300

atc ggc gaa gtc ggt tat caa aaa ggc ggc aca tcg cct aaa gaa ggc        960
Ile Gly Glu Val Gly Tyr Gln Lys Gly Gly Thr Ser Pro Lys Glu Gly
305                 310                 315                 320

ttt gat acg gct ggg ttt atc caa tat gtc tac aaa acg gcg gca gga       1008
Phe Asp Thr Ala Gly Phe Ile Gln Tyr Val Tyr Lys Thr Ala Ala Gly
                325                 330                 335

gtg gag ctt ccg agg tat gct gac aaa caa tac agc acg ggt aag aaa       1056
Val Glu Leu Pro Arg Tyr Ala Asp Lys Gln Tyr Ser Thr Gly Lys Lys
            340                 345                 350

att acc aaa cag gag ctt gag cct gga gac atc gtc ttc ttt aaa gga       1104
Ile Thr Lys Gln Glu Leu Glu Pro Gly Asp Ile Val Phe Phe Lys Gly
        355                 360                 365

acc act gtt atg aat ccc gcc atc tat atc gga aac ggc cag gtc gtt       1152
Thr Thr Val Met Asn Pro Ala Ile Tyr Ile Gly Asn Gly Gln Val Val
    370                 375                 380

ctt gtc acc ttg tct gcc ggt gta acg aca gca gat atg gag acg agc       1200
Leu Val Thr Leu Ser Ala Gly Val Thr Thr Ala Asp Met Glu Thr Ser
385                 390                 395                 400

gcc tat tgg aaa gat aaa tac gcc gga agc gtc aga att gag tag           1245
Ala Tyr Trp Lys Asp Lys Tyr Ala Gly Ser Val Arg Ile Glu
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<213>  Bacillus licheniformis DSM 13

<220>
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<221> misc_feature
<222> (1)..(3)
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<400> 10

Leu Ile Lys Lys Ala Ala Asn Lys Lys Leu Val Leu Phe Cys Gly Ile
1 5 10 15

Ala Val Leu Trp Met Ser Leu Phe Leu Thr Asn His Asn Asp Val Arg
20 25 30

Ala Asp Thr Ile Gly Glu Lys Ile Ala Glu Thr Ala Arg Gln Leu Glu
35 40 45

Gly Ala Lys Tyr Ser Tyr Gly Gly Glu Lys Pro Lys Thr Gly Phe Asp
50 55 60

Ser Ser Gly Phe Val Gln Tyr Val Phe Gln Ser Leu Asp Ile Thr Leu
65 70 75 80

Pro Arg Thr Val Lys Glu Gln Ser Thr Leu Gly Ser Ser Val Gly Arg
85 90 95

Gln Gln Leu Glu Lys Gly Asp Leu Val Phe Phe Lys Asn Ala Glu Leu
100 105 110

Glu Ser Asp Gly Pro Thr His Val Ala Ile Tyr Leu Gly Asn Asp Gln
115 120 125

Ile Ile His Ser Thr Lys Ser Asn Gly Val Val Val Thr Lys Leu Glu
130 135 140

Gly Ser Ser Tyr Trp Ser Ser Gly Tyr Phe Lys Ala Lys Arg Ile Thr
145 150 155 160

Lys Glu Pro Glu Ile Ser Met Asp Pro Val Val Gln Lys Ala Lys Ser
165 170 175

Tyr Val Gly Val Pro Tyr Val Phe Gly Gly Asn Ser Pro Asp Leu Gly
180 185 190

Phe Asp Cys Ser Gly Leu Thr Gln Tyr Val Phe Arg Glu Val Leu Gly
195 200 205

Val Tyr Leu Pro Arg Ser Ala Glu Gln Gln Trp Ala Val Gly Gln Lys

```
    210                 215                 220

Val Lys Leu Glu Asp Ile Arg Pro Gly Asp Val Leu Phe Phe Ser Asn
225                 230                 235                 240

Thr Tyr Lys Pro Gly Ile Ser His Asn Gly Ile Tyr Ala Gly Gly Gly
                245                 250                 255

Arg Phe Ile His Ala Ser Arg Ser Asn Lys Val Thr Ile Ser Tyr Leu
            260                 265                 270

Ser Ala Ser Tyr Trp Gln Lys Lys Phe Thr Gly Val Arg Arg Phe Asp
        275                 280                 285

Asn Met Ser Leu Pro Lys Asn Pro Ile Val Ser Glu Ala Ile Arg His
    290                 295                 300

Ile Gly Glu Val Gly Tyr Gln Lys Gly Gly Thr Ser Pro Lys Glu Gly
305                 310                 315                 320

Phe Asp Thr Ala Gly Phe Ile Gln Tyr Val Tyr Lys Thr Ala Ala Gly
                325                 330                 335

Val Glu Leu Pro Arg Tyr Ala Asp Lys Gln Tyr Ser Thr Gly Lys Lys
            340                 345                 350

Ile Thr Lys Gln Glu Leu Glu Pro Gly Asp Ile Val Phe Phe Lys Gly
        355                 360                 365

Thr Thr Val Met Asn Pro Ala Ile Tyr Ile Gly Asn Gly Gln Val Val
    370                 375                 380

Leu Val Thr Leu Ser Ala Gly Val Thr Thr Ala Asp Met Glu Thr Ser
385                 390                 395                 400

Ala Tyr Trp Lys Asp Lys Tyr Ala Gly Ser Val Arg Ile Glu
                405                 410
```

Patentkrav

1. Fremgangsmåde til reduktion af slim, som skyldes poly-gamma-glutamat, kendetegnet ved undertrykkelse af funktionen i det af genet ywtA kodede protein YwtA (CapC, PgsC) med en aminosyresekvens, som er i det mindste 94% identisk med den i SEQ ID NO. 6 anførte aminosyresekvens, som et i dannelsen af polyaminosyrer impliceret enzym eller som underenhed af et sådant enzym.

2. Fremgangsmåde ifølge krav 1, kendetegnet ved, at funktionen af proteinet YwtA er undertrykt under fermenteringen af mikroorganismen.

3. Fremgangsmåde ifølge krav 1 eller 2, kendetegnet ved en reduktion af det slim, som skyldes polyaminosyrer, til 50%.

4. Fremgangsmåde ifølge et af kravene 1, 2 eller 3, kendetegnet ved, at mikroorganismen
a) er en bakterie og/eller
b) er en gram-negativ bakterie eller
c) er en gram-positiv bakterie.

5. Fremgangsmåde ifølge krav 4, kendetegnet ved, at mikroorganismen er udvalgt blandt en af slægterne Escherichia, Klebsiella, Pseudomonas, Xanthomonas, Bacillus, Staphylococcus eller Corynebacterium.

6. Fremgangsmåde ifølge et af de foregående krav, kendetegnet ved følgende trin til undertrykkelse af funktionen af det af genet ywtA kodede protein YwtA:
a) udvælgelse af to områder på sekvensen SEQ ID NO. 5,
b) kloning af områderne i en vektor, således af de flankerer en del, som koder for et ikkeaktivt protein, eller således at de følger direkte efter hinanden, idet det derimellem liggende område udelades,
c) deletering af genet ywtA med den i trin b) fremstillede vektor og

d) påvisning af gen-deletionen.

7. Fremgangsmåde ifølge et af de foregående krav, kendetegnet ved, at funktionen af det af genet ywtA kodede protein YwtA (CapC, PsgC) er undertrykt ved hjælp af anvendelse af en nukleinsyre med en deletions- eller insertionsmutation, fortrinsvis omfattende de randsekvenser, som omfatter hver især i det mindste 70 til 150 nukleinsyrepositioner, af det for proteinet kodende område.

8. Fremgangsmåde til fremstilling af et genanvendeligt stof ved hjælp af fermentering af en mikroorganisme, kendetegnet ved, at dannelsen af poly-gamma-glutamat under fermenteringen er reduceret af mikroorganismen ved hjælp af undertrykkelse af funktionen af det af genet ywtA kodede protein YwtA (CapC, PgsC) med en aminosyresekvens, som er i det mindste 94% identisk med den i SEQ ID NO. 6 anførte aminosyresekvens, som et i dannelsen af polyaminosyrer impliceret enzym eller som underenhed af et sådant enzym.

9. Fremgangsmåde ifølge krav 8, kendetegnet ved, at funktionen af det af genet ywtA kodede protein YwtA (CapC, PgsC) er undertrykt ved hjælp af en fremgangsmåde med følgende trin:
a) udvælgelse af to områder på sekvensen SEQ ID NO. 5,
b) kloning af områderne i en vektor, således at de flankerer en del, som koder for et ikkeaktivt protein, eller således at de følger direkte efter hinanden, idet det derimellem liggende område udelades,
c) deletering af genet ywtA med den i trin b) fremstillede vektor og
d) påvisning af gen-deletionen.

10. Fremgangsmåde ifølge krav 8 eller 9, kendetegnet ved, at funktionen af det af genet ywtA kodede protein YwtA (CapC, PsgC) er undertrykt ved hjælp af anvendelse af en nukleinsyre med en deletions- eller insertionsmutation, fortrinsvis omfattende de randsekvenser, som omfatter hver især i det

mindste 70 til 150 nukleinsyrepositioner, af det for proteinet kodende område.

11. Fremgangsmåde ifølge krav 8, 9 eller 10, kendetegnet ved,
5 at det genanvendelige stof er et naturligt stof, et kosttilskudsmiddel eller en farmaceutisk relevant forbindelse eller et enzym.

12. Fremgangsmåde ifølge krav 11, kendetegnet ved, at enzymet
10 er udvalgt af gruppen af α-amylaser, proteaser, cellulaser, lipaser, oxidoreduktaser, peroxidaser, laccaser, oxidaser og hemicellulaser.

13. Anvendelse af en nukleinsyre kodende for et i dannelsen
15 af poly-gamma-glutamat impliceret protein YwtA (CapC, PgsC) med en aminosyresekvens, som er i det mindste 94% identisk med den i SEQ ID NO. 6 anførte aminosyresekvens, eller hver især dele heraf til undertrykkelse af funktionen af proteinet YwtA (CapC, PgsC) som et i dannelsen af polyaminosyrer impliceret
20 enzym eller som underenhed af et sådant enzym.

14. Anvendelse af en nukleinsyre kodende for et i dannelsen af poly-gamma-glutamat impliceret protein YwtA (CapC, PgsC) med en aminosyresekvens, som er i det mindste 94% identisk med
25 den i SEQ ID NO. 6 anførte aminosyresekvens, eller hver især dele heraf til reduktion af det slim, som skyldes poly-gamma-glutamat, til 50% under fermenteringen af en mikroorganisme.

15. Anvendelse ifølge krav 13 eller 14, kendetegnet ved, at
30 nukleinsyren har sekvensen SEQ ID NO. 5.

16. Anvendelse af en nukleinsyre med en deletions- eller insertionsmutation omfattende de randsekvenser, som hver især omfatter i det mindste 70 til 150 nukleinsyrepositioner, af
35 det for et protein YwtA (CapC, PsgC) med aminosyresekvensen, som er i det mindste 94% identisk med den i SEQ ID NO. 6 anførte aminosyresekvens, kodende område til reduktion af det slim, som skyldes poly-gamma-glutamat under fermenteringen af

en mikroorganisme.

17. Mikroorganisme, hvor det for et i dannelsen af poly-gamma-glutamat impliceret genprodukt kodende gen ywtA
5 funktionelt er inaktiveret, idet den kodende nukleotidsekvens ywtA har en nukleotidsekvens, som er i det mindste 94% identisk med den i SEQ ID NO. 5 anførte nukleotidsekvens.

18. Mikroorganisme ifølge krav 17, idet det drejer sig om
10 Bacillus licheniformis.

Figure 1

```
                1                                                     50
B.l. ywtA   ATGTTTGGAT CAGATTTATA TATCGCCCTC ATTTTAGGAG TCTTACTCAG
B.s. ywtA   ATGTTCGGAT CAGATTTATA CATCGCACTA ATTTTAGGTG TACTACTCAG

            51                                                   100
B.l. ywtA   TTTGATTTTT GCAGAGAAAA CGGGAATTGT ACCAGCCGGC CTCGTCGTAC
B.s. ywtA   TTTAATTTTT GCGGAAAAAA CAGGGATCGT GCCGGCAGGA CTTGTTGTAC

            101                                                  150
B.l. ywtA   CGGGTTATTT GCGACTTGTC TTCAATCAGC CGATTTTCAT GCTGCTCGTT
B.s. ywtA   CGGGATATTT AGGACTTGTG TTTAATCAGC CGGTCTTTAT TTTACTTGTT

            151                                                  200
B.l. ywtA   CTTTTTGTCA GTTTGCTGAC GTATGTCATC GTGAAATTCG GACTTTCCAA
B.s. ywtA   TTGCTAGTGA GCTTGCTCAC GTATGTCATT GTGAAATACG GTTTATCCAA

            201                                                  250
B.l. ywtA   AATTATGATT CTATACGGAC GCAGAAAATT CGCAGCAATG CTGATTACGG
B.s. ywtA   ATTTATGATT TTGTACGGAC GCAGAAAATT CGCTGCCATG CTGATAACAG

            251                                                  300
B.l. ywtA   GAATTCTTTT GAAAATCGGT TTTGATTTTA TATATCCGGT GATGCCGTTT
B.s. ywtA   GGATCGTCCT AAAAATCGCG TTTGATTTTC TATACCCGAT TGTACCATTT

            301                                                  350
B.l. ywtA   GAGATTGCCG AATTCAGGGG AATCGGAATC ATCGTGCCGG GGCTGATCGC
B.s. ywtA   GAAATCGCAG AATTTCGAGG AATCGGCATC ATCGTGCCAG GTTTAATTGC

            351                                                  400
B.l. ywtA   CAATACCATT CAAAGACAGG GATTAACGAT TACGCTTGGA AGTACGCTTT
B.s. ywtA   CAATACCATT CAGAAACAAG GTTTAACCAT TACGTTCGGA AGCACGCTGC

            401                                                  450
B.l. ywtA   TATTGAGCGG AGCAACATTC GTCATTATGT ATGCTTACTA TCTAATCTAA
B.s. ywtA   TATTGAGCGG AGCGACCTTT GCTATCATGT TTGTTTACTA CTTAATT...
```

Figure 2

```
               1                                                  50
B.l. YwtA   MFGSDLYIAL ILGVLLSLIF AEKTGIVPAG LVVPGYLGLV FNQPIFMLLV
B.s. YwtA   MFGSDLYIAL ILGVLLSLIF AEKTGIVPAG LVVPGYLGLV FNQPVFILLV

            51                                                100
B.l. YwtA   LFVSLLTYVI VKFGLSKIMI LYGRRKFAAM LITGILLKIG FDFIYPVMPF
B.s. YwtA   LLVSLLTYVI VKYGLSKFMI LYGRRKFAAM LITGIVLKIA FDFLYPIVPF

            101                                              149
B.l. YwtA   EIAEFRGIGI IVPGLIANTI QRQGLTITLG STLLLSGATF VIMYAYYLI
B.s. YwtA   EIAEFRGIGI IVPGLIANTI QKQGLTITFG STLLLSGATF AIMFVYYLI
```