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(54) Title: IMPROVED MEVALONATE KINASE

(57) Abstract: The present invention relates to modified mevalonate kinases that are less sensitive to feedback inhibition, and to polynucleotides encoding them. The invention further pertains to vectors comprising these polynucleotides and host cells containing such vectors. The invention provides a process for producing the modified enzyme and for producing isoprenoid compounds using the modified enzymes.

Improved mevalonate kinase

The present invention provides modified mevalonate kinases that are less sensitive to feedback inhibition. The modified enzymes and polynucleotides encoding the same can be used for the production of isoprenoid compounds, for the treatment of disorders that are characterized by decreased mevalonate kinase activity, and for diagnostic purposes.

Mevalonate kinase (Mvk) is an essential enzyme in the mevalonate pathway which leads to the production of numerous cellular isoprenoids. Isopentenyl diphosphate (IPP), the product of the mevalonate pathway, and the isomeric compound, dimethylallyl diphosphate (DMAPP), are the fundamental building blocks of isoprenoids in all organisms. The isoprenoids include more than 23,000 naturally occurring molecules of both primary and secondary metabolism. The chemical diversity of this natural product class reflects their wide-ranging physiological roles in all living systems. Isoprenoids include, *e.g.*, hopane triterpenes, ubiquinones and menaquinones in bacteria, carotenoids, plastoquinones, mono-, sesqui-, di-, and tri-terpenes, and the prenyl side chains of chlorophylls in plants, and heme A, quinones, dolichols, sterols/steroids and retinoids in mammals. In addition, isoprenoids are involved in isopentenyl tRNAs, in protein prenylation and in cholesterol modification of, *e.g.*, the hedgehog class of cell signaling proteins.

In terms of regulation, HMG-CoA reductase is considered broadly to be the rate-determining enzyme in the mevalonate pathway (*e.g.*, Goldstein and Brown, Nature 343, 425-430, 1990; Weinberger, Trends Endocrinol. Metab. 7, 1-6, 1996; Hampton *et al.*, Trends Biochem. Sci. 21, 140-145, 1996; Houten *et al.*, J. Biol. Chem. 278, 5736-5743, 2003). In line with this view, supplementation of the culture medium with mevalonate has been shown to stimulate carotenoid production in both *Phaffia rhodozyma* (Calo *et al.*, Biotechnol. Lett. 17, 575-578, 1995) and *Haematococcus pluvialis* (Kobayashi *et al.*, J. Ferment. Bioeng. 71, 335-339, 1991). Increasing evidence in recent years, however,

indicates that mevalonate kinase is subject to feedback inhibition by, *e.g.*, the downstream products geranyldiphosphate, farnesyldiphosphate and geranylgeranyldiphosphate. This feedback inhibition may also contribute to regulation and rate limitation of the mevalonate pathway and, thus, of isoprenoid biosynthesis in general.

In humans, the importance of mevalonate kinase was demonstrated by the identification of its deficiency as the biochemical and molecular cause of the inherited human disorders mevalonic aciduria and hyperimmunoglobulinemia D and periodic fever syndrome (Houten *et al.*, 2000; Nwokoro *et al.*, Mol. Genet. Metab. 74, 105-119, 2001). The pathophysiology of these disorders is not yet understood, but eventually will give insight into the in vivo role of mevalonate kinase and isoprenoid biosynthesis with respect to the acute phase response and fever. Mevalonate kinase deficiency also seems to be involved, *e.g.*, in Zellweger syndrome and in rhizomelic chondrodysplasia punctata, a disorder of peroxisomal biogenesis wherein a subset of peroxisomal enzymes, including mevalonate kinase, is not transported into peroxisomes (Kelley and Herman, Annu. Rev. Genomics Hum. Genet. 2, 299-341, 2001). Finally, mevalonate kinase was proposed to play a role in cellular proliferation, cell cycle regulation and/or cellular transformation (see Graef *et al.*, Virology 208, 696-703, 1995; Hinson *et al.*, J. Biol. Chem. 272, 26756-26760, 1997).

All mevalonate kinases investigated so far are feedback-inhibited by downstream products of the pathway, *e.g.* farnesyl pyrophosphate or geranylgeranyl pyrophosphate.

Thus, it is an object of the present invention to provide a modified mevalonate kinase which is less sensitive or resistant to feedback inhibition or with sensitivity to feedback inhibition which is reduced relative to that of the non-modified mevalonate kinase, *i.e.* having improved catalytical properties. Feedback-resistant mevalonate kinase enzymes may have industrial potential, *e.g.*, (1) in the biotechnological production of all kinds of isoprenoid compounds (*e.g.*, carotenoids, coenzyme Q10, vitamin D, sterols, etc.), (2) as diagnostic enzymes for, *e.g.*, enzymatic measurement of mevalonate concentrations in biological fluids, or (3) as therapeutic enzymes for lowering mevalonate concentrations in patients with mevalonic aciduria. Feedback-resistant mevalonate kinases are particularly suited for biotechnological production of isoprenoids, since they may allow a larger flux through the mevalonate pathway and, thus, higher isoprenoid productivity.

In particular, the present invention relates to a modified mevalonate kinase which exhibits a sensitivity to feedback inhibition which is reduced in comparison to the corresponding non-modified mevalonate kinase wherein

(i) the amino acid sequence of the modified mevalonate kinase contains at least one mutation when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase and

(ii) the at least one mutation is at one or more amino acid position(s) selected from the group consisting of amino acid positions corresponding to positions 55, 59, 66, 83, 106, 111, 117, 142, 152, 158, 218, 231, 249, 367 and 375 of the amino acid sequence of *Saccharomyces cerevisiae* mevalonate kinase as shown in SEQ ID NO:1.

Any enzyme capable of catalyzing the phosphorylation of mevalonate (mevalonic acid) to 5-phosphomevalonate (5-phosphomevalonic acid), or of mevalonate analogues (as, *e.g.*, described by Wilde and Eggerer, Eur. J. Biochem. 221, 463-473, 1994) to the corresponding phosphorylated compounds and which exhibits sensitivity to feedback inhibition may be used as mevalonate kinase for the purpose of the present invention.

The term "wild-type enzyme" or "wild-type mevalonate kinase" thus means any mevalonate kinase which exhibits sensitivity to feedback inhibition which may serve as starting point for designing (more) feedback resistant mutants according to the present invention. Such wild-type enzymes may be for instance mevalonate kinases/mevalonate kinase sequences derivable from nature or variants of synthetic mevalonate kinases, which can be made (more) feedback resistant by any of the teachings of the present invention. Examples of amino acid sequences of such mevalonate kinases include those which can be found in publicly available databases, such as for instance Swiss-Prot. Preferred are such wild-type enzymes which are homologous or identical to any one of the amino acid sequences shown in Fig. 1 or Table 3, including *e.g.* SEQ ID NOs:1 and 6, or SEQ ID NO:8. Homologous refers to a mevalonate kinase that is at least about 60% identical, preferably at least about 70% identical, more preferably at least about 80% identical, even more preferably at least about 90% identical, most preferably at least about 95% identical to one or more of the amino acid sequences as shown in Fig. 1, including *e.g.* SEQ ID NOs:1 and 6, or SEQ ID NO:8. The terms "wild-type mevalonate kinase" and "non-modified mevalonate kinase" are used interchangeably herein.

If required, a suitable phosphate donor may be added to afford phosphorylation of mevalonate (or mevalonate analogues). As phosphate donors for mevalonate kinase, different compounds may be conceivable, as for instance ATP, TTP, ITP, GTP, UTP, or CTP (see Gibson *et al.*, Enzyme 41, 47-55, 1989). The most preferred phosphate donor is ATP (adenosine 5'-triphosphate).

The term "% identity", as known in the art, means the degree of relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match

between strings of such sequences. "Identity" may be readily determined by known methods of sequence alignments, such as *e.g.*, with the program GAP (GCG Wisconsin Package, version 10.2, Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752, USA) using for instance the following parameters: gap creation penalty 8, gap extension penalty 2 (default parameters); with the program "PILEUP" (GCG Wisconsin Package, version 10.2, Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752, USA) using for instance the following parameters: gap creation penalty 12, gap extension penalty 4, and blosum62.cmp matrix (default parameters); or with the program ClustalW (Version 1.7, EMBL, Heidelberg, Germany) using BLOSUM exchange matrix. Such sequence alignments are routinely performed by the man skilled in the art (*e.g.*, Cho *et al.*, J. Biol. Chem. 276, 12573-12578, 2001).

With "at least one mutation" it is meant that the modified mevalonate kinase of the present invention may contain one or more mutations, *i.e.*, one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, etc. (*i.e.* and more) mutations, including at least one at a position mentioned above.

For the purpose of the present invention a "mutant", "mutant enzyme", or "mutant mevalonate kinase" may be any variant derivable from a given wild-type enzyme/mevalonate kinase that is (more) feedback resistant or having a reduced sensitivity to feedback inhibition than the respective wild-type enzyme. The mutant(s) may be obtained by any method known in the art, such as for instance site-directed mutagenesis, saturation mutagenesis, random mutagenesis/directed evolution, chemical or UV mutagenesis of entire cells/organisms, designing synthetic genes, and/or by in vitro (cell-free) translation (see, *e.g.*, Jermutus *et al.*, Curr. Opin. Biotechnol. 9, 534-548, 1998; Betton, Curr. Prot. Pept. Sci. 4, 73-80, 2003; Martin *et al.*, Biotechniques 31, 948-953, 2001). It is not relevant how the mutant(s) is/are obtained.

As used herein, the term "feedback inhibition" includes any inhibition of enzymatic activity of mevalonate kinase by a metabolite downstream of mevalonate in isoprenoid biosynthesis. Metabolites downstream of mevalonate in isoprenoid biosynthesis include but are not limited to 5-phosphomevalonate, isopentenyl diphosphate (IPP), 3,3-dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), farnesol, dolichol phosphate, and phytyl-pyrophosphate (Dorsey and Porter, J. Biol. Chem. 243, 4667-4670, 1968; Flint, Biochem. J. 120, 145-150, 1970; Gray and Kekwick, Biochim. Biophys. Acta 279, 290-296, 1972; Hinson *et al.*, J. Lipid Res. 38, 2216-2223, 1997). Feedback inhibition of mevalonate kinase may be based on allosteric regulation of mevalonate kinase by binding to the enzyme of the metabolite downstream of mevalonate in isoprenoid biosynthesis.

Preferably, the feedback inhibition is feedback inhibition by farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP). Sensitivity to feedback inhibition means for instance sensitivity to inhibition to physiologically or industrially relevant concentrations of a downstream product of the mevalonate pathway, *e.g.*, FPP or GGPP.

According to the present invention the modified mevalonate kinase exhibits sensitivity to feedback inhibition which is reduced in comparison to the corresponding non-modified mevalonate kinase. Preferably, the sensitivity to feedback inhibition of the modified mevalonate kinase of the invention is reduced by at least about 5%, more preferably at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% in comparison to the corresponding non-modified mevalonate kinase (for measurement and quantification of feedback resistance, see below). Thus, in other words, the modified mevalonate kinase of the invention may exhibit a feedback resistance of at least about 5%, preferably at least about 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% when compared with the corresponding non-modified mevalonate kinase.

"Feedback resistance" as used herein may be any increase in resistance to "feedback inhibition" (as defined above). Feedback resistance can be analyzed in different ways known to those skilled in the art. An appropriate example of such an analysis is shortly described herein: mevalonate kinase activity is measured in an activity assay at non-saturating concentrations of for instance ATP (or of another phosphate donor) and mevalonate (or mevalonate analogue), *i.e.*, at for instance ATP (or phosphate donor) and mevalonate (or mevalonate analogue) concentrations around which the reaction rate is sensitive to changes of these substrate concentrations, *e.g.*, at concentrations around the respective K_m values of the enzyme under investigation for these substrates. The activities of both wild-type mevalonate kinase and of a variant/mutant of this enzyme are measured under otherwise identical conditions both in the absence and presence of a relevant concentration of a feedback inhibitor, *i.e.*, at a concentration of feedback inhibitor affording significant inhibition of the wild-type mevalonate kinase. If the extent of inhibition (*e.g.*, % inhibition) by the feedback inhibitor is lower for the mutant than for the wild-type enzyme, then the mutant is feedback resistant in the context of the present patent application. Once a feedback resistant variant/mutant has been identified, the same procedure as described above may be applied to identify further improved mutants, *i.e.*, mutants that are even more feedback resistant. Feedback resistance (%) is calculated as follows: if (a) and (b) are the measured mevalonate kinase activities of the wild-type enzyme in the absence and presence, respectively, of the feedback inhibitor (*e.g.*, FPP), and if (c) and (d) are the measured mevalonate kinase activities of the mutant enzyme in the absence and presence, respectively, of the same feedback inhibitor, then % feedback resistance is:

$$\% \text{ resistance} = 100 \cdot ((d/c)-(b/a))/(1-(b/a))$$

Preferably, the feedback resistance refers to the experimental conditions described in Example 1 of this application. Approximately 3-30 mU/ml (corresponding to ca. 40-400 ng/ml of *Saccharomyces cerevisiae* mevalonate kinase), preferably ca. 10-20 mU/ml of mevalonate kinase activity, and optionally 1 μM FPP may be present in the assay mixture, and the reaction may be carried out at 25°C.

The amino acid sequence of a modified mevalonate kinase of the invention contains at least one mutation when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase. The at least one mutation may be for instance an addition, deletion and/or substitution. Preferably, the at least one mutation is an amino acid substitution wherein a given amino acid present in the amino acid sequence of the non-modified mevalonate kinase is replaced with a different amino acid in the amino acid sequence of the modified mevalonate kinase of the invention. The amino acid sequence of a modified mevalonate kinase may contain at least one amino acid substitution when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase. In further embodiments, the modified mevalonate kinase contains at least two, at least three, at least four or at least five substitutions when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase. In other embodiments of the invention, the modified mevalonate kinase contains one to fifteen, one to twelve, one to ten, one to seven, one to five, one to four, two to fifteen, two to twelve, two to ten, two to seven, two to five, two to four, three to fifteen, three to twelve, three to ten, three to seven, three to five or three to four amino acid substitutions when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase.

According to the present invention the at least one mutation is at one or more amino acid position(s) selected from the group consisting of amino acid positions corresponding to positions 55, 59, 66, 83, 106, 111, 117, 142, 152, 158, 218, 231, 249, 367 and 375 of the amino acid sequence of *Saccharomyces cerevisiae* mevalonate kinase as shown in SEQ ID NO:1. Any combination of mutations at these amino acid positions of SEQ ID NO:1, *i.e.* a mutation at positions corresponding to at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen or to at least all 15 of the amino acid positions mentioned above may be selected as target for the at least one mutation to generate a modified mevalonate kinase as defined above. Preferably, the present invention provides a modified mevalonate kinase originating from *S. cerevisiae*, wherein the amino acid sequence of said modified mevalonate kinase comprises at least one mutation, said mutation(s) including one or more mutation(s) at positions 55, 59, 66, 83, 106, 111, 117,

142, 152, 158, 218, 231, 249, 367 and/or 375 as shown in SEQ ID NO:1, wherein SEQ ID NO:1 represents the wild-type amino acid sequence.

Since a modified mevalonate kinase as of the present invention contains at least one mutation at one or more amino acid position(s) as defined above, it may contain further mutation(s) at amino acid position(s) besides the one(s) listed above.

In one aspect, the present invention relates to a modified mevalonate kinase which exhibits a sensitivity to feedback inhibition which is reduced in comparison to the corresponding non-modified mevalonate kinase wherein

(i) the amino acid sequence of the modified mevalonate kinase comprises one or more mutation(s) when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase and

(ii) the one or more mutation(s) is/are at one or more amino acid position(s) selected from the group consisting of amino acid positions corresponding to positions 55, 59, 66, 83, 106, 111, 117, 142, 152, 158, 218, 231, 249, 367 and 375 of the amino acid sequence of *Saccharomyces cerevisiae* mevalonate kinase as shown in SEQ ID NO:1.

Any combination of these positions shown in SEQ ID NO:1, *i.e.* two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen or all 15 positions corresponding to the positions mentioned above may be selected as target for mutations to generate said modified mevalonate kinase.

In one embodiment the at least one mutation is at one or more amino acid position(s) selected from the group consisting of amino acid positions corresponding to positions 55, 59, 66, 117, and 152 of the amino acid sequence of *Saccharomyces cerevisiae* mevalonate kinase as shown in SEQ ID NO:1.

The modified mevalonate kinase may contain only a single mutation, such as for instance a single amino acid substitution, when compared to the corresponding non-modified mevalonate kinase. Preferably, the single mutation is at a position selected from the group consisting of positions corresponding to the amino acid positions 55, 59, 66, 117, and 152 of SEQ ID NO:1. More preferably, the single mutation is an amino acid substitution, such as for instance P55L, F59S, N66K, C117S, or I152M. Most preferably, the substitution is F59S, *i.e.* a substitution/replacement of phenylalanine with serine on a position corresponding to position 59 of SEQ ID NO:1.

The modified mevalonate kinase may contain at least two mutations, such as for instance two amino acid substitutions, when compared to the corresponding non-modified

mevalonate kinase. Preferably, one of the at least two mutations, *e.g.* amino acid substitutions, is at an amino acid position corresponding to a position of SEQ ID NO:1 which is selected from position 55, 66, 83, 106, 111, 117, 152, 218, 249, and/or 375. In case of two mutations, *e.g.* amino acid substitutions, it is preferred that the two mutations are at positions corresponding to combinations of positions 55/117, 66/152, 83/249, 111/375 or 106/218 of SEQ ID NO:1. More preferably, the two mutations consist of one or two amino acid substitution(s), even more preferably two amino acid substitutions. Most preferred are combinations of two amino acid substitutions/replacements corresponding to combinations of positions of SEQ ID NO:1 which are selected from P55L/C117S, N66K/I152M, K83E/S249P, H111N/K375N or L106P/S218P.

In a particularly preferred embodiment, the modified mevalonate kinase contains two amino acid substitutions corresponding to the combination N66K/I152M of the amino acid sequence shown in SEQ ID NO:1. More preferably, the two amino acid substitutions are N66K and I152M in the non-modified *S. cerevisiae* mevalonate kinase amino acid sequence as shown in SEQ ID NO:1.

The modified mevalonate kinase may contain at least four mutations, such as for instance four amino acid substitutions, when compared to the corresponding non-modified mevalonate kinase. Preferably, one of the at least four mutations, *e.g.* amino acid substitutions, is at an amino acid position corresponding to a position of SEQ ID NO:1 which is selected from position 142, 158, 231, and 367. In case of four mutations, *e.g.* amino acid substitutions, it is preferred that the four mutations are at positions corresponding to a combination of positions 142/158/231/367 of SEQ ID NO:1. More preferably, the four mutations consist of one, two, three or four amino acid substitutions, even more preferably four amino acid substitutions. Most preferred is a combination of four amino acid substitutions corresponding to positions 142/158/231/367 of SEQ ID NO:1 which is I142N/L158S/L231I/T367S.

Most preferred are the combinations of mutations disclosed in Table 1 (see infra). The amino acid positions identified in the examples may be transferred to mevalonate kinases of different origin.

A modified mevalonate kinase of the invention may be obtained by introducing a mutation to the corresponding non-modified mevalonate kinase.

The non-modified mevalonate kinase may be of eukaryotic or prokaryotic origin, such as for instance animals including humans, plants, algae, fungi including yeast, and bacteria. Preferably, the non-modified mevalonate kinase is selected from fungi including yeast or from bacteria, more preferably selected from the group consisting of *Aspergillus*,

Saccharomyces, *Paracoccus*, *Rhodobacter* and *Phaffia*. Even more preferred are *Aspergillus niger*, *Saccharomyces cerevisiae*, *Paracoccus zeaxanthinifaciens*, *Rhodobacter sphaeroides*, such as *R. sphaeroides* ATCC 35053, or *Phaffia rhodozyma*, with *Saccharomyces cerevisiae* being most preferred.

In one aspect of the present invention, the non-modified mevalonate kinase is feedback inhibited by FPP. Feedback inhibition of the non-modified mevalonate kinase by FPP may be for instance at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, as determined by a method known to the skilled person, such as for instance as described by Popják (Meth. Enzymol. 15, 393-, 1969), Gibson *et al.* (Enzyme 41, 47-55, 1989), Hinson *et al.* (J. Lipid Res. 38, 2216-2223, 1997), Schulte *et al.* (Anal. Biochem. 269, 245-254, 1999), or Cho *et al.* (J. Biol. Chem. 276, 12573-12578, 2001). A particular assay is described in Example 1 using different FPP concentrations.

The modified mevalonate kinase of the invention may comprise foreign amino acids, preferably at its N- or C-terminus. "Foreign amino acids" mean amino acids which are not present in a native (occurring in nature) mevalonate kinase, such as for instance a stretch of at least about 3, preferably at least about 5 and more preferably at least about 7 contiguous amino acids which are not present in a native mevalonate kinase. Suitable stretches of foreign amino acids include but are not limited to "tags" that facilitate purification of the recombinantly produced modified mevalonate kinase. Examples of such tags include but are not limited to a His_6 tag, a FLAG tag, a myc tag, and the like.

In another embodiment the modified mevalonate kinase may contain one or more deletion(s), *e.g.* two deletions, when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase. Preferably, the deletions affect N- or C-terminal amino acids of the corresponding non-modified mevalonate kinase and do not significantly reduce the functional properties, *e.g.*, the specific activity, of the enzyme.

The modified mevalonate kinase of the invention usually is a non-naturally occurring mevalonate kinase. The specific activity of the modified mevalonate kinase may be for instance at least about 10%, preferably at least 20%, 30%, 35%, 40%, 50%, 60%, 70%, 80%, 90%, 100% and even more, such as for instance about 150%, 200% and more of the specific activity of the corresponding non-modified mevalonate kinase.

Methods for measuring specific activity are known to the man skilled in the art. The specific activity may for instance be determined via measuring the consumption of NADH. Suitable conditions for such measurement may be those as *e.g.* outlined in Example 1 except that, typically, saturating substrate concentrations are used or, in the case of enzyme

inhibition at high substrate concentrations, at substrate concentrations that provide maximal activity under the particular experimental conditions.

The invention further relates to a polynucleotide comprising a nucleotide sequence which codes for a modified mevalonate kinase according to the invention. Any polyribonucleotide or polydeoxyribonucleotide such as for instance unmodified RNA or DNA or modified RNA or DNA may be used as polynucleotide. Polynucleotides include but are not limited to single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. As used herein, a polynucleotide may further include DNA or RNA that comprises one or more unusual base(s), *e.g.*, inosine, or one or more modified base(s), *e.g.*, tritylated bases.

A polynucleotide of the invention may easily be obtained by modifying a polynucleotide sequence which codes for a non-modified mevalonate kinase, *e.g.* constructed by starting from genomic or cDNA sequences coding for mevalonate kinases known in the state of the art [for sequence information see, *e.g.*, the relevant sequence databases, for example Genbank (Intelligenetics, California, USA), European Bioinformatics Institute (Hinston Hall, Cambridge, GB), NBRF (Georgetown University, Medical Centre, Washington DC, USA) and Vecbase (University of Wisconsin, Biotechnology Centre, Madison, Wisconsin, USA)] by mutagenesis methods known in the art, such as for instance introducing mutations such as, *e.g.*, additions, deletions and/or substitutions into the nucleotide sequences coding for non-modified mevalonate kinases by for instance site-directed mutagenesis and PCR-based methods (see *e.g.* Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor Laboratory Press, New York).

The principles of the polymerase chain reaction (PCR) method are outlined, *e.g.*, by White *et al.*, Trends Genet. 5, 185-189, 1989, whereas improved methods are described, *e.g.*, in Innis *et al.* [PCR Protocols: A guide to Methods and Applications, Academic Press, Inc. (1990)].

The generation of modified mevalonate kinases may be performed by site-directed mutagenesis, a method which is originally outlined by Hutchison and Edgell (J. Virol. 8, 181-189, 1971), involving the annealing of a synthetic oligonucleotide carrying the desired nucleotide substitution, deletion or addition to a target region of a single-stranded DNA sequence wherein the mutation should be introduced (for review see Smith, Annu. Rev. Genet. 19, 423-462, 1985; and for improved methods see references 2-6 in Stanssen *et al.*, Nucl. Acids Res. 17, 4441-4454, 1989). DNA as starting material can be isolated by

methods known in the art and described, *e.g.*, in Sambrook *et al.* (Molecular Cloning) from the respective strains/organisms. It is, however, understood that DNA encoding a mevalonate kinase to be constructed/mutated in accordance with the present invention may also be prepared on the basis of a known DNA sequence, *e.g.* by construction of a synthetic gene by methods known in the art (as described, *e.g.*, in EP 747 483 and by Lehmann *et al.*, Prot. Eng. 13, 49-57, 2000).

A non-limiting example of a polynucleotide encoding a modified mevalonate kinase according to the invention is shown in SEQ ID NO:5.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living microorganism is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition and still be isolated in that such vector or composition is not part of its natural environment.

An isolated polynucleotide or nucleic acid as used herein may be a DNA or RNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5'-end and one on the 3'-end) in the naturally occurring genome of the organism from which it is derived. Thus, in one embodiment, a nucleic acid includes some or all of the 5'-non-coding (*e.g.*, promoter) sequences that are immediately contiguous to the coding sequence. The term "isolated polynucleotide" therefore includes, for example, a recombinant DNA that is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding an additional polypeptide that is substantially free of cellular material, viral material, or culture medium (when produced by recombinant DNA techniques), or chemical precursors or other chemicals (when chemically synthesized). Moreover, an "isolated nucleic acid fragment" is a nucleic acid fragment that is not naturally occurring as a fragment and would not be found in the natural state. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides.

An isolated polypeptide may be a polypeptide which is substantially free of other polypeptides. An isolated polypeptide may be for instance greater than 80% pure, preferably greater than 90% pure, more preferably greater than 95% pure, and most preferably greater than 99% pure. Purity may be determined according to methods known in the art, *e.g.*, by SDS-PAGE and subsequent protein staining. Protein bands may be quantified by for instance densitometry. Further methods for determining the purity are within the level of ordinary skill.

In yet another embodiment the invention pertains to a vector or plasmid comprising a polynucleotide according to the invention. The vector or plasmid preferably comprises at least one marker gene. The vector or plasmid may further comprise regulatory elements operably linked to the polynucleotide of the invention. The term "operably linked" as used herein refers for instance to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence, *i.e.*, the coding sequence is under the transcriptional control of the promoter. Coding sequences may be operably linked to regulatory sequences in sense or antisense orientation. The term "expression" denotes the transcription of a DNA sequence into mRNA and/or the translation of mRNA into an amino acid sequence. The term "overexpression" means for instance the production of a gene product in a modified organism (*e.g.*, modified by transformation or transfection) that exceeds levels of production in the corresponding non-modified organism.

Integration of DNA sequences encoding modified mevalonate kinases as of the present invention into vectors for overexpressing the encoded polypeptides in an appropriate host system may be performed by methods known in the art and described in, *e.g.*, Sambrook *et al.* (s.a.). Either the DNA sequences themselves or a vector/plasmid comprising a DNA sequence as of the present invention may be used to transform the suitable host systems of the invention to get (over-) expression of the encoded polypeptide. Suitable host systems useful for the present invention may be selected from eukaryotic or prokaryotic cells, for instance cells of animals including humans, plants, bacteria, or fungi including yeast. Examples of such host cells include but are not limited to cells selected from streptococci, staphylococci, enterococci, cyanobacteria, yeast (*e.g. Saccharomyces*), basidiomycetes, gymnosperms, angiosperms, or cell-lines such as for instance *Drosophila* S2, *Spodoptera* Sf9, CHO, COS, HeLa, 3T3, BHK, HK293 [human kidney 293 cell line???], and CV-1.

Suitable methods for the expression in plants are described, *e.g.*, by Pen *et al.* in Bio/Technology 11, 811-814, 1994 or in EP 449 375, preferably in seeds as described, *e.g.*, in EP 449 376. Some suitable examples of promoters and terminators include those from nopaline synthase (nos), octopine synthase (ocs) and cauliflower mosaic virus

(CaMV) genes. One type of efficient plant promoter that may be used is a high-level plant promoter. Such promoters, in operable linkage with the genetic sequences of the present invention should be capable of promoting expression of the present gene product. High level plant promoters that may be used in this invention include for instance the promoter of the small subunit (ss) of the ribulose-1,5-bisphosphate carboxylase, for example from soybean (Berry-Lowe *et al.*, J. Mol. Appl. Genet. 1, 483-498, 1982), and the promoter of the chlorophyll a/b binding protein.

A fungal host cell within the scope of the present invention may be for instance selected from Aspergilli, such as *Aspergillus niger* or *Aspergillus oryzae*, *Trichoderma*, such as *Trichoderma reesei*, *Saccharomyces*, such as *Saccharomyces cerevisiae*, *Pichia*, such as *Pichia pastoris*, or *Hansenula*, such as *Hansenula polymorpha*, preferably *H. polymorpha* DSM 5215. A bacterial host cell within the scope of the present invention may be for instance selected from *Paracoccus*, such as *Paracoccus zeaxanthinifaciens*, *Rhodobacter*, such as *R. sphaeroides*, *Escherichia*, such as *E. coli*, *Bacillus*, such as *Bacillus subtilis*, *Streptomyces*, such as *Streptomyces lividans* (see *e.g.* Anné and van Mellaert in FEMS Microbiol. Lett. 114, 121-128, 1993). Preferred strains of *E. coli* which may be used are selected from *e.g.*, *E. coli* K12 strains, such as for instance M15 (described as DZ 291 by Villarejo *et al.* in J. Bacteriol. 120, 466-474, 1974), HB 101 (ATCC No. 33694) or *E. coli* SG13009 (Gottesman *et al.*, J. Bacteriol. 148, 265-273, 1981). The man skilled in the art knows that such suitable hosts may be available from any known depository authority, as listed for instance in the Journal "Industrial Property" (vol. 1, pages 29-40, 1991) or in the Official Journal of the European Patent Office (vol. 4, pages 155/156, 2003).

Depending on the host system, different vectors may be used, said vectors comprising a polynucleotide according to the invention. Non-limiting examples of vectors which may be used for expression in fungi are known in the art and described *e.g.* in EP 420 358, or by Cullen *et al.* (Bio/Technology 5, 369-376, 1987), Ward (in Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi, Marcel Dekker, New York, 1991), Upshall *et al.* (Bio/Technology 5, 1301-1304, 1987), Gwynne *et al.* (Bio/Technology 5, 71-79, 1987), or Punt *et al.* (J. Biotechnol. 17, 19-34, 1991), and for yeast by Sreekrishna *et al.* (J. Basic Microbiol. 28, 265-278, 1988; Biochemistry 28, 4117-4125, 1989), Hitzemann *et al.* (Nature 293, 717-722, 1981) or in EP 183 070, EP 183 071, EP 248 227, EP 263 311. Non-limiting examples of vectors which may be used for expression in *E. coli* are mentioned, *e.g.*, by Sambrook *et al.* [s.a.] or by Fiers *et al.* in Proc. 8th Int. Biotechnol. Symp. [Soc. Franc. de Microbiol., Paris (Durand *et al.*, eds.), pp. 680-697, 1988], Bujard *et al.* (in Meth. Enzymol., eds. Wu and Grossmann, Academic Press, Inc., Vol. 155, 416-433, 1987), or Stüber *et al.* (in Immunological Methods, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. IV, 121-152, 1990). Non-limiting

examples of vectors which may be used for expression in Bacilli are known in the art and described, *e.g.* in EP 207 459 or EP 405 370, by Yansura and Henner in Proc. Natl. Acad. Sci. USA 81, 439-443 (1984), or by Henner, Le Grice and Nagarajan in Meth. Enzymol. 185, 199-228, 1990. Non-limiting examples of vectors which may be used for expression in *H. polymorpha* are known in the art and described, *e.g.*, in Gellissen *et al.*, Biotechnology 9, 291-295, 1991.

Either such vectors already carry regulatory elements, *e.g.* promoters, or the DNA sequences of the present invention can be engineered to contain such elements. Suitable promoter elements which may be used are known in the art and are, *e.g.*, for *Trichoderma reesei* the cbh1- (Haarki *et al.*, Biotechnology 7, 596-600, 1989) or the pki1-promoter (Schindler *et al.*, Gene 130, 271-275, 1993), for *Aspergillus oryzae* the amy-promoter [Christensen *et al.*, Abstr. 19th Lunteren Lectures on Molecular Genetics F23 (1987); Christensen *et al.*, Biotechnology 6, 1419-1422, 1988; Tada *et al.*, Mol. Gen. Genet. 229, 301-306, 1991], for *Aspergillus niger* the glaA- (Cullen *et al.*, Bio/Technology 5, 369-376, 1987; Gwynne *et al.*, Bio/Technology 5, 713-719, 1987; Ward in Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi, Marcel Dekker, New York, 83-106, 1991), alcA- (Gwynne *et al.*, Bio/Technology 5, 718-719, 1987), suc1- (Boddy *et al.*, Curr. Genet. 24, 60-66, 1993), aphA- (MacRae *et al.*, Gene 71, 339-348, 1988; MacRae *et al.*, Gene 132, 193-198, 1993), tpiA- (McKnight *et al.*, Cell 46, 143-147, 1986; Upshall *et al.*, Bio/Technology 5, 1301-1304, 1987), gpdA- (Punt *et al.*, Gene 69, 49-57, 1988; Punt *et al.*, J. Biotechnol. 17, 19-37, 1991) and the pkiA-promoter (de Graaff *et al.*, Curr. Genet. 22, 21-27, 1992). Suitable promoter elements which may be used for expression in yeast are known in the art and are, *e.g.*, the pho5-promoter (Vogel *et al.*, Mol. Cell. Biol. 9, 2050-2057, 1989; Rudolf and Hinnen, Proc. Natl. Acad. Sci. USA 84, 1340-1344, 1987) or the gap-promoter for expression in *Saccharomyces cerevisiae*, and *e.g.* the aox1-promoter for *Pichia pastoris* (Koutz *et al.*, Yeast 5, 167-177, 1989; Sreekrishna *et al.*, J. Basic Microbiol. 28, 265-278, 1988), or the FMD promoter (Hollenberg *et al.*, EPA No. 0299108) or MOX promoter (Ledeboer *et al.*, Nucleic Acids Res. 13, 3063-3082, 1985) for *H. polymorpha.*

Suitable promoters include natural and synthetic promoters as for instance described in Giacomini *et al.* (Gene 144, 17-24, 1994). Appropriate teachings for expression of a claimed (mutant) mevalonate kinase in bacteria, either by appropriate plasmids or through integration of mevalonate kinase-encoding DNA sequences into the chromosomal DNA, can be found in many places, *e.g.*, US patent No. 6,322,995.

The invention further relates to a method/process for producing a modified mevalonate kinase of the invention comprising:

(a) culturing a host cell of the invention under conditions that allow expression of the modified mevalonate kinase of the invention; and

(b) recovering the modified mevalonate kinase from the cells or from the medium.

The modified mevalonate kinase of the invention may be prepared from genetically engineered host cells.

For recombinant production of the polypeptides of the invention, host cells may be genetically engineered to incorporate polynucleotides or vectors or plasmids of the invention. Introduction of a polynucleotide or vector into the host cell may be effected by methods described in many standard laboratory manuals such as for instance calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, ballistic introduction and infection [for reference, see, *e.g.*, Davis *et al.*, Basic Methods in Molecular Biology (1986), and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y. (1989)].

Any system or vector suitable to maintain, propagate or express polynucleotides and/or to express a polypeptide in a host may be used for expression/production of the mevalonate kinases of the invention, including, among others, those described supra.

In recombinant expression systems in eukaryotes, for secretion of a translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

Polypeptides of the invention may be recovered and purified from recombinant cell cultures by well-known methods including for instance ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, and hydroxyapatite chromatography. In one embodiment, high performance liquid chromatography is employed for purification. Well-known techniques for protein refolding may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification. Methods of protein purification are described in, *e.g.*, Deutscher, Protein Purification, Academic Press, New York, 1990; and Scopes, Protein Purification, Springer Verlag, Heidelberg, 1994.

A variety of culture methodologies may be applied to produce the proteins of the present invention. For example, large-scale production of a specific gene product, overexpressed

from a recombinant microbial host may be produced by for instance batch, fed-batch, continuous or semi-continuous culture methodologies. Details of the various culture methods may be found in, *e.g.*, Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, Mass., or Deshpande, Appl. Biochem. Biotechnol. 36, 227-234, 1992.

The fermentation media may contain suitable carbon substrates including but not limited to monosaccharides such as glucose and fructose, oligosaccharides such as lactose or sucrose, polysaccharides such as starch or cellulose or mixtures thereof and unpurified mixtures from renewable feedstocks. It is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon containing substrates and is depending on the choice of organism.

The invention further relates to a method/process for the preparation of a modified mevalonate kinase having reduced sensitivity to feedback inhibition, comprising the following steps:

(a) providing a polynucleotide encoding a first mevalonate kinase which exhibits sensitivity to feedback inhibition;

(b) introducing one or more mutation(s) into the polynucleotide sequence such that the mutated polynucleotide sequence encodes a second mevalonate kinase which contains at least one amino acid mutation when compared to the first mevalonate kinase wherein the at least one amino acid mutation is at one or more amino acid position(s) selected from the group consisting of amino acid positions corresponding to positions 55, 59, 66, 83, 106, 111, 117, 142, 152, 158, 218, 231, 249, 367 and 375 of the amino acid sequence of *Saccharomyces cerevisiae* mevalonate kinase as shown in SEQ ID NO:1;

(c) optionally inserting the mutated polynucleotide in a vector or plasmid;

(d) introducing the polynucleotide of step (b) or (c) into a suitable host cell; and

(e) culturing the host cell under conditions that allow expression of the modified mevalonate kinase having reduced sensitivity to feedback inhibition.

The preferred embodiments of this method correspond to the preferred embodiments of the modified mevalonate kinase, the polynucleotides encoding them, the vectors and plasmids, the host cells, and the methods described herein. The first and second mevalonate kinase correspond to the non-modified and modified mevalonate kinase, respectively (see supra).

The invention further relates to a method or process for producing an isoprenoid comprising:

(a) culturing the host cell of the invention in a suitable medium under conditions that allow expression of the modified mevalonate kinase in the host cell; and

(b) optionally separating the isoprenoid from the medium.

Preferably, a modified mevalonate kinase of the present invention is used for increasing the production of an isoprenoid.

Such a method/process may be used for the biotechnological production of any type of isoprenoid or isoprenoid compound. Any metabolite(s) and prenylated macromolecule(s) derivable from mevalonate may be used as isoprenoid in the context of this patent application. These isoprenoids may be generated from either natural or non-natural pathways (*i.e.*, pathways not occurring in nature, but engineered biotechnologically), preferably biochemical pathways. Non-limiting examples of such isoprenoids include hopane triterpenes, quinones, carotenoids, mono-, sesqui-, di-, and triterpenes, the prenyl side chains of chlorophylls, heme A, dolichols, sterols/steroids, retinoids, and rubber or rubber derivatives, preferably natural rubber (= *cis*-1,4-polyisoprene; Mooibroek & Cornish, Appl. Microbiol. Biotechnol. 53, 355-365, 2000).

Quinones within the scope of this invention may be selected from *e.g.* ubiquinone (= coenzyme Q), menaquinone, plastoquinones and anthraquinones, preferably coenzyme Q6, coenzyme Q7, coenzyme Q8, coenzyme Q9, coenzyme Q10 or coenzyme Q11, and most preferably coenzyme Q10 (Clarke, Protoplasma 213, 134-147, 2000; Han *et al.*, Plant Cell Tissue Organ Culture 67, 201-220, 2001; Kawamukai, J. Biosci. Bioeng. 94, 511-517, 2002). Carotenoids within the scope of this invention may be selected from *e.g.* phytoene, lycopene, α-, β- and γ-carotene, lutein, zeaxanthin, β-cryptoxanthin, adonixanthin, echinenone, canthaxanthin, astaxanthin and derivatives thereof (Misawa & Shimada, J. Biotechnol. 59, 169-181, 1998; Miura *et al.*, Appl. Environ. Microbiol. 64, 1226-1229, 1998; Hirschberg, Curr. Opin. Biotechnol. 10, 186-191, 1999; Margalith, Appl. Microbiol. Biotechnol. 51, 431-438, 1999; Schmidt-Dannert, Curr. Opin. Biotechnol. 11, 255-261, 2000; Sandmann, Arch. Biochem. Biophys. 385, 4-12, 2001; Lee & Schmidt-Dannert, Appl. Microbiol. Biotechnol. 60, 1-11, 2002). Sterols within the scope of this invention may be selected from *e.g.* ergosterol, cholesterol, hydrocortisone (Ménard Szczebara *et al.*, Nature Biotechnol. 21, 143-149, 2003), vitamin D, 25-hydroxy-vitamin D3, dietary phytosterols (Ling & Jones, Life Sci. 57, 195-206, 1995) and natural surfactants (Holmberg, Curr. Opin. Colloid. Interface Sci. 6, 148-159, 2001) and derivatives thereof.

Suitable host cells for the production of isoprenoids or isoprenoid compounds as defined above may be all types of organisms that are amenable to genetic modification such as, for instance, bacteria, fungi including yeast, algae, plants or animal cells including human

cells. These host cells may be the same as defined above for the expression of a modified mevalonate kinase of the present invention. Methods of genetic and metabolic engineering are known to the man skilled in the art (*e.g.*, Verpoorte *et al.*, Biotechnol. Lett. 21, 467-479, 1999; Verpoorte *et al.*, Transgenic Res. 9, 323-343, 2000; Barkovich & Liao, Metab. Eng. 3, 27-39, 2001). Similarly, (potentially) suitable purification methods for isoprenoids and isoprenoid compounds are well known in the art.

The method/process for biotechnological production of an isoprenoid according to the present invention may be performed by for instance whole-cellular fermentation processes as described above, permeabilized host cells, crude cell extracts, cell extracts clarified from cell remnants by any suitable method, *e.g.*, centrifugation or filtration, or even reconstituted reaction pathways with isolated enzymes. Also combinations of such processes are in the scope of the present invention. In the case of cell-free biosynthesis (such as for instance with reconstituted reaction pathways), it is irrelevant how the enzyme has been prepared, such as, for instance, by isolation from a host cell, by in vitro transcription/translation, or by still other means known in the art.

The production of an isoprenoid, such as for instance coenzyme Q10, using a modified Mvk as described above may be increased by for instance at least about 1%, 2%, 5%, 10%, 15%, 20%, 30% or more when compared to the production of the same isoprenoid using a non-modified Mvk. One way of measuring the concentration of isoprenoid compounds in a given sample is described in Example 5.

Another aspect of the invention is the use of a modified mevalonate kinase of the invention or a polynucleotide of the invention for the manufacture of a medicament for the treatment of a disorder associated with decreased activity of mevalonate kinase. Such disorders include but are not limited to mevalonic aciduria, or hyperimmunoglobulinemia D and periodic fever syndrome. It is preferred that a modified mevalonate kinase of the invention is administered as a therapeutic enzyme. Suitable modes of administration may be for instance oral, parenteral, intraperitoneal and/or subcutaneous administration. The modified mevalonate kinases of the invention may be formulated as pharmaceutical compositions (*e.g.* granules, enzyme crystals, tablets, pills, capsules, injections, solutions, and the like) comprising at least one such enzyme alone or in admixture with for instance pharmaceutically acceptable carriers, excipients and/or diluents. The pharmaceutical compositions may be formulated in accordance with a conventional method. Specific dose levels for any particular patient may be employed depending upon a variety of factors including for instance the activity of specific compounds employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy.

The polynucleotides of the invention may be used in a gene therapy protocol.

Yet another aspect of the invention is the use of a modified mevalonate kinase of the invention or a polynucleotide of the invention for determining the concentration of mevalonate in biological fluids. Non-limiting examples of biological fluids include blood, serum, plasma, cerebrospinal fluid, urine, tears, sweat, as well as any other intracellular, intercellular and/or extracellular fluids.

It is an object of the present invention to provide a polynucleotide comprising a nucleic acid sequence coding for a modified mevalonate kinase as described above, a vector, preferably an expression vector, comprising such a polynucleotide, a host cell which has been transformed by such a polynucleotide or vector, a process for the preparation of a mevalonate kinase of the present invention wherein the host cell as described above is cultured under suitable culture conditions and the mevalonate kinase is isolated from such host cell or the culture medium by methods known in the art, and a process for the biotechnological production of isoprenoid(s) based on a host cell which has been transformed by such a polynucleotide or vector, and/or which may have stably integrated such a polynucleotide into its chromosome(s).

It is also an object of the present invention to provide (i) a DNA sequence which codes for a mevalonate kinase carrying at least one of the specific mutations of the present invention and which hybridizes under standard conditions with any of the DNA sequences of the specific modified mevalonate kinases of the present invention, or (ii) a DNA sequence which codes for a mevalonate kinase carrying at least one of the specific mutations of the present invention but, because of the degeneracy of the genetic code, does not hybridize but which codes for a polypeptide with exactly the same amino acid sequence as a DNA sequence which hybridizes under standard conditions with any of the DNA sequences of the specific modified mevalonate kinases of the present invention, or (iii) a DNA sequence which is a fragment of such DNA sequences which maintains the activity properties of the polypeptide of which it is a fragment.

"Standard conditions" for hybridization mean in the context the conditions which are generally used by a man skilled in the art to detect specific hybridization signals and which are described, *e.g.* by Sambrook *et al.*, "Molecular Cloning", second edition, Cold Spring Harbor Laboratory Press 1989, New York, or preferably so-called stringent hybridization and non-stringent washing conditions or more preferably so-called stringent hybridization and stringent washing conditions a man skilled in the art is familiar with and which are described, *e.g.*, in Sambrook *et al.* (s.a.). A specific example of stringent hybridization conditions is overnight incubation (*e.g.*, 15 hours) at 42°C in a solution comprising 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate

(pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml of denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at about 65°C for 3x 10 min.

It is furthermore an object of the present invention to provide a DNA sequence which can be obtained by the so-called polymerase chain reaction method (PCR) by suitable primers designed on the basis of the specifically described DNA sequences of the present invention. It is understood that a so obtained DNA sequence codes for a mevalonate kinase with at least the same mutation(s) as the ones from which they are designed and show comparable activity properties.

The various embodiments of the invention described herein may be cross-combined.

Figure 1: Multiple sequence alignment calculated with the program ClustalW (version 1.82, EMBL, Heidelberg, Germany) of mevalonate kinase sequences from various sources (accession numbers and sequence ID numbers, respectively, of the corresponding amino acid/nucleotide sequences are indicated): mouse (Swiss-Prot accession no. Q9R008/Genbank accession no. AF137598), rat (Swiss-Prot accession no. P17256/Genbank accession no. M29472), human (H_sapiens; Swiss-Prot accession no. Q03426/Genbank accession no. M88468), *Phaffia rhodozyma* (P_rhodozyma; SEQ ID NOs:8/9), *Schizosaccharomyces pombe* (S_pombe; Swiss-Prot accession no. Q09780/Genbank accession no. AB000541), *Saccharomyces cerevisiae* (yeast; Swiss-prot accession P07277/Genbank accession no. NP013935; SEQ ID NOs:1/2), *Aeropyrum pernix* (A_pernix; Swiss-Prot accession no. Q9Y946/Genbank accession no. AP000064), *Pyrococcus abyssi* (P_abyssi; Swiss-Prot accession no. Q9V187/Genbank accession no. AJ248284), *Pyrococcus horikoshii* (P_horikoshii; Swiss-Prot accession no. O59291/Genbank accession no. AB009515), *Pyrococcus furiosus* (P_furiosus; Swiss-Prot accession no. Q8U0F3/Genbank accession no. AE010263), *Methanobacterium thermoautotrophicum* (M_thermoautotrophicum; Swiss-Prot accession no. Q50559/Genbank accession no. U47134), *Archaeoglobus fulgidus* (A_fulgidus; Swiss-Prot accession no. O27995/Genbank accession no. AE000946), *Methanococcus jannaschii* (M_jannaschii; Swiss-Prot accession no. Q58487/Genbank accession no. U67551), and *Paracoccus zeaxanthinifaciens* (P_zeaxanthinifaciens; SEQ ID NOs:6/7). The *Saccharomyces cerevisiae* mevalonate kinase amino acid sequence (SEQ ID NO:1) is used as the reference for amino acid numbering to which the positions of the other sequences, *e.g.* the ones named above, are referred to (see also Table 3). Amino acids corresponding to positions 55, 59, 66, 83, 106, 111, 117, 142, 152, 158, 218, 231, 249, 367 and 375 of SEQ ID NO:1 are highlighted.

The following non-limiting examples further illustrate the invention.

Example 1: Measurement of mevalonate kinase activity and of inhibition by feedback inhibitors

For preparing mevalonate as substrate, 130 mg of DL-mevalonate lactone (FLUKA Chemie AG, Buchs, Switzerland) were dissolved in 5.5 ml of 0.2 M KOH and incubated for 15 min at 50°C. The solution was then adjusted to pH 7.0 by addition of 0.1 M HCl at room temperature (RT). Except if stated otherwise, the assay mixture consisted of 100 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 1 mM ATP, 2 mM $MgCl_2$, 1 mM mevalonate, 0.5 mM phosphoenolpyruvate (PEP), 0.32 mM NADH, 20 U/ml pyruvate kinase and 27 U/ml lactate dehydrogenase (Sigma-Aldrich, St. Louis, MO, USA). Inhibition was tested by adding 1 μM FPP.

Purification of His_6-tagged mevalonate kinase and of His_6-tagged mevalonate kinase mutant enzymes was done with Ni-NTA chromatography using the QIA*express* system/reagents of Qiagen. Upon addition of purified (His_6-tagged) mevalonate kinase, enzymatic reaction reflected by consumption of NADH was followed by photometric measurement at 340 nm. One unit (1 U) of mevalonate kinase activity catalyzes the phosphorylation of 1 μmol of mevalonate per min.

Example 2: Generation of feedback-resistant mutants of *Saccharomyces cerevisiae* mevalonate kinase

The cDNA of mevalonate kinase from *Saccharomyces cerevisiae* (SEQ ID NO:2) is amplified by PCR using primer Mvk-SphI containing an *Sph*I restriction site (SEQ ID NO:10) along with a sequence of His_6 as well as a piece of the 5'-end sequence of mevalonate kinase without the ATG start codon, and primer Mvk-HindIII containing the 3'-end sequence of mevalonate kinase including the stop codon and a *Hind*III restriction site (SEQ ID NO:11). The PCR reaction is run according to the supplier's protocol (1 cycle at 94.5°C for 30 sec; 25 cycles at 94.5°C for 30 sec, 55°C for 30 sec, 70°C for 3 min) using Turbo-Pfu polymerase of Stratagene (La Jolla, CA, USA). After purification by agarose gel electrophoresis, the PCR product is digested by *Sph*I and *Hind*III and ligated into pQE-80L (Qiagen, Hilden, Germany) digested with the same enzymes, resulting in pQE-80L-His_6-Mvk. Plasmid pQE-80L contains a T5 promoter regulated by a *lac* operator element, which can be *cis*-inhibited by the *lac* repressor also encoded by pQE-80L. The plasmid is then transformed into *E. coli* DH5α (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. Upon addition of 100 μM IPTG at an OD_{600nm} of 0.6 during exponential growth of *E. coli*, His_6-tagged mevalonate kinase is induced at 30°C for 4 h by shaking at 250 rpm.

Site-directed mutagenesis of His_6-tagged mevalonate kinase is achieved by the so-called "two step PCR" using Turbo-Pfu DNA polymerase of Stratagene (La Jolla, CA, USA). The first PCR (see above for conditions) is performed with one of the primers represented by SEQ ID NOs:12-26 containing the mutated codons as the first primer and primer pQE-5' (SEQ ID NO:27) corresponding to a piece of sequence at the 5'-end of the multiple cloning sites (MCS) of pQE-80L as the second primer. The template is pQE-80L-His_6-Mvk. The PCR product is purified by agarose gel electrophoresis and used as a primer for the second PCR reaction together with the primer pQE-3' (SEQ ID NO:28) encompassing a piece of the 3'-end sequence of the MCS, with wild-type pQE-80L-His_6-Mvk as template. The PCR product (1.4 kb) is purified by agarose gel electrophoresis and digested by *Sph*I and *Hind*III, with which the His_6-Mvk is subcloned into pQE-80L. Finally, the digested fragment is purified by agarose electrophoresis and ligated into pQE-80L linearized by the same restriction enzymes, resulting in a mutated pQE-80L-His_6-Mvk.

Example 3: Feedback resistance of *Saccharomyces cerevisiae* mevalonate kinase mutants

Preparation of mevalonate and activity measurement was exactly performed as described in Example 1. 1 μM FPP was used for inhibition assays performed with the *Saccharomyces cerevisiae* mevalonate kinase wild-type enzyme and its mutants.

Feedback resistance (%) was calculated according to the following formula:

$$\% \text{ resistance} = 100 \cdot ((d/c)-(b/a))/(1-(b/a))$$

wherein (a) and (b) refer to the measured mevalonate kinase activities of the wild-type enzyme in the absence and presence, respectively, and (c) and (d) refer to the measured mevalonate kinase activities of the mutant enzyme in the absence and presence, respectively, of FPP. The results are shown in Tab. 1, wherein WT represents the non-mutated mevalonate kinase containing a His_6-tag (SEQ ID NO:3).

Table 1. Impact of mutagenesis of the *Saccharomyces cerevisiae* mevalonate kinase on the specific activity and the feedback resistance of the enzyme.

Mutant	Specific activity (% of wild-type)	Feedback resistance (%)
WT	100	0
P55L, C117S	60	58
F59S	31	56

N66K, I152M	148	67
K83E, S249P	78	37
H111N, K375N	87	65
L106P, S218P	42	24
I142N, L158S, L231I, T367S	61	58

Example 4: Saturated mutagenesis of *Saccharomyces cerevisiae* mevalonate kinase at amino acid residues/positions previously identified to have an impact on the resistance of the enzyme to feedback inhibition

Saturated mutagenesis is done in the same way as described in Example 2, except that the mutagenesis primer is synthesized in a way that the codons subject to saturated mutagenesis are made of randomized sequence.

Example 5: Improved production of coenzyme Q10 using a feedback inhibition-resistant mevalonate kinase

To test the *in vivo* effect of mutation N66K/I152M on the production of coenzyme Q10, the DNA encoding *S. cerevisiae* mevalonate kinase mutant N66K/I152M (SEQ ID NO:5) is introduced into *Paracoccus zeaxanthinifaciens* and compared to the CoQ10 production in *P. zeaxanthinifaciens* carrying the DNA encoding the wild-type *S. cerevisiae* mevalonate kinase (SEQ ID NO:2).

Plasmid construction

The plasmid pQE-80L-His_6-Mvk (see Example 3) was introduced into *E. coli* according to Example 2. *E. coli* strains were grown at 37°C in LB medium (Becton Dickinson, Sparks, MD, USA). For maintenance of plasmids in recombinant *E. coli* strains, ampicillin (100 μg/ml) and/or kanamycin (25-50 μg/ml, depending on the experiment) were added to the culture medium. Agar (1.5% final concentration) was added for solid media. Liquid cultures were grown in a rotary shaker at 200 rpm.

Plasmid pBBR-K-mev-op-R114 was constructed to contain the mevalonate operon, including its promoter region, from *P. zeaxanthinifaciens* R114, inserted between the *Sac*I and *Nsi*I sites of plasmid pBBR1MCS-2 (Kovach *et al.*, Gene 166, 175-176, 1995). The cloned mevalonate operon corresponds to the sequence from nucleotides 2469 to 9001 of the sequence having the GenBank/EMBL accession number AJ431696. Between the *Sac*I site and the mevalonate operon sequence there is a short linker sequence, which is derived

from plasmid pCR®2.1-TOPO (Invitrogen, Carlsbad, CA, USA) and corresponds to the sequence from the *SacI* site to the PCR fragment insertion site.

Introduction of a *ddsA* gene from *P. zeaxanthinifaciens* strain ATCC 21588 under the control of the *crtE* promoter region between the *Ecl136* II and the *SpeI* sites of pBBR-K-mev-op-R114 resulted in pBBR-K-mev-op-R114-P*crtE-ddsA*$_{wt}$ (for construction of pBBR-K-P*crtE* see Example 6 of WO 02/099095). The DNA sequence of the *ddsA* gene of strain ATCC 21588 (*ddsA*$_{wt}$) is shown in SEQ ID NO:29, the corresponding amino acid sequence is depicted as SEQ ID NO:30.

Plasmids according to pBBR-K-mev-op-R114-P*crtE-ddsA*$_{wt}$, are constructed wherein the mevalonate operon, including its promoter region, from *P. zeaxanthinifaciens* R114 (see above) is replaced by either the DNA encoding the wild-type *S. cerevisiae* mevalonate kinase (SEQ ID NO:2) resulting in pBBR-K-mev-op-(*S. cerevisiae mvk*)-P*crtE-ddsA*$_{wt}$ or the DNA encoding *S. cerevisiae* mevalonate kinase mutant N66K/I152M (SEQ ID NO:5) resulting in pBBR-K-mev-op-(*S. cerevisiae mvk*-N66K/I152M)-P*crtE-ddsA*$_{wt}$.

Construction of recombinant *P. zeaxanthinifaciens* strains

P. zeaxanthinifaciens strains are grown at 28°C. The compositions of the media used for cultivation of *P. zeaxanthinifaciens* are described below. All liquid cultures of *P. zeaxanthinifaciens* grown in flasks are shaken in a rotary shaker at 200 rpm unless specified otherwise. Agar (2% final concentration) is added for solid medium. When media are sterilized by autoclaving, glucose is added (as a concentrated stock solution) after sterilization to achieve the desired final concentration. F-Medium contains (per liter distilled water): 10 g tryptone, 10 g yeast extract, 30 g NaCl, 10 g D-glucose·H_2O, 5 g $MgSO_4 \cdot 7H_2O$. The pH is adjusted to 7.0 before sterilization by filtration or autoclaving. Medium 362F/2 contains (per liter distilled water): 33 g D-glucose·H_2O, 10 g yeast extract, 10 g tryptone, 5 g NaCl, 2.5 g $MgSO_4 \cdot 7H_2O$. The pH of the medium is adjusted to 7.4 before sterilization by filtration or autoclaving. Following sterilization, 2.5 ml each (per liter of final solution) of microelements solution, NKP solution and CaFe solution are added. The latter three solutions are sterilized by filtration. Microelements solution contains (per liter distilled water): 80 g $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 6 g $ZnSO_4 \cdot 7H_2O$, 2 g $MnSO_4 \cdot H_2O$, 0.2 g $NiSO_4 \cdot 6H_2O$, 6 g EDTA. NKP solution contains (per liter distilled water): 250 g K_2HPO_4, 300 g $(NH_4)_2PO_4$. CaFe solution contains (per liter distilled water): 75 g $CaCl_2 \cdot 2H_2O$, 5 g $FeCl_3 \cdot 6H_2O$, 3.75 ml concentrated HCl.

Preparation of electrocompetent cells of *P. zeaxanthinifaciens* strain R114 and electroporation are performed as follows: 100 ml F-medium is inoculated with 1.5 ml of a stationary phase culture of *P. zeaxanthinifaciens* strain R114 and grown at 28°C, 200 rpm

until an optical density at 660 nm of about 0.5 is reached. The cells are harvested by centrifugation for 15 minutes at 4°C, 7000 x g and washed twice in 100 ml ice-cold HEPES buffer, pH 7. The final pellet is resuspended in 0.1 ml ice-cold HEPES buffer, pH 7 and the cells are either used immediately for electroporation or glycerol is added to a final concentration of 15% and the cells are stored in 50 µl aliquots at -80°C. One to five µl plasmid DNA is added in salt-free solution and electroporations are performed at 18 kV/cm and 129 Ohms in ice-cooled 1-mm cuvettes. Pulse lengths are typically between 4 and 5 milliseconds. One ml of F-medium is added and the cells are incubated for 1 hour at 28°C. Dilutions are spread onto F-agar plates containing 25-50 µg/ml kanamycin and incubated at 28°C. Putative transformants are confirmed to contain the desired plasmid by PCR analysis.

Culture conditions for evaluating coenzyme Q10 production

Coenzyme Q10 production is tested in fed-batch cultivations of *P. zeaxanthinifaciens* strains R114/pBBR-K-mev-opR114-P*crtE-ddsA*$_{wt}$, R114/pBBR-K-mev-op-(*S. cerevisiae mvk*)-P*crtE-ddsA*$_{wt}$ and R114/pBBR-K-mev-op-(*S. cerevisiae mvk*-N66K/I152M)-P*crtE-ddsA*$_{wt}$. All cultures are initiated from frozen cell suspensions (stored as 25% glycerol stocks at -80°C). The precultures for the fed-batch fermentations are prepared in duplicate 2-liter baffled shake flasks containing 200 ml of 362F/2 medium each. Two milliliters of thawed cell suspension are used as inoculum for each flask. The initial pH of the precultures is 7.2. The precultures are incubated at 28°C with shaking at 250 rpm for 28 h, after which time the optical density at 660 nm (OD_{660}) is between 14 and 22 absorbance units, depending on the strain used. Main cultures are grown in Biostat ED Bioreactors (B. Braun Biotech International, Melsungen, Germany) containing medium having the following composition (per liter distilled water): 25 g D-glucose·H_2O, 17 g yeast extract (Tastone 900), 4.0 g NaCl, 6.25 g $MgSO_4 \cdot 7H_2O$, 0.5 g $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 0.038 g $ZnSO_4 \cdot 7H_2O$, 0.013 g $MnSO_4 \cdot H_2O$, 0.001 g $NiSO_4 \cdot 6H_2O$, 0.47 g $CaCl_2 \cdot 2H_2O$, 0.062 g $FeCl_3 \cdot 6H_2O$, 0.01 g niacin, 0.5 g NH_4Cl, 0.1 ml antifoam, 3.5 ml KP solution. The composition of KP solution is (per liter distilled water): 250 g K_2HPO_4, 200 g $NaH_2PO_4 \cdot 2H_2O$, 100 g $(NH_4)_2HPO_4$. Kanamycin (50 mg/l final concentration) is added to the medium for plasmid-carrying strains. The feeding solution used in all processes has the following composition (per liter distilled water): 550 g D-glucose·H_2O, 18.25 ml KP solution. The initial volume in the bioreactor (after inoculation) is 8.0 l. Precultures are diluted as needed with sterile water such that addition of 400 ml to the bioreactor results in an initial OD_{660} value of 0.5. Fermentation conditions are automatically controlled as follows: 28°C, pH 7.2 (pH controlled with addition of 28% NH_4OH), dissolved oxygen controlled at a minimum of 40% relative value by agitation, minimum agitation of 300 rpm and an aeration rate of 1 v.v.m. (relative to final volume). The cultivations proceed

under these conditions without addition of feed solution for about 20 h (batch phase) followed by the feeding phase. After this time, a decrease in agitation speed, cessation of base consumption, a sharp pH increase and a decrease in CO_2 production are the indication that the initial glucose is exhausted and the feeding is started. A standard feed profile is defined as follows (from feeding start point): ramp from 50 g/h to 80 g/h in 17 h, continue at 80 g/h for 7 h, then ramp down to 55 g/h in 11 h, and continue at 55 g/h for the rest of the fermentation (total fermentation time = 70 h). The final volumes of the main cultures are about 10 liters.

Analytical methods

400 µl of whole cultivation broth are transferred to a disposable 15 ml polypropylene centrifuge tube. Four milliliters of stabilized extraction solution (0.5 g/l 3,5-di-*tert*-butyl-4-hydroxytoluene) in 1:1 (v/v) (DMSO / tetrahydrofuran) are added and the samples are mixed for 20 min in a laboratory shaker (IKA, Germany) to enhance extraction. Finally, the samples are centrifuged and the supernatants transferred to amber glass vials for analysis by reverse phase HPLC. This method was developed for the simultaneous determination of ubiquinones and their corresponding hydroquinones, with a clear separation of CoQ10 from the carotenoids zeaxanthin, phytoene, β-cryptoxanthin, β-carotene and lycopene. Chromatography is performed using an Agilent 1100 HPLC system (Agilent Technologies, USA) equipped with a temperature-controlled autosampler and a diode array detector. The method parameters were as follows:

Column	YMC Carotenoid C30 column 3 micron, steel, 150 mm x 3.0 mm I.D. (YMC Europe GmbH, Dinslaken, Germany, Part No. CT99S031503QT)
Guard column	Security Guard C18 (ODS, Octadecyl) 4 mm length x 3.0 mm I.D. (Phenomenex, Torrance, CA, USA, Part No. AJO-4287)
Typical column pressure	60 bar at start
Flow rate	0.5 ml/min
Mobile phase	Mixture of acetonitrile(A):methanol(B):TBME(C)

Gradient profile

Time (min)	%A	%B	%C
0	60	15	25
13	60	15	25
20	0	0	100
22	60	15	25
25	60	15	25

Post time	4 min
Injection volume	10 µl
Column temperature	15°C
Detection	Three wavelengths were used for detection of specific compounds according to Table 2.

Table 2. HPLC retention times and wavelengths used

Compound	Wavelength (nm)	Retention times (min)
Zeaxanthin (Z-isomers)	450	4.2, 6.4
E-Zeaxanthin	450	5.2
Phytoene	280	7.7
β-Cryptoxanthin	450	8.6
Ubiquinol 10	210	11.4
Coenzyme Q10	210	12.8
β-Carotene	450	14.5
Lycopene	450	22.0

Calculations: Calculations are based on peak areas.

Coenzyme Q10 production results

Under the fed-batch cultivation conditions described above, the final concentration of coenzyme Q10 produced by *P. zeaxanthinifaciens* strain R114/pBBR-K-mev-op-(*S. cerevisiae mvk*-N66K/I152M)-P*crtE-ddsA*$_{wt}$ is at least 5% higher than observed for strain R114 carrying plasmid pBBR-K-mev-op-(*S. cerevisiae mvk*)-P*crtE-ddsA*$_{wt}$.

Example 6: Identification of corresponding residues in mevalonate kinases that are homologous to *Saccharomyces cerevisiae* mevalonate kinase

A multiple amino acid sequence alignment of different mevalonate kinases was calculated as shown in Figure 1. The sequence named as "Mvk_yeast" corresponds to SEQ ID NO:1, the sequence named as "Mvk-P-zeaxanthinifaciens" corresponds to SEQ ID NO:6.

The following residues corresponding to specific amino acid positions of the amino acid sequence of *Saccharomyces cerevisiae* mevalonate kinase (SEQ ID NO:1) were identified

(for further reference to the source of Mvk, *i.e.* name of the organism, including accession numbers of the respective sequences see legend to Figure 1):

Table 3. Amino acid residues from various organisms (see Fig. 1) corresponding to the positions 55, 59, 66, 83, 106, 111, 117, 142, 152, 158, 218, 231, 249, 367, 375 of *S. cerevisiae* mevalonate kinase (Yeast; SEQ ID NO:1).

Source of Mvk	Amino acid position														
Yeast	P55	F59	N66	K83	L106	H111	C117	I142	I152	L158	S218	L231	S249	T367	K375
Mouse	P54	I58	G65	-	P98	E105	A111	P139	Y149	A155	-	L224	S242	E349	K357
Rat	P54	I58	A65	-	P98	E105	A111	P139	Y149	A155	-	L224	S242	E349	K357
H-sapiens	P54	I58	A65	-	P98	E105	A111	P139	Y149	A155	-	L224	N242	E349	K357
P_rhodozyma	T61	F65	D72	-	G106	E113	A119	M146	L156	L162	-	I236	D254	S362	M370
S_pombe	S53	T57	Q64	-	E98	I103	C109	L134	I144	T150	T211	L221	S239	K343	K351
A_pernix	R44	K45	S52	-	-	-	-	P104	S114	L120	-	R176	R194	V295	V303
P_abyssi	E51	I55	V62	-	A96	-	-	V112	V122	G128	-	H181	S199	-	V307
P_horikoshii	E51	I55	V62	-	S96	-	-	V112	V122	G128	-	P181	S199	-	V307
P_furiosus	E49	I53	V60	-	A94	-	-	V111	V121	G127	-	H180	P198	-	V306
M_thermoautotr.	P49	I53	-	-	-	-	-	A91	L101	G107	-	Q161	E179	-	V282
A_fulgidus	-	I44	-	-	R72	-	-	I87	V97	A103	-	E156	S170	-	-
M_jannaschii	N49	K53	N60	-	L87	-	-	I105	I115	K121	-	E181	K199	-	-
P_zeaxanthinifac.	A57	G61	K68	-	A102	L109	P115	I143	I153	V159	-	P210	R228	A340	H348

Claims

1. A modified mevalonate kinase which exhibits a sensitivity to feedback inhibition which is reduced in comparison to the corresponding non-modified mevalonate kinase wherein

(i) the amino acid sequence of the modified mevalonate kinase contains at least one mutation when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase and

(ii) the at least one mutation is at one or more amino acid position(s) selected from the group consisting of amino acid positions corresponding to positions 55, 59, 66, 83, 106, 111, 117, 142, 152, 158, 218, 231, 249, 367 and 375 of the amino acid sequence of *Saccharomyces cerevisiae* mevalonate kinase as shown in SEQ ID NO:1.

2. A modified mevalonate kinase according to claim 1 which consists of one or more mutations at one or more amino acid position(s) which are selected from the group consisting of amino acid positions corresponding to positions 55, 59, 66, 83, 106, 111, 117, 142, 152, 158, 218, 231, 249, 367 and 375 of the amino acid sequence of *Saccharomyces cerevisiae* mevalonate kinase as shown in SEQ ID NO:1.

3. A modified mevalonate kinase according to claim 1 or 2 wherein the non-modified mevalonate kinase is from *Saccharomyces cerevisiae*.

4. A modified mevalonate kinase according to any one of claims 1 to 3 wherein said feedback inhibition is feedback inhibition by farnesyl diphosphate or geranylgeranyl diphosphate.

5. A modified mevalonate kinase according to any one of claims 1 to 4 wherein the modified mevalonate kinase exhibits a feedback resistance of at least 10% in comparison to the corresponding non-modified mevalonate kinase.

6. A modified mevalonate kinase according to any one of claims 1 to 5 wherein the at least one mutation is at least one amino acid substitution.

7. A modified mevalonate kinase according to any one of claims 1 to 6 wherein the modified mevalonate kinase comprises two substitutions when compared with the amino acid sequence of the corresponding non-modified mevalonate kinase, preferably two substitutions at the amino acid positions corresponding to amino acid positions 66 and 152 of the sequence as shown in SEQ ID NO:1.

8. A modified mevalonate kinase according to claim 7 wherein the substitution at the amino acid position corresponding to position 66 of the sequence as shown in SEQ ID NO:1 consists of the replacement of asparagine with lysine and the substitution at the amino acid position corresponding to position 152 of the sequence as shown in SEQ ID NO:1 consists of the replacement of isoleucine with methionine.

9. A modified mevalonate kinase according to any one of claims 1 to 8 wherein the amino acid sequence of the corresponding non-modified mevalonate kinase is selected from the group consisting of the amino acid sequences as shown in Figure 1.

10. A modified mevalonate kinase according to any one of claims 1 to 9 wherein the amino acid sequence of the non-modified mevalonate kinase is SEQ ID NO:1.

11. A polynucleotide comprising a nucleotide sequence which codes for a modified mevalonate kinase according to any one of claims 1 to 10.

12. A polynucleotide according to claim 11 wherein the nucleotide sequence which codes for a modified mevalonate kinase is the nucleotide sequence SEQ ID NO:5.

13. A vector or plasmid comprising a polynucleotide according to claim 11 or 12.

14. A host cell comprising a polynucleotide according to any one of claims 11 to 13.

15. A host cell according to claim 14 which is selected from the group consisting of *Escherichia*, *Paracoccus*, *Rhodobacter*, and *Saccharomyces*.

16. A process for producing a modified mevalonate kinase according to any one of claims 1 to 10 comprising:

(a) culturing a host cell according to claim 14 or 15 in a suitable medium and under conditions that allow expression of the modified mevalonate kinase; and

(b) recovering the modified mevalonate kinase from the cells or from the medium.

17. A process for the preparation of a mevalonate kinase having reduced sensitivity to feedback inhibition, comprising the following steps:

(a) providing a polynucleotide encoding a first mevalonate kinase which exhibits sensitivity to feedback inhibition;

(b) introducing one or more mutation(s) into the polynucleotide sequence such that the mutated polynucleotide sequence encodes a second mevalonate kinase which is a modified mevalonate kinase according to any one of claims 1 to 10;

(c) optionally inserting the mutated polynucleotide in a vector or plasmid;

(d) introducing the polynucleotide of step (b) or (c) into a suitable host cell; and

(e) culturing the host cell under conditions that allow expression of the modified mevalonate kinase having reduced sensitivity to feedback inhibition.

18. A process for producing an isoprenoid comprising:

(a) culturing a host cell according to claim 14 or 15 in a suitable medium under conditions that allow expression of the modified mevalonate kinase; and

(b) optionally separating the isoprenoid from the medium.

19. The use of a modified mevalonate kinase according to any one of claims 1 to 10 or a polynucleotide according to claim 11 or 12 for the manufacture of a medicament for the treatment of a disorder associated with decreased activity of mevalonate kinase.

20. The use of a modified mevalonate kinase according to any one of claims 1 to 10 or a polynucleotide according to claim 11 or 12 for determining the concentration of mevalonate in biological fluids.

***

Figure 1.

```
Mvk_mouse                    --MLSEALLVSAPGKVILHGEHAVVHGKVALAAALN-LRTFLLLRP---- 43
Mvk_rat                      --MLSEVLLVSAPGKVILHGEHAVVHGKVALAVALN-LRTFLVLRP---- 43
Mvk_H_sapiens                --MLSEVLLVSAPGKVILHGEHAVVHGKVALAVSLN-LRTFLRLQP---- 43
Mvk_P_rhodozyma              ----KEEILVSAPGKVILFGEHAVGHGVTGIAASVD-LRCYALLSPTATT 45
Mvk_S_pombe                  ---MSKSLIVSSPGKTILFGEHAVVYGATALAAAVS-LRSYCKLQT---- 42
Mvk_A_pernix                 ---MRRAARASAPGKVIIVGEHFVVRGSLAIVAAIG-------------- 33
Mvk_P_abyssi                 ---MPRLVLASAPAKIILFGEHSVVYGKPAIASAID-LRTYVRAEF---- 42
Mvk_P_horikoshii             ---MVKYVLASAPAKVILFGEHSVVYGKPAIASAIE-LRTYVRAQF---- 42
Mvk_P_furiosus               -----MKVIASAPAKVILFGEHSVVYGKPAIAAAID-LRTFVEAEL---- 40
Mvk_M_thermoautotrophicum    -----MKSSASAPAKAILFGEHAVVYSKPAIAAAID-RRVTVTVSE---- 40
Mvk_A_fulgidus               -------MIASAPGKIILFGEHAVVYGRHAVVSAIN-LRCRVSVRK---- 38
Mvk_M_jannaschii             -------MIIETPSKVILFGEHAVVYGYRAISMAIDLTSTIEIKETQ--- 40
Mvk_P_zeaxanthinifaciens     MSTGRPEAGAHAPGKLILSGEHSVLYGAPALAMAIARYTEVWFTPLG--- 47
Mvk_yeast                    ---MSLPFLTSAPGKVIIFGEHSAVYNKPAVAASVSALRTYLLISE---- 43

Mvk_mouse                    -----QSNGKVSVNLPNIGIKQVWDVGMLQR-LDTSFLE------QGDVS 81
Mvk_rat                      -----QSNGKVSLNLPNVGIKQVWDVATLQL-LDTGFLE------QGDVP 81
Mvk_H_sapiens                -----HSNGKVDLSLPNIGIKRAWDVARLQS-LDTSFLE------QGDVT 81
Mvk_P_rhodozyma              TTSSSLSSTNITISLTDLNFTQSWPVDSLPWSLAPDWTE------ASIPE 89
Mvk_S_pombe                  ----TNN-NEIVIVMSDIGTERRWNLQSLPWQHVTVEN------VQHPAS 81
Mvk_A_pernix                 --------RRLRVTVRSGGKGIVLESSMLGR---------------HSAP 60
Mvk_P_abyssi                 -------NDSGNIKIEAHDIKTPGLIVSFSEDKIYFETD------YGKAA 79
Mvk_P_horikoshii             -------NDSGNIKIEAHDIKTPGLIVSFSEDKIYFETD------YGKAA 79
Mvk_P_furiosus               -------IREKKIRIEAHDIKVPGLTVSFSENEIYFETD------YGKAA 77
Mvk_M_thermoautotrophicum    -------SSSTHVTIPSLGIRHS------SER---------------PSG 62
Mvk_A_fulgidus               -------SDR-------FLIRSS-----LGES--------------GLDY 55
Mvk_M_jannaschii             -------EDEIILNLNDLNKSLGLNLNEIKN-------------INPNNF 70
Mvk_P_zeaxanthinifaciens     ------IGEGIRTTFANLSGGATYSLKLLSGFKSRLDRR------FEQFL 85
Mvk_yeast                    ----SSAPDTIELDFEDISENHKWSINDFNAITEDQVNSQKLAKAQQATD 89

Mvk_mouse                    VPTLEQLEKLKKMGDLPRDRAGNEGMALLAFLYLYLAICRKQRTLPSLDM 131
Mvk_rat                      APTLEQLEKLKKVAGLPRDCVGNEGLSLLAFLYLYLAICRKQRTLPSLDI 131
Mvk_H_sapiens                TPTSEQVEKLKEVAGLPDDCAVTERLAVLAFLYLYLSICRKQRALPSLDI 131
Mvk_P_rhodozyma              SLCPTLLAEIERIAGQGGNGGEREKVATMAFLYLLVLLSKGKPSEP-FEL 138
Mvk_S_pombe                  SPNLDLLQGLGELLKNEENG--LIHSAMLCTLYLFTSLS---SPSQGCTL 126
Mvk_A_pernix                 LPGQGAAAKVSPVLEP--------------YIAVLRSLAARGYSVVPHTI 96
Mvk_P_abyssi                 EVLSYVRHAIELVLEEADKR-------------------------TGVSV 104
Mvk_P_horikoshii             EVLSYVRYAIELALEESDKR-------------------------VGIDV 104
Mvk_P_furiosus               EVLSYVREAINLVLEEADKKN------------------------VGIKV 103
Mvk_M_thermoautotrophicum    GILDYIGRCLELYHDA-----------------------------SPLDI 83
Mvk_A_fulgidus               QRHPYVVQAVKRFGELRNIP-------------------------GAEI 79
Mvk_M_jannaschii             GDFKYCLCAIKNTLDYLNIEP-----------------------KTGFKI 97
Mvk_P_zeaxanthinifaciens     NGDLKVHKVLTHPDDLAVYALASLLHDKPPGTAAMPGIGAMHHLPRPGEL 135
Mvk_yeast                    GLSQELVSLLDPLLAQESES--FEYHAAFEFLYMFVCLC---PHAKNIKF 134

Mvk_mouse                    VVWSELPPGAGLGSSAAYSVCLAAAALLTACEEVSNPLKDGVSVSRWPEED 181
Mvk_rat                      MVWSELPPGAGLGSSAAYSVCVAAAALLTACEEVTNPLKDRGSIGSWPEED 181
Mvk_H_sapiens                VVWSELPPGAGLGSSAAYSVCLAAAALLTVCEEIPNPLKDGDCVNRWTKED 181
Mvk_P_rhodozyma              TARSALPMGAGLGSSAALSTSLALVFLLHFSHLSPTTTGRE--STIPTAD 186
Mvk_S_pombe                  TISSQVPLGAGLGSSATISVVVATSLLLAFGNIEPPS----SNSLQNNKA 172
Mvk_A_pernix                 LVESGIPPRAGLGSSAASMVAYALSYSAMHGDP---------------LS 131
Mvk_P_abyssi                 SITSQIPVGAGLGSSAAVAVATIGAVSKLLDLELS---------------- 139
Mvk_P_horikoshii             SITSQIPVGAGLGSSAAVAVATIGAVSRLLGLELS---------------- 139
Mvk_P_furiosus               SITSQIPVGAGLGSSAAVAVATIGAVSKLLGLELS---------------- 138
Mvk_M_thermoautotrophicum    RVEMEIPAGSGLGSSAALTVALIGALDRYHGRDHG---------------- 118
Mvk_A_fulgidus               EIESEIPIGSGLGSSAAVIVATIAALNAEFDGDMD---------------- 114
Mvk_M_jannaschii             NISSKIPISCGLGSSASITIGTIKAVSGFYNKELK---------------- 132
Mvk_P_zeaxanthinifaciens     GSRTELPIGAGMGSSAAIVAATTVLFETLLDRPKT---------------- 170
Mvk_yeast                    SLKSTLPEGAGLGSSASESVSLAEAMAYLGGLIGSND----LEKLSEN-D 179

Mvk_mouse                    LKSINKWAFEGERVIHGNPSGVDNAVSTWGGALRFQ---------QGTMS 222
Mvk_rat                      LKSINKWAYEGERVIHGNPSGVDNSVSTWGGALRYQ---------QGKMS 222
Mvk_H_sapiens                LELINKWAFQGERMIHGNPSGVDNAVSTWGGALRYH---------QGKIS 222
Mvk_P_rhodozyma              TEVIDKWAFLAEKVIHGNPSGIDNAVSTRGGAVAFKR--KIEGKQEGGME 234
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Figure 1 (continued)

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Mvk_S_pombe                 LALIEAWSFLGECCIHGTPSGIDNAVATNGGLIAFRKATAHQSAMK---E 219
Mvk_A_pernix                AEDLYSVAMEGEKIAHGKPSGVDVTIAVRGGVLAYRR-------GENPVD 174
Mvk_P_abyssi                KEEIAKMGHKVELLVQGASSGIDPTVSAIGGFLYYK----------QGEF 179
Mvk_P_horikoshii            KEEIAKLGHKVELLVQGASSGIDPTVSAVGGFLYYK----------QGKF 179
Mvk_P_furiosus              KEEIAKMGHKTELLVQGASSGIDPTVSAIGGFIFYE----------KGKF 178
Mvk_M_thermoautotrophicum   PGETAARAHRVEVDVQGAASPLDTAISTYGGLVYLDS---------QRRV 159
Mvk_A_fulgidus              KEAIFQMAKQVEIDVQGRASGIDPFISTFGGSWLFP----------ERRK 154
Mvk_M_jannaschii            DDEIAKLGYMVEKEIQGKASITDTSTITYKGILEIKN---NKFRKIKGEF 179
Mvk_P_zeaxanthinifaciens    PEQRFDRVRFCERLKHGKAGPIDAASVVRGGLVRVGG----------N 208
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Mvk_H_sapiens               SLKRSPALQILLTNTKVPRNTRALVAGVRNRL-LKFPEIVAPLLTSIDAI 271
Mvk_P_rhodozyma             AIKSFTSIRFLITDSRIGRDTRSLVAGVNARL-IQEPEVIVPLLEAIQQI 283
Mvk_S_pombe                 FLKPKDTLSVMITDTKQPKSTKKLVQGVFELK-ERLPTVIDSIIDAIDGI 268
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Mvk_P_horikoshii            EPLPFMELPIVVGYTGSTGSTKELVAMVRKRY-EEMPELVEPILEAMGKL 228
Mvk_P_furiosus              EHLPFMELPIVVGYTGSSGPTKELVAMVRKRY-EEMPELIVPILEAMGKV 227
Mvk_M_thermoautotrophicum   RQFEADLGDLVIAHLDYSGETARMVAGVAERF-RRFPDIMGRIMDTVESI 208
Mvk_A_fulgidus              VEMPFKFFVINFG----SRSTAEMVAKVAELR-ERHPEVVDKIFDAIDAI 199
Mvk_M_jannaschii            EEFLKNCKFLIVYAEKRKKKTAELVNEVAKIE------NKDEIFKEIDKV 223
Mvk_P_zeaxanthinifaciens    GPGSISSFDLPEDHDLVAGRGWYWVLHGRPVSGTGECVSAVAAAHGRDAA 258
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Mvk_H_sapiens               SLECERVLG--EMGEAP-------APEQYLVLEELIDMNQHHLNALGVGH 312
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Mvk_A_fulgidus              SLEASDVGSAER-------------------LEELIAINQSLLRAIGVSN 230
Mvk_M_jannaschii            IDEALKIKNKED-------------------FGKLMTKNHELLKKLNIST 254
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Mvk_A_pernix                LEIETLVYRMRSAG-ALGAKLTGAG-----WGGCVIGLFKEGEVERGLES 301
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Mvk_P_horikoshii            KKLGELVYAAR-TAGAIGAKLTGAG-----GGGCMYALAP------GRQR 305
Mvk_P_furiosus              KKLSELVYAAR-VAGALGAKITGAG-----GGGCMYALAP------NKQR 304
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Mvk_A_fulgidus              PEIDRTIAELE-RMG-LNAKITGAG-----GGGCIFGLFK------GEKP 267
Mvk_M_jannaschii            PKLDRIVDIGN--RFGFGAKLTGAG-----GGGCVIILVN---------- 287
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Mvk_rat                     AKQALT-GCGFDCWETSIGAPGVSMHSATSIEDP---------------- 388
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Mvk_P_rhodozyma             LMETLV-QSSFAPYIARVGGSGVGFLSSTKADPEDGENRL------KDGL 411
Mvk_S_pombe                 CKESLLAHKN-SIYDVQLGGPGVSVVTDSDS-------------FFPQYE 385
Mvk_A_pernix                VVESSS-----QAFTASIAEEGARLEEF---------------------- 324
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Mvk_P_horikoshii            EVATAIKIAGGIPMITRVSREGLRIEEVSR--------------------- 335
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Figure 1 (continued)

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Mvk_M_jannaschii              -------EEKEKELLKELNKEDVRIFNCRMMN---------- 312
Mvk_P_zeaxanthinifaciens      RHPDLDWAPLRMSRTGAAPGPAPRAQPLPGQG---------- 378
Mvk_yeast                     F[illegible]KKLQDDFSYETFETDLGGTGCCLLSAKNLNKDLKIKSLVFQLFENKTT 423

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Mvk_rat                       VRQALG-L---------- 395
Mvk_H_sapiens                 VQQALDGL---------- 396
Mvk_P_rhodozyma               VGTEIDELDRWALKTGRWS- 430
Mvk_S_pombe                   SDFDFKKLNLLSKFNKYYI- 404
Mvk_A_pernix                  ----------
Mvk_P_abyssi                  ----------
Mvk_P_horikoshii              ----------
Mvk_P_furiosus                ----------
Mvk_M_thermoautotrophicum     ----------
Mvk_A_fulgidus                ----------
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/013282

A. CLASSIFICATION OF SUBJECT MATTER
C12N9/12 C12N15/54 C12N1/19 C12N1/21 C12Q1/48
C12P23/00 A61K38/45

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12Q C12P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00/01649 A (DCV, INC. DOING BUSINESS AS BIO-TECHNICAL RESOURCES; EASTMAN CHEMICAL) 13 January 2000 (2000-01-13) abstract page 19, line 24 - page 20, line 7 page 22, line 13 - line 26 claims 28,30	

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 March 2006	31/03/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ferreira, R

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/013282

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOUTEN S M ET AL: "Biochemical and genetic aspects of mevalonate kinase and its deficiency" BIOCHIMICA AND BIOPHYSICA ACTA. MOLECULAR AND CELL BIOLOGY OF LIPIDS, ELSEVIER, AMSTERDAM, NL, vol. 1529, no. 1-3, 15 December 2000 (2000-12-15), pages 19-32, XP004277444 ISSN: 1388-1981 the whole document	
A	WO 02/099095 A (ROCHE VITAMINS AG; BERRY, ALAN; BRETZEL, WERNER; HUEMBELIN, MARKUS; LO) 12 December 2002 (2002-12-12) abstract page 5, line 24 - line 34 claims 13-15	
A	HUMBELIN M ET AL: "Genetics of isoprenoid biosynthesis in Paracoccus zeaxanthinifaciens" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, ELSEVIER, AMSTERDAM, NL, vol. 297, no. 1-2, 4 September 2002 (2002-09-04), pages 129-139, XP004388023 ISSN: 0378-1119 the whole document	
P,X	WO 2004/111214 A (DSM IP ASSETS B.V; BAI, RENYUNAN; HUEMBELIN, MARKUS; LEHMANN, MARTIN;) 23 December 2004 (2004-12-23) the whole document	1-20

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2005/013282

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		BR 9911873 A	27-03-2001
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