

Yeast cell

The present invention relates to the use of orthologous promoters in yeast cells.

Recombinant proteins such as biopharmaceuticals or industrially relevant biocatalysts are produced most commonly by means of heterologous gene expression in microorganisms. *Escherichia coli*, *Saccharomyces cerevisiae* and filamentous fungi have been used frequently and for a long time for recombinant protein production. In the last two decades, the methylotrophic yeasts *Komagataella (Pichia) pastoris*, *Komagataella (Pichia) phaffii* (Pp), *Komagataella Kurtzmanii*, *Ogataea (Hansenula) polymorpha* (Hp), *Candida boidinii* (Cb) and *Ogataea (Pichia) methanolica* (Pm) have become established as efficient alternative production strains. These strains make it possible to achieve high expression rates for heterologous proteins with a high cell density. Of the aforementioned four yeast species, *P. pastoris* (*Komagataella phaffii*) has in the meantime been used most commonly for heterologous protein production.

All methylotrophic yeasts have strictly regulated strong promoters which are involved in the regulation of expression of genes of methanol utilization (MUT). Promoters of genes of methanol utilization are usually repressed on repressing carbon sources such as glucose and are greatly upregulated in the presence of methanol as a carbon source. If the repressing carbon source is depleted or in the presence of a non-repressing carbon source, then the promoter is activated by derepression, whereby the strength of this effect can vary greatly between species and even within the same organism. The promoter of the alcohol oxidase-1-gene in *P. pastoris* GS115 (PPpAOX1), for example, has only a 2-4% activity under derepressing conditions in comparison with methanol-induced conditions. In contrast thereto the promoter of the orthologous gene (methanol oxidase, MOX) in *H. polymorpha* (PHpMOX) has an activity of up to 70% under derepressing conditions in comparison with methanol-induced conditions. Also the promoters of the orthologous gene in *C. boidinii* (alcohol oxidase 1, AOD1) and *P. methanolica* (methanol oxidase 1/2, MOD1/2) have a comparable behavior.

Induction of expression with toxic and flammable methanol is undesirable especially on a large industrial scale for reasons of operational safety so that strong derepressed promoters constitute a favorable alternative. Accordingly PPpAOX1 variants, alternative promoters and novel MUT promoters with different derepressing properties have been developed recently to enable a methanol-free protein expression on an industrial scale. Since the rates of expression of such promoters are usually much lower in comparison with methanol-induced promoters, one object of the present invention is to make available alternative possibilities for inducible and strong methanol-free overexpression of recombinant proteins in yeasts such as *P. pastoris*.

This object is achieved with a yeast cell of the Komagataella genus comprising an orthologous promoter of a methylotrophic yeast cell or a variant thereof that can be induced by derepression, wherein the orthologous promoter is an orthologous formate dehydrogenase (FMD) or a methanol oxidase (MOX) promoter of a methylotrophic yeast cell; in this process, the orthologous promoter in the methylotrophic yeast cell is capable of controlling the expression of polypeptides under derepressing conditions.

This object is also achieved with a yeast cell of the Komagataella (Pichia) genus comprising an orthologous formate dehydrogenase (FMD) promoter and/or a methanol oxidase (MOX) promoter of a methylotrophic yeast cell or variants of these two promoters, wherein the original regulation profile of the orthologous promoter in yeast cells of the Komagataella genus is retained.

It has surprisingly been found that promoters capable of controlling the expression of polypeptides under derepressing conditions in other methylotrophic yeast cells, which preferably do not belong to the Komagataella (Pichia) genus, are capable of controlling the expression of polypeptides under derepressing conditions (for example, increasing expression in comparison with non-derepressing conditions), also have comparable properties in yeast cells of the Komagataella (Pichia) genus.

Furthermore, it has surprisingly been found that a formate dehydrogenase (FMD) promoter and/or a methanol oxidase (MOX) promoter of a methylotrophic yeast cell that does not occur nat-

urally in a yeast cell of the *Komagataella* genus and/or in the same yeast cell has special properties in such a cell. An orthologous FMD and/or MOX promoter is significantly stronger in *Komagataella* cells under both derepressing conditions and under methanol-induced conditions than all the naturally occurring promoters and *Komagataella* that are involved in the regulation of the expression of genes of methanol utilization ("MUT promoters") and have been tested so far. Thus, an orthologous FMD and/or MOX promoter is significantly stronger under derepressing conditions than the CAT1 and GAP promoters occurring naturally in *Komagataella* cells, for example. Orthologous FMD and/or MOX promoters are surprisingly even just as strong as the AOX (AOX1 and AOX2) promoters occurring naturally in *Komagataella* under methanol-induced conditions under the screening conditions used under derepressing conditions than the AOX promoters used under methanol-inducing conditions. Such effects can usually be intensified under controlled C-source doses in a bioreactor experiment. Orthologous FMD and/or MOX promoters can replace the AOX promoters generally used in *Komagataella*. Essentially identical or even higher protein expression yields can be achieved in this way in comparison with traditional methanol-induced expression systems but without using any methanol as the induction agent. It is surprising here that a formate dehydrogenase (FMD) promoter of a methylotrophic yeast cell (for example, of *H. polymorpha*) which is also significantly derepressed in this yeast cell (for example, in *H. polymorpha*), retains this regulation profile even in another methylotrophic yeast cell (for example, *P. pastoris*). In contrast thereto earlier studies have shown that in a transfer of promoters between methylotrophic yeasts, the regulation profile of the foreign promoter is not transferred (for example, the *P. pastoris* AOX1 promoter, for example, is not stringently repressed in *H. polymorpha* as it is naturally in *P. pastoris*; see, for example, W.C. Raschke et al. Gene 177 (1996):163-167 and L. Rodriguez et al. Yeast 12 (1996):815-822). Accordingly, the current opinion in the technical world is that different types of regulation between methylotrophic yeast cells do not occur due to the promoter sequence but instead due to different regulation mechanisms in the yeast cells (see, for ex-

ample, F.S. Hartner et al. Microb. Cell Fact 5 (2006):39-59). However, it has surprisingly been found that the strong activation of a formate dehydrogenase (FMD) promoter of a methylotrophic yeast cell (for example, of *H. polymorpha*) due to derepression can be transferred not only to other methylotrophic yeast cells, such as, for example, *Komagataella phaffii*, but instead even exceeds the technical properties of the strong homologous promoters such as that of the AOX1 gene and CAT1 gene.

Use of orthologous promoter sequences also has other technical advantages. For example, the possibility of homologous recombination is reduced by their use, resulting in a higher genetic stability of the expression strains.

"Yeast cell of the *Komagataella* genus" includes all yeast cells of this genus, such as *Komagataella kurtzmanii*, *Komagataella pastoris*, *Komagataella phaffii*, *Komagataella populi*, *Komagataella pseudopastoris*, *Komagataella ulmi* and *Komagataella* sp. 11-1192. "Yeast cells of the *Komagataella* genus" naturally also include those from specific strains of the genus as mentioned above, such as, for example, *Komagataella pastoris* GS115, X-33, KM71, KM71H, CBS7435 or NRLL Y11430, CBS704, BG10, BG11 and/or other derivatives of these strains.

The term "orthologous", as used herein, relates to nucleic acid or amino acid molecules from different species, which at least have functional homology with corresponding nucleic and amino acid molecules of other species. "Orthologs" come from different organisms which occur due to generation and are also derived from a common predecessor. The sequences of the "orthologs" can vary significantly among one another, but the biological and/or biochemical function thereof is usually not affected (for example, AOX from *Komagataella pastoris* is orthologous with MOX from *Hansenula polymorpha* and vice versa, FMD from *Hansenula polymorpha* is orthologous to FDH1 in *Komagataella pastoris* and vice versa).

The term "promoter", as used herein, includes at least one transcription initiation start site, a binding site for a nucleic acid polymerase complex and additional nucleotides so that these two elements can be functionally active and may retain the original regulation profile of the starting cell of the ortholo-

gous promoter in yeast cells of the *Komagataella* genus. These additional nucleotides may form transcription factor binding sites, for example. A "promoter inducible by derepression" is a promoter that is activated under derepressing conditions (see below), so that nucleic acid molecules operably linked to it are transcribed so that they code for heterologous or homologous polypeptides.

The orthologous promoters according to the invention, i.e. the orthologous FMD and/or MOX promoter, preferably comprise between 50 and 2000, even more preferably between 100 and 1000, even more preferably between 150 and 800 nucleotides from the region before the start codon (upstream from the 5' end) of the region of the corresponding gene comprising the promoter and coding for a protein/polypeptide, preferably the region of the FMD and/or MOX gene which codes for FMD and/or MOX which may comprise 1 to 1000, preferably 1 to 900, even more preferably 1 to 800 nucleotides. The orthologous promoter, preferably the orthologous FMD and/or MOX promoter, comprises preferably nucleotides 1 to 1000, preferably 1 to 900, even more preferably 1 to 800, upstream from the 5' end of the region of the gene that codes for the polypeptide, preferably the region of the FMD and/or MOX gene that codes for FMD and/or MOX.

"Variants" of the orthologous promoter of the invention, preferably of the orthologous formate dehydrogenase (FMD) promoter and/or of the methanol oxidase (MOX) promoter, include nucleic acid molecules, which differ in one or more (for example, 2, 3, 4, 5, 10, 15, 20, 25, 50) nucleotides from the naturally occurring orthologous promoters, preferably the orthologous FMD and/or MOX promoters. Such promoter variants are at least 80%, preferably at least 90%, even more preferably at least 95%, even more preferably at least 98% identical to the corresponding regions of the naturally occurring promoters.

The variants of orthologous promoters that can be used according to the invention may comprise deletions, substitutions and insertions in comparison with the naturally occurring promoters, preferably FMD and/or MOX promoters. The variants of the promoters also have the property of enabling expression of proteins under derepressing conditions. Variants are preferably

used, which are capable of expressing under derepressing conditions at least 50%, preferably at least 60%, even more preferably at least 70%, even more preferably at least 80%, even more preferably at least 90%, even more preferably at least 100%, even more preferably at least 120%, even more preferably at least 150%, of the amount of protein that would be expressed by a yeast cell of the *Komagataella* genus including a naturally occurring orthologous promoter, preferably an orthologous FMD promoter and/or an orthologous MOX promoter.

Methods of identifying and producing promoter variants are sufficiently well known. Mutations are usually introduced into the promoter, whereupon a test is performed showing whether and how the properties (for example, expression rate of a model protein) of the promoter variants have changed.

"Variants" of the orthologous promoter of the present invention, preferably of the orthologous formate dehydrogenase (FMD) promoter and/or of the orthologous methanol oxidase (MOX) promoter, also include promoter variants which include the regulatory elements of the naturally occurring orthologous promoter or variants thereof as defined above (differing in one or more, for example, 2, 3, 4, 5, 10, 15, 20, 25, 50 nucleotides from the naturally occurring sequence) and an alternative minimal promoter and/or core promoter. The minimal promoter and/or core promoter is part of a promoter that contains only the general promoter elements which are necessary for transcription (TATA box and transcription start). Therefore, the regulatory elements of the variants of the orthologous promoters according to the invention include preferably between 100 and 1000, even more preferably between 150 and 800 nucleotides from the region upstream from the start codon (upstream from the 5' end) without 20 to 100, preferably without 25 to 80, even more preferably without 30 to 70, nucleotides directly before the starting point of the transcription.

"Identity" and "identical", respectively, refer to the degree of correspondence between two or more nucleic acid and/or amino acid sequences which can be determined by the correspondence between the sequences. The percentage of "identity" is derived from the percentage of identical regions in two or more

sequences, taking into account gaps or other sequence particulars (i.e., % identity refers to the number of identical positions/total number of positions x 100). A particularly preferred method for determining identity is the BLAST program of the National Centre for Biotechnology Information (NCBI) (see S. Altschul et al., J Mol Biol 215 (1990):403-410 among others). The BLOSUM62 algorithm is preferably used with the parameters "gap" "existence":11 and "extension":1.

The term "methylophilic yeast cells", as used herein, includes yeast cells capable of growing on culture media containing as carbon source substances with only one carbon atom, for example methanol.

"Derepressing conditions", as used in culturing the yeast cells according to the invention, means that the yeast cells are first cultured in the presence of a repressing carbon source (e.g. glucose) until this carbon source has been mostly or entirely consumed. After reducing the concentration of the repressing carbon source (e.g. glucose), the cells are in derepressing conditions with respect to the repressing carbon source and glucose, respectively. The strength of the repression effects may depend on the type of carbon source.

According to a preferred embodiment of the present invention the orthologous FMD and/or the orthologous MOX promoter is operably linked to a nucleic acid molecule coding for a heterologous or homologous polypeptide.

The orthologous promoter may be operably linked to a nucleic acid molecule coding for a heterologous (not originating from *Komagataella*) or homologous polypeptide (originating from *Komagataella*) and can thus influence the expression of this polypeptide and/or control it. The resulting polypeptide includes at least 5, preferably at least 10, even more preferably at least 50 amino acid residues and thus includes molecules, which are also referred to as polypeptides or proteins.

The nucleic acid molecule codes preferably for polypeptides such as antibodies or fragments thereof, enzymes, structural proteins, etc.

"Operably linked", as used herein, means that the nucleic acid molecule coding for a heterologous or homologous polypep-

tide is linked to the promoter in a way which permits expression of the nucleotide sequence in a yeast cell according to the invention. The promoter is thus operably linked to a coding nucleic acid sequence when this has an influence on the transcription of the coding sequence.

According to another preferred embodiment of the present invention, the heterologous or homologous polypeptide comprises a signal peptide, in particular a secretion signal peptide.

To secrete a recombinant homologous or heterologous polypeptide from the yeast cell, the polypeptide encoded by the nucleic acid molecule includes a signal peptide.

The term "signal peptide", as used herein, refers to a peptide linked to the C-terminus or N-terminus of the polypeptide, which controls the secretion of the polypeptide. The signal sequence used in the present invention may be a polynucleotide which codes for an amino acid sequence which initiates the transport of a protein through the membrane of the endoplasmic reticulum (ER). The nucleic acid sequence of these signal sequences may correspond to the natural sequence of the original host cell or may be codon-optimized. The non limited examples of the signal sequence include MF-alpha ("mating factor alpha" signal sequence), the signal sequence of the CBH2 protein from *Trichoderma reesei*, the signal sequence of the xylanase A from *Thermomyces lanuginosus*, Kl killer toxin signal, the signal peptide for invertase secretion, the signal sequence of the killer toxin from *Kluyveromyces lactis*, the signal sequence of the killer toxin from *Pichia acaciae*, the signal sequence of the killer toxin from *Hanseniaspora uvarum* and from *Pichia (Hansenula) anomala* or variants thereof as described for example, by Cereghino et al. (Gene 519 (2013):311-317). The preferred signal sequence of the invention is MF-alpha ("mating factor alpha" signal sequence).

According to a particularly preferred embodiment of the present invention, the orthologous FMD promoter and/or the orthologous MOX promoter, originates from a methylotrophic yeast cell selected from the group consisting of the genera *Hansenula* (Ogataea), *Candida*, *Komagataella* and *Pichia*.

According to another preferred embodiment of the present invention, the methylotrophic yeast cell is selected from the group consisting of *Hansenula polymorpha*, *Candida boidinii*, *Pichia methanolica*, *Komagataella pastoris*, *Komagataella phaffii*, *Komagataella pseudopastoris*, *Komagataella ulmi* and *Komagataella* sp. 11-1192.

The orthologous FMD and/or MOX promoter and optionally the nucleic acid molecule operably linked thereto, coding for the heterologous or homologous polypeptide, can be present in the genome, as an extrachromosomal nucleic acid construct on a plasmid with autonomously replicating sequence (ARS) or as a vector/expression cassette integrated into the genome.

The orthologous FMD and/or MOX promoter and optionally the nucleic acid molecule operably linked thereto may be present extrachromosomally or integrated into the genome of the yeast cell according to the invention.

According to a particularly preferred embodiment of the present invention, the orthologous promoter comprises or consists of a nucleic acid sequence SEQ ID No. 1 or SEQ ID No. 2 or a variant thereof.

SEQ ID No. 1 (FMD promoter):

```
AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC
```

SEQ ID No. 2 (MOX promoter):

```
CGACGCGGAGAACGATCTCCTCGAGCTGCTCGCGGATCAGCTTGTGGCCCGTAATGGAACCAG
CGCGACGGCAGCTCCTTGCGGACCACGGTGGCTGGCGAGCCCAGTTTGTGAACGAGGTGCTTT
```

AGAACGTCTCGCGCAAAGTCCAGTGTGATGAATGTCCTCCTCGGACCAATTCAGCATGTTCT
 CGAGCAGCCATCTGTCTTTGGAGTAGAAGCGTAATCTCTGCTCCTCGTTACTGTACCGGAAGAG
 GTAGTTTGCCTCGCCGCCATAATGAACAGGTTCTCTTTCTGGTGGCCTGTGAGCAGCGGGGAC
 GTCTGGACGGCGTCGATGAGGCCCTTGAGGCGCTCGTAGTACTTGTTCGCGTCGCTGTAGCCGG
 CCGCGGTGACGATACCCACATAGAGGTCCTTGGCCATTAGTTTGTGAGGTGGGGCAGGATGGG
 CGACTCGGCATCGAAATTTTTGCCGTCGTCGTACAGTGTGATGTCACCATCGAATGTAATGAGC
 TGCAGCTTGCATCTCGGATGGTTTTTGAATGGAAGAACCGCGACATCTCCAACAGCTGGGCCG
 GTTTGAGAATGAGCCGGACGTCGTTGAACGAGGGGGCCACAAGCCGGCGTTTGTGATGGCGCG
 GCGCTCGTCCTCGATGTAGAAGGCCTTTTCCAGAGGCAGTCTCGTGAAGAAGCTGCCAACGCTC
 GGAACCAGCTGCACGAGCCGAGACAATTCGGGGGTGCCGGCTTTGGTCATTTCAATGTTGTCGT
 CGATGAGGAGTTTCGAGGTCGTGGAAGATTTCCGCGTAGCGGCGTTTGCCTCAGAGTTTACCAT
 GAGGTCGTCCACTGCAGAGATGCCGTTGCTCTTCACCGCGTACAGGACGAACGGCGTGGCCAGC
 AGGCCCTTGATCCATTCTATGAGGCCATCTCGACGGTGTTCCTTGAGTGCGTACTCCACTCTGT
 AGCGACTGGACATCTCGAGACTGGGCTTGCTGTGCTGGATGCACCAATTAATTGTTGCCGCATG
 CATCCTTGACCCGCAAGTTTTTAAAACCCACTCGCTTTAGCCGTCGCGTAAACTTGTGAATCT
 GGCAACTGAGGGGGTTCTGCAGCCGCAACCGAACTTTTCGCTTCGAGGACGCAGCTGGATGGTG
 TCATGTGAGGCTCTGTTTGTGCGTAGCCTACAACGTGACCTTGCTTAACCGGACGGCGCTAC
 CCACTGCTGTCTGTGCCTGCTACCAGAAAATCACCAGAGCAGCAGAGGGCCGATGTGGCAACTG
 GTGGGGTGTGCGACAGGCTGTTTCTCCACAGTGCAAATGCGGGTGAACCGGCCAGAAAGTAAAT
 TCTTATGCTACCGTGCAGTGAAGTCCGACATCCCCAGTTTTTGGCCTACTTGATCACAGATGGGG
 TCAGCGCTGCCGCTAAGTGTACCCAACCGTCCCCACACGGTCCATCTATAAATACTGCTGCCAG
 TGCACGGTGGTGACATCAATCTAAAGTACAAAAACAAA

According to a particularly preferred embodiment of the pre-
 sent invention the variant of SEQ No. 1 comprises or consists of
 SEQ ID No. 27. SEQ ID No. 27 has the following nucleic acid se-
 quence:

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
 GCGGCGAX₁TCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGA
 TCCCACCGCGTTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATA
 GTCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAA
 TATTTTCTTAAACATGCAATCGGCTGCCCCGCX₂ACGGGAAAAGAATGACTTTGGCACTCTTC
 ACCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCA
 GAAAAAX₃AGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGG
 AGTCTACATCAAGCTGTCTCCGATACACTCGACTACCAX₄CCGGGTCTCTCX₅X₆X₇X₈X₉X₁₀X₁₁X₁₂X₁₃X₁₄X₁₅X₁₆X₁₇X₁₈CACX₁₉, wherein

X₁ is adenine or no nucleotide, X₂ is adenine or guanine, X₃ is cytosine or thymine, X₄ is thymine or guanine, X₅ is adenine or cytosine, X₆ is guanine or cytosine, X₇ is adenine or cytosine, X₈ is guanine or cytosine, X₉ is adenine, guanine or cytosine, X₁₀ is guanine or cytosine, X₁₁ is guanine or cytosine, X₁₂ is guanine or cytosine, X₁₃ is guanine or cytosine, X₁₄ is adenine or cytosine, X₁₅ is adenine or cytosine, X₁₆ is thymine or cytosine, X₁₇ is guanine or cytosine, X₁₈ is guanine or cytosine, X₁₉ is a core promoter of an orthologous promoter, preferably of an FMD and/or MOX promoter, particularly preferred a nucleic acid sequence selected from the group consisting of TATAAATAC-CGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC (SEQ ID No. 28), TATA-TAAACTGGTGATAATTCCTTCGTTCTGAGTTCATCTCATACTCAAACCTATATTAAAC-TACAACA (SEQ ID No. 29), TATAAATACAAGACGAGTGCGTCCTTTTCTA-GACTCACCCATAAAACAAATAATCAATAAAT (SEQ ID No. 30), TATAAATACTGCC-TACTTGTCCTCTATTCTTCATCAATCACATC (SEQ ID No. 31), CGATAGGG-CAGAAATATATAAAGTAGGAGGTTGTATACCAAATATACCAACGCAGTACAAGCAACTCTT-GGTTTAAACGGAAGAAACAATTCTTGAACATTTACAACAAAGAAGGTACCGTAACATTAA-TAATCGGAAGGGT (SEQ ID No. 32), GTAATCTTTCGGTCAATTGTGATCTCTCTT-GTAGATATTTAATAGGACGGCCAAGGTAGAAAAAGATACATAACTAGTTAG-CAAACCTTCAATTGCTTAAGTTACAAGTGCAATCCATATCTTAAAGTTATTACATTATTTATA (SEQ ID No. 33) and CCTCCTCTAGGTTTATCTATAAAAGCTGAAGTCGTTA-GAATTTTTTCATTTAAAGCATAATCAAACATCTAGATTCTGAATCGATAAAAAGCAGATA-GAAGTTATTAAGATTATAGGTTACATTCTAGAGTAGTATAGGAAGGTA (SEQ ID No. 34), in particular SEQ ID No. 28, SEQ ID No. 29, SEQ ID No. 30 and SEQ ID No. 31, in particular SEQ ID No. 28. At least one nucleotide within SEQ ID No. 27 is different at the corresponding position of SEQ ID No. 1, thus resulting in a variant of SEQ ID No. 1.

It turned surprisingly out that at least one, preferably at least 2, 3, 4, 5, 6, 7, 8, 9 or 10, point mutations (insertions and/or substitutions) within SEQ ID No. 1 (see SEQ ID No. 27) result in a promoter variant exhibiting superior effects compared to a promoter region consisting of or comprising SEQ ID No. 1. Yeast cells comprising such promoters operably linked to a nucleic acid molecule encoding for a polypeptide show at least the same or even an increased expression rate, at least within

the first 24 hours of culturing, compared to yeast cells carrying a promoter consisting of SEQ ID No. 1. Therefore, it is particularly preferred to modify SEQ ID No. 1 at one or more of the positions indicated in its corresponding nucleic acid sequence SEQ ID No. 27 as X₁ to X₁₈ and X₁ to X₁₉.

Mutations of one or more (2, 3, 4, 5, 6 or 7) of nucleotides X₁, X₃, X₄, X₅, X₉, X₁₆ and X₁₇ of SEQ ID No. 27 resulting in a nucleotide sequence different from SEQ ID No. 1 are preferred since such promoters show also an increased polypeptide and protein expression compared to the use of SEQ ID No. 1 after 48 hours of cultivation under derepressing conditions.

Particularly preferred are mutations of one or more (2, 3, 4 or 5) of nucleotides X₁, X₄, X₉, X₁₆ and X₁₇ of SEQ ID No. 27 resulting in a nucleotide sequence different from SEQ ID No. 1 since such promoters show also an increased polypeptide and protein expression compared to the use of SEQ ID No. 1 after 72 hours of cultivation using methanol, for instance, as carbon source.

As mentioned above X₁₉ of SEQ ID No. 27 can be the core promoter naturally occurring in SEQ ID No. 1 (i.e. TATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC (SEQ ID No. 28)) or an alternative core promoter. Particularly preferred core promoters comprise or consist of SEQ ID No. 29, SEQ ID No. 30 and SEQ ID No. 31. All these core promoters show in combination with SEQ ID No. 1 or SEQ ID No. 27 (the naturally occurring core promoter is substituted with one of these alternative core promoters at position X₁₉ of SEQ ID No. 27) a significantly enhanced polypeptide expression rate compared to the promoter encoded by SEQ ID No. 1 under derepressing conditions.

Particularly preferred variants of SEQ ID No. 1 are selected from the group consisting of the following nucleic acid sequences:

SEQ ID NO. 35 (v1; see example 2):

```
AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGAATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGA
TCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGGACATCCATCAAATATACCAGGCGC
```

CAACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATA
GTCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAA
TATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTC
ACCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCA
GAAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGG
AGTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGG
CACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 36 (v2):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCGACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCA
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 37 (v3):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCA
AAAAATAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 38 (v4):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT

CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGGGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 39 (v5) :

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCAGCCGGGTCTCTCAGAGAGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 40 (v6) :

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCCGAGAGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 41 (v7) :

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 42 (v8) :

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGCGAGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 43 (v9) :

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGACAGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 44 (v10) :

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCCCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGCGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 45 (v11):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCCCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGACGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 46 (v12):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCCCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGCGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 47 (v13):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGCGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 48 (v14):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGCAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 49 (v15):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGCATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 50 (v16):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGACTGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 51 (v17):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAACGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 52 (v18):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA

GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATCGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 53 (v19):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGCC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

SEQ ID NO. 54 (v20):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACTATATAAACTGGTGATAATTCCTTCGTTCTGAGTTCCATCTCATACTCAAACCTATATTAAAA
CTACAACA

SEQ ID NO. 55 (v21):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA

CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACTATAAATAACAAGACGAGTGCGTCCTTTTCTAGACTCACCCATAAACAAATAATCAATAAAT

SEQ ID NO. 56 (v22):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACTATAAATACTGCCTACTTGTCTCTATTCTTCATCAATCACATC

Variants of the FMD promoter consisting of or comprising
SEQ ID No. 1 showing a reduced expression rate under
derepression comprise or consist of the following sequences:

SEQ ID NO. 57 (v23):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACCGATAGGGCAGAAATATATAAAGTAGGAGGTTGTATACCAAATATACCAACGCAGTACAAGC
AACTCTTGGTTTAAACGGAAGAAACAATTCTTCAACATTTACAACAAAGAAGGTACCGTAACA
TTAATAATCGGAAGGGT

SEQ ID NO. 58 (v24):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACGTAATCTTTGGTCAATTGTGATCTCTCTTGTAGATATTTAATAGGACGGCCAAGGTAGAAA
AAGATACATAACTAGTTAGCAAACCTTCAATTGCTTAAGTTACAAGTGCAATCCATATCTTAAAG
TTATTACATTATTTATA

SEQ ID NO. 59 (v25):

AATGTATCTAAACGCAAACCTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCGACATCCATCAAATATAACCAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACCCTCTCTAGGTTTATCTATAAAAGCTGAAGTCGTTAGAATTTTTCATTTAAAGCATAATCA
AACATCTAGATTGCAATCGATAAAAAGCAGATAGAAGTTATTAAGATTATAGGTTACATTCTAG
AGTAGTATAGGAAGGTA

According to a further preferred embodiment of the present invention the variant of SEQ ID No.1 is selected from the group consisting of SEQ ID No. 35, SEQ ID No. 37, SEQ ID No. 39, SEQ ID No. 40, SEQ ID No. 44, SEQ ID No. 51, SEQ ID No. 52, SEQ ID No. 54, SEQ ID No. 55 and SEQ ID No. 56.

Another aspect of the present invention relates to a method for producing a heterologous polypeptide, comprising the step of culturing a yeast cell according to the present invention.

The yeast cell according to the invention, comprising an orthologous FMD and/or MOX promoter, is suitable in particular for overexpression of homologous or heterologous polypeptides. Because of the excellent properties, it is possible with the yeast cell according to the invention to express a polypeptide and/or protein under derepressing conditions as well as under methanol-induced conditions or suitable alternative inducing conditions and optionally to secrete it from the cell.

According to a preferred embodiment of the present invention, during cultivation, the expression of the heterologous polypeptide is induced under derepressing conditions or its expression rate is increased.

Promoter derepression can be achieved by a reduced feeding rate with a repressing carbon source (C source: e.g., glucose, glycerol) or by using a non-repressing C source (e.g., sorbitol). The repressing C source can achieve its properties through direct repression or through repressing properties of metabolites of the C source. The feed rate with repressing C sources can approach zero in the extreme case. Additional induction effects due to other compounds such as fatty acids, formaldehyde or formic acid are also possible.

To increase protein yield during cultivation and/or during its expression, methanol is preferably added during the culturing under derepressing conditions.

Those skilled in the art are sufficiently familiar with the general cultivation conditions, such as temperature, medium, etc. (see for example, Krainer FW et al. *Microbial Cell Factories* 11 (2012):22).

The present invention will be defined in greater detail on the basis of the following figures and examples but without being limited to them.

Fig. 1 shows the fluorescence intensities of a green fluorescent reporter protein (an improved variant of the green fluorescent protein (GFP)) in culturing yeast cells of the *Komagataella* genus in which a nucleic acid coding for the green fluorescent protein is operably linked to orthologous and endogenous promoters. The orthologous promoters (and endogenous promoters from *P. pastoris* as reference) were operably linked to the GFP

reporter gene and transformed as vectors in *P. pastoris*. The strains were cultured for 60 hours on minimal medium (BMD1) in microtiter plates with 96 deep wells (deep well plate (DWP)) and then induced with methanol. The fluorescence of the reporter protein and OD 600 (as a measure of biomass) was measured under glucose-repressed conditions (16 h), derepressed conditions (60 h) and measured at various points in time after methanol induction. The fluorescence measurements were normalized with respect to the OD 600 values. Averages and standard deviations of four transformants each are shown in the figure.

Fig. 2 shows the curve of measurements of protein expression over time. Selected strains from Fig. 1 were cultured in shaking flasks. The protein fluorescence (Fig. 2A; ratio RFU/OD600; RFU = relative fluorescence unit), while the OD600 (Fig. 2B) and the amount of glucose (Fig. 2C) were measured over time. The glucose concentration at the start of the measurements was 55.5 mM (10 g/L). The averages (MV) and standard deviations of three transformants each are shown.

Figs. 3A to 3C show that the orthologous HpFMD promoter is also capable of upregulating the expression of other reporter proteins such as horseradish per oxidase (HRP) (Fig. 3A), lipase B from *Candida antarctica* (CalB) (Fig. 3B) and a hydroxynitrile lyase from *manihot esculenta* (MeHNL) (Fig. 3C). The strains were cultured in DWPs in minimal medium to the point of glucose depletion after 60 h and then additionally induced with methanol. HRP and CalB enzyme activities were measured in the culture supernatant. The activity of MeHNL was measured using digested cells. Averages and standard deviations of four transformants each are shown.

Fig. 4 shows reporter protein fluorescence of the HpFMD promoter (P_FMD) and the AOX1 promoter (P_AOX1) wild type sequence promoters tested. The strain background is the *P. pastoris* Bgl1 KU70. Cultivation was done in deep well plate (DWP). Reporter protein fluorescence and OD600 were measured under glucose derepressed (24 and 48 h) and two different time points of methanol induction (72 and 96 h). The strain harboring the FMD promoter was used as reference strains for testing various promoter variants.

EXAMPLES:**Example 1:****Materials and methods****Cloning the promoters**

The orthologous promoters were amplified by means of PCR and cloned before a GFP reporter gene. To do so, the reporter plasmid pPpT4mutZeoMlyI-intARG4-eGFP-BmrIstuffer (T. Vogl et al. ACS Synth Biol. 2015, DOI: 10.1021/acssynbio.5b00199; published on 22 November 2015).

This plasmid is based on the pPpT4 vector, which was described by L. Näättsaari et al. (PLoS One 7 (2012): e39720). The promoters were cloned seamlessly (i.e., without any restriction enzyme cleavage sites or linker sequences between the promoter and the start codon) to obtain the natural context. Primers were designed on the basis of literature references (HpFMD promoters (H. Song et al. Biotechnol Lett 25 (2003):1999-2006; A.M. Ledebøer et al. Nucleic Acids Res 13 (1985):3063-3082), CbAOD1 promoter (H. Yurimoto et al. Biochim Biophys Acta 1493 (2000):56-63), CbFLD1 promoter (B. Lee et al. Microbiology 148 (2000): 2697-704), Pm MOD1 and MOD2 promoters (C.K. Raymond et al. Yeast 14 (1998):11-23; T. Nakagawa et al. J Biosci Bioeng 91 (2001):225-7; T. Nakagawa et al. Yeast 23 (2006):15-22). The primer sequences used are given in Table A:

Table A: Primers for amplification of the orthologous promoters

Name	Sequence	SEQ ID No.
HpFMDfwd	AATGTATCTAAACGCAAACCTCCGAGCTG	3
HpFMDrev	GATTTGATTGATGAAGGCAGAGAGCGCAAG	4
HpMOXfwd	TCGACGCGGAGAACGATCTCCTCGAGCT	5
HpMOXrev	TTTGTTTTTGTACTTTAGATTGATGTCACCACCGTGCACTGG CAG	6
PmMOD1fwd	CGAGATGGTACATACTTAAAAGCTGCCATATTGAG	7
PmMOD1rev	TTTGAGAAATTAATAGTAAGATTTTTTTTTTCGTAAAAGTTTT GATTGAGTTAATTC	8
PmMOD2fwd	GGATCCACTACAGTTTACCAATTGATTACGCCAATAG	9
PmMOD2rev	TTTGAATTTTAGTTTTAGATAGATAAATATAATTTTCAATCC TGTTATAAAATAGTATAT	10

The alternative reporter vectors, containing HRP (isoenzyme A2A; L. Näätsaari et al. BMC Genomics 15 (2014):227), CalB and MeHNL downstream from the corresponding promoters, were taken from US 2015/0011407 or created by installing the eGFP reporter gene that had been cut from the above-mentioned eGFP vectors (restriction enzymes NheI and NotI) and the PCR products of HRP, CalB and MeHNL were installed seamlessly by recombinant cloning. The primers indicated in Table B were used for the PCR amplifications.

Primer	Sequence	SEQ ID No.
pHpFMD-MFalpha-Gib	cttgcgctctctgccttcacatcaatcaatcatg agattcccatctattttcaccgctgtc	15
AOX1TT-NotI-CalB	caaatggcattctgacatcctcttgagcggccg cttatggggtcacgataaccggaacaag	16
AOX1TT-NotI-HRPA2A	caaatggcattctgacatcctcttgagcggccg cttaggatccgttaactttcttgcaatcaagtc	17
seq-pHpHMD- 149..126fwd	actggtgtccgccaataagaggag	18
pHpFMD-MeHNL	cttgcgctctctgccttcacatcaatcaatcatg	19

	gttactgctcacttcgtcttgattcac	
AOX1TT-NotI-MeHNL	caaatggcattctgacatcctcttgagcggccg cttaagcgtaagcgtcggcaacttcctg	20
pCAT1-MeHNL-Gib	cacttgctctagtcaagacttacaattaaaatg gttactgctcacttcgtcttgattcac	21

The HRP and CalB vectors mentioned in the literature where therefore used as PCR templates (US 2015/0011407 and T. Vogl et al. (ACS Synth Biol. 2015, DOI:10.1021/acssynbio.5b00199; published on 22 November 2015). The MeHNL sequence was optimized for the *P. pastoris* codon and designed as a synthetic double-stranded DNA fragment with overhangs to the AOX1 promoter and terminator (see Table B). This fragment was used as a template for PCRs. The following sequence was used:

cgacaacttgagaagatcaaaaaacaactaattattgaaagaattccgaaacg**ATG**GTTACTGC
TCACTTCGTCTTGATTCACACTATCTGTTCATGGTGCTTGGATCTGGCACAAGTTGAAGCCAGCA
TTGGAGAGAGCTGGACATAAGGTTACCGCTCTTGATATGGCTGCATCTGGTATTGATCCTCGTC
AAATCGAACAAATCAATTCATTCGACGAGTACTCAGAGCCACTGCTGACCTTCTTGGAAAAGTT
GCCTCAAGGTGAAAAGGTGATCATCGTTGGTGAATCCTGTGCTGGATTGAACATTGCCATTGCA
GCTGATAGATATGTCGATAAGATCGCTGCTGGTGTCTTCCACAACTCTCTGTTACCAGATACTG
TTCCTCTCCATCTTACACTGTCGAGAAGTTGTTAGAATCATTTCCAGATTGGAGAGATACTGA
ATACTTTACTTTTCACTAACATCACTGGAGAGACTATCACCACCATGAACTTGGATTGCTTTTG
TTGAGAGAAAACCTTTTACCAAGTGTACTGATGGTGAATACGAATTGGCCAAGATGGTTATGA
GAAAGGGTTCTTTGTTTTCAGAATGTTCTTGACAAAAGACCAAAGTTACCGGAAAAGGGTTACGG
TTCTATCAAGAAGGTCTACATCTGGACTGATCAGGACAAGATCTTCCTGCCAGACTTCCAAAGA
TGGCAAATCGCAAACTACAAACCAGATAAGGTCTACCAAGTCCAAGGTGGTGATCACAAGTTAC
AATTGACCAAGACCGAAGAGGTCGCTCACATCTTGACAGGAAGTTGCCGACGCTTACGCT**TAA**gc
ggccgctcaagaggatgtcagaatgccatttgctg (SEQ ID No. 22)

The protein coding sequence here is large and the start and stop codon is shown in bold font, while overhangs to the vector for recombinant cloning are written in lower case letters, EcoRI and NotI, which are cleavage sites typically used for cloning in the pPpT4 vector family, are underlined.

The same forward primer (pHpFMD-MFalpha-Gib) was also used for PCR amplification of the HRP and CalB genes because the two genes are fused to an MFalpha signal sequence. Genes cloned in

the vectors were sequenced by using primers that bind to the AOX1 terminator and the respective promoters (seq-pHpHMD-149..126fwd for the HpFMD promoter).

Strains, materials, fluorescence measurements and enzyme assays

Enzymatic HRP and CalB activity were determined with the substrates 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) and p-nitrophenyl butyrate (p-NPB) according to protocols in Krainer FW (Microb Cell Fact 11 (2012):22).

For the transformations of all promoter comparisons with GFP, the CBS7435 wild type strain was used. HRP and CalB plasmids were transformed into the mutS strain (L. Näätsaari et al. (PLoS One (2012); 7:e39720) because it has a higher protein expression (F.W. Krainer et al. Microb Cell Fact 11 (2012):22). For MeHNL activity measurements, the cells were lysed by Y-PER digestion according to the manufacturer's instructions (Thermo Fisher Scientific, Y-PER™ Yeast Protein Extraction Reagent) and the activity was measured using a "mandelonitrile cyanogenase assay," as described by R. Wiedner et al. Comput Struct Biotechnol J10 (2014):58-62) (final mandelonitrile concentration 15mM).

Results

Six heterologous promoters of HpFMD, HpMOX, CbFLD1, CbAOD1, PmMOD1 and PmMOD2 genes were tested in *P. pastoris*. The promoters were compared with the methanol-inducible AOX1 promoter, the constitutional GAP promoter and the derepressed/methanol-inducible CAT1 promoter in *P. pastoris*, namely the orthologous promoters were amplified by genomic DNA PCR and cloned in vectors with GFP as reporter gene. The following promoter sequences were used:

HpFMD (SEQ ID No. 1):

```
AATGTATCTAAACGCAAACTCCGAGCTGGAAAAATGTTACCGGCGATGCGCGGACAATTTAGAG
GCGGCGATCAAGAAACACCTGCTGGGCGAGCAGTCTGGAGCACAGTCTTCGATGGGCCCGAGAT
CCCACCGCGTTCTGGGTACCGGGACGTGAGGCAGCGCACATCCATCAAATATACAGGCGCC
AACCGAGTCTCTCGGAAAACAGCTTCTGGATATCTTCGCTGGCGGCGCAACGACGAATAATAG
TCCCTGGAGGTGACGGAATATATATGTGTGGAGGGTAAATCTGACAGGGTGTAGCAAAGGTAAT
ATTTTCCTAAAACATGCAATCGGCTGCCCCGCAACGGGAAAAAGAATGACTTTGGCACTCTTCA
```

CCAGAGTGGGGTGTCCCGCTCGTGTGTGCAAATAGGCTCCCACTGGTCACCCCGGATTTTGCAG
AAAAACAGCAAGTTCCGGGGTGTCTCACTGGTGTCCGCCAATAAGAGGAGCCGGCAGGCACGGA
GTCTACATCAAGCTGTCTCCGATACACTCGACTACCATCCGGGTCTCTCAGAGAGGGGAATGGC
ACTATAAATACCGCCTCCTTGCGCTCTCTGCCTTCATCAATCAAATC

HpMOX (SEQ ID No. 2):

CGACGCGGAGAACGATCTCCTCGAGCTGCTCGCGGATCAGCTTGTGGCCCGGTAATGGAACCAG
GCCGACGGCAGCTCCTTGCGGACCACGGTGGCTGGCGAGCCCAGTTTGTGAACGAGGTCGTTT
AGAACGTCTCGCAAAGTCCAGTGTGAGATGAATGTCTCCTCGGACCAATTCAGCATGTTCT
CGAGCAGCCATCTGTCTTTGGAGTAGAAGCGTAATCTCTGCTCCTCGTTACTGTACCGGAAGAG
GTAGTTTGCCTCGCCGCCATAATGAACAGGTTCTCTTTCTGGTGGCCTGTGAGCAGCGGGGAC
GTCTGGACGGCGTCGATGAGGCCCTTGAGGCGCTCGTAGTACTTGTTGCGCTCGCTGTAGCCGG
CCGCGGTGACGATACCCACATAGAGGTCCTTGGCCATTAGTTTGTAGAGGTGGGGCAGGATGGG
CGACTCGGCATCGAAATTTTTGCCGTCGTCGTACAGTGTGATGTCACCATCGAATGTAATGAGC
TGCAGCTTGCATCTCGGATGGTTTTTGAATGGAAGAACCGCGACATCTCCAACAGCTGGGCCG
TGTTGAGAATGAGCCGGACGTCGTTGAACGAGGGGGCCACAAGCCGGCGTTTGTGATGGCGCG
GCGCTCGTCCTCGATGTAGAAGGCCTTTTCCAGAGGCAGTCTCGTGAAGAAGCTGCCAACGCTC
GGAACCAGCTGCACGAGCCGAGACAATTCGGGGGTGCCGGCTTTGGTCATTTCAATGTTGTCGT
CGATGAGGAGTTTCAGGTCGTGGAAGATTTCCGCGTAGCGGCGTTTTGCCTCAGAGTTTACCAT
GAGGTCGTCCACTGCAGAGATGCCGTTGCTCTTCACCGCGTACAGGACGAACGGCGTGGCCAGC
AGGCCCTTGATCCATTCTATGAGGCCATCTCGACGGTGTTTCTTGAGTGCGTACTCCACTCTGT
AGCGACTGGACATCTCGAGACTGGGCTTGCTGTGCTGGATGCACCAATTAATTGTTGCCGCATG
CATCCTTGACCCGCAAGTTTTTAAACCCACTCGCTTTAGCCGTCGCGTAAACTTGTGAATCT
GGCAACTGAGGGGGTTCTGCAGCCGCAACCGAACTTTTCGCTTCGAGGACGCAGCTGGATGGTG
TCATGTGAGGCTCTGTTTGTGCGTAGCCTACAACGTGACCTTGCCTAACCGGACGGCGCTAC
CCACTGCTGTCTGTGCCTGCTACCAGAAAAATCACCAGAGCAGCAGAGGGCCGATGTGGCAACTG
GTGGGGTGTGCGACAGGCTGTTTCTCCACAGTGCAAATGCGGGTGAACCGGCCAGAAAGTAAAT
TCTTATGCTACCGTGCAGTGAATCCGACATCCCCAGTTTTTGCCTACTTGATCACAGATGGGG
TCAGCGCTGCCGCTAAGTGTACCCAACCGTCCCCACACGGTCCATCTATAAATACTGCTGCCAG
TGCACGGTGGTGACATCAATCTAAAGTACAAAAACAAA

CbFLD1 (SEQ ID No. 23):

GGATCCCTTCAACAGCGGAGTCTCAAGCAGTGGCTATTATCAGTGTATTTAATTACTGATGCAT
TGTATTATAGTGCATACATAGTTAATAATTACTCTCTGTTATCATTGAAAATTTTGAAATTCTC
ACTCTCACGCAGTGCAAACTTTGCCATAATTGAGTAAGTGAACGCAATATTTAGGCTACATAT
TTTGGATTCCCTTAAGTATGTAATCAAAGATCATTCATACTGCCATCTTATAATATTGGAGTAT

TATTATGTTGCTATACTGTTCTACCTGTTTATTCTATTGTATGCGTCTAAATCTTTCCATCAGT
TTCTATACTATCTTTTCGTTTGCAATGAAATATTACTCCAATTTCGCTTGTTTCAACTCGCTTGCC
TTCTCTCTTGCCCTCTTTTTTTCTTTTCATTTTATCGTTGTTTAAACGGTATATAAATATGTAA
CGTTGTCGCTTAGTTTTGAGAAATCACTTTTGTTGCTCTCAATTCTGTTTTGACATCTTAAGGT
TAGTCAATTGATTGAATCAACTACACTAAATCATATTTATCTATTTTTTTATTCCACAAAA

CbAOD1 (SEQ ID No. 24):

GGAGTATACGTAAATATATAATTATATATAATCATATATATGAATACAATGCAATGAAAGTGAA
TATGATAAGATTGAAATAATAACAAACAGCGATAAATATATCTCAAAATGGAGTTACACAACAA
ATAATAATAAAATATAAATTATAAATTATAAATTATAAAAGAATAAAAAATAAACCCCACTAAT
TTATTTTATTAAAAGATAGATTGGTATCTTTACTTAATAACAATTCTGAACTTTATTCACTTA
ATTTTATTTAACTTATTTAATTTATTTTTACCCAGTTTTTTCAGTACAATGCAGCTCCGAAACT
TTATTTGGCTGTGATTTGGCTGTGATTTGGCTGTGATTTGGCTTGCTGGCTGGCTGGAATTG
TCTCCTGCAGGAATTGCTCGGGGTCCGGTTCTCCCGCTGGCTGGCTATTTGGCGGGCTGGCTAT
TTGGCGGGCTGGCTGGCTGGCTGCTCTGCCATCTGCTGTGGCCACCCCGCATCTCTGGATGCAC
GCCGTGCAGCTGGACGTGCGTCTACCCTGCAGCCGTGTGCCTTATTTCCCAATCTCCCAATCTC
TCAATCTGCCAGTCAGCCAAAACACCGGCCAGGCAGGCAGGCAGGCAGGCAGGCAGGCAGTGAA
GCCTTCCCACGCCCCACTCCGCATAAACATCCCCAGCAGTTTCCCCAGCAGTTTCCCCAGCTTT
TCAATTTAATAAAAATAGCCTGTTTCTGTTTCTGTTTTATATTATACAATTTTTTTATCCTAATAA
TTACTCTTTTCGGGAATTAAATAATAATTATATCATATACCCATATCACATTTTACTATATTTAC
TATCTATAAATAAATTCATATTATAATATTAATTTATATTGCTTAATTAAAATGCTCTTTTCC
ATCATCATCATCATCATCATCACGAGTTTTTCGGTTATCAATACTCTTTTCATTAATTTCTA
GAATTTCAATTATTTATTTTTTATTGACTGGAAATTTTCAATCAATTTTATTTATTTTATTTAT
TTATTTTCATATTCTTAGATTTAACTTTTTAGATGACCGCTATTTTACTTACTTACTTACTGT
TGTTTTATATTATGATAAGAATTAATTTTCATATTTATGATGATGATGATGTAAATTTAACCTA
GTATACTATTTTAAAGTTATCACTATCTTTTAGTGCTGGCATTTTTTATTCTATTTTCATATAT
GTATATACGTAAATTAAGTATCATCACGCTGCTTACTGTACGTTTAAAATGTGGAGATGGAAAT
AGAGATGGGGATGAAGATGAAGATGATGAGAATTATAAACCATTTCATTCAATCAATCAATA
TAACCTATAAAAAAATTTATATTTAAATGAATTAATTTCTTTATTTTAATAATATCGTTAATT
CTTTTAAATTCTATTTTATTTTAATTCTTTCTTTATCATAGTTATCATATAACAATTATATAAC
ATAGATACACAATTATTATTTTATTATCATATTATTTTTTAAAATATTGATTATTTTTTAAAAA
ATATCTTAATTAATTAATTTTTTACGAATATACAAATTTTAACTTACTTTTTTTAACGAATT
TTAACGAACTTTTAAAAAAACAAAAAATAAATAAATTTATTTTCAATA

PmMOD1 (SEQ ID No. 25):

CGAGATGGTACATACTTAAAAGCTGCCATATTGAGGAACTTCAAAGTTTTATCTGTTTTTAGAA
TAAAAGACGATTGTTGTAACAAAACGTTGTGCCTACATAAACTCAAATTAATGGAAATAGCCT
GTTTTGAAAAATACACCTTCTTAAGTACTGACAAAGTTTTGTAAATGACTATCGAACAAGCCA
TGAAATAGCACATTTCTGCCAGTCACTTTTAACACTTTCCTGCTTGCTGGTTGACTCTCCTCAT
ACAAACACCCAAAAGGGAACTTTAGTGTGGGGACACTTGACATCTCACATGCACCCAGATT
AATTTCCCCAGACGATGCGGAGACAAGACAAAACAACCTTTGTCCTGCTCTTTTCTTTCTCAC
ACCGCGTGGGTGTGTGCGCAGGCAGGCAGGCAGGCAGCGGGCTGCCTGCCATCTCTAATCGCTG
CTCCTCCCCCTGGCTTCAAATAACAGCCTGCTGCTATCTGTGACCAGATTGGGACACCCCCCT
CCCCTCCGAATGATCCATCACCTTTTGTGCTACTCCGACAATGATCCTTCCCTGTCATCTTCTG
GCAATCAGCTCCTTCAATAATTAAATCAAATAAGCATAAATAGTAAAATCGCATACAAACGTCA
TGAAAAGTTTTATCTCTATGGCCAACGGATAGTCTATCTGCTTAATTCATCCACTTTGGGAAC
CGTTCTCTCTTTACCCCAGATTCTCAAAGCTAATATCTGCCCCTTGTCTATTGTCTTTCTCCG
TGTAACAAGCGGAGCTTTTGCCTCCCATCCTCTTGCTTTGTTTCGGTTATTTTTTTTTCTTTTGA
AACTCTTGGTCAAATCAAATCAAACAAAACCAAACCTTCTATTCCATCAGATCAACCTTGTTCA
ACATTCTATAAATCGATATAAATATAACCTTATCCCTCCCTTGTTTTTTACCAATTAATCAATC
TTCAAATTTCAAATATTTTCTACTTGCTTTATTACTCAGTATTAACATTTGTTTAAACCAACTA
TAACTTTTAACTGGCTTTAGAAGTTTTATTTAACATCAGTTTCAATTTACATCTTTATTTATTA
ACGAAATCTTTACGAATTAACCTCAATCAAACTTTTACGAAAAAAAATCTTACTATTAATTTCT
TCAA

PmMOD2 (SEQ ID No. 26):

GGATCCACTACAGTTTACCAATTGATTACGCCAATGTGTTTATTTACCAAGTAATTACAAAAC
TGAGATTTGGTTATGTCATTATGTATTTTCGGCAATGGCTGTAATTTAACTGGATTAGGGTTA
ATTAACGTTTAGCCTACGAAAGCGGCTAGCTTTTATTTCTGCTTTTGTTTTGAGCCCGTTTCTA
ATTCCAATCTTTGCAATTTGTTCCATCTTTTAAAATTAAGTGCTCTTTTCTAATCTGATAAAG
ATAAGCCATCGTAGAGTAAGTAAAACAAAATAATGTACTGTATATTAAGCGGAAAACTTGGA
AAGTCGTATGATGTTGAAGGAGCAAAGAATGACTAATATTAGGAGATTTAAGCAAACAATGTTG
AGGGGAACAGGACGATTAACCCCTTATAGAGGAAGCGTCTTTGATGTTGGAAGGGGGAGGGGTC
AAAAGCACTGAGCAGTGCTAATTAGTAACCAATTTCTGTAAGCAATGAACTTGTTGCTATTGG
AAATACTATTAAGTAATACAAGGTACAGACTAATGGGGGTGAGCCGGTAGTTCAGGCTATCTTA
TAGACAGACTATTCCGGATTGTCTAATCATTGGTGCACCTGGTTAATAATTATCAGTCAACTCT
TTTACGGTGCTGATAGGTCTTTGCGAACTTGCCCTTGTTGAATTTGGTTGTTAATCAAACGTGT
CTGTATTTTCATGTCATACTACTATTGATATTATTAATGTTACTTACTCATCTGGCCATTTAACA
GGTTTGAAGCTTTAATGCTCTTAACCTAACAGCAATCCATCACCGTCAACCTTAACCCCCCTGGT
GCTTGCTGTCTTTATCCTTCGTATCTTTTTCATGTTGCACCGCCCTGTTCTTATACGGTTGTT
CCCCCATAGGCTAACTTCTCTGTTTCCGACCATCTCTGCAATAACAAAGAATTCTATACGCTTA
CACTATAATCATACAATGACTCTACATGCCATTTTCACTTTACTTACTTGCCATCGGAAGATAC

TGAATCAGAAAGCCATAGTAACTACATAACTTCAAAACACACCCTTTTTACAGATTAGTTACAA
 TTTTGTCAATGTTTGTGTTGATAACCCAAGGTGGAACGTTTCCAGTTAGACCTGTTTAATCCAAC
 TCACTTTACCACCCCAAACTTTCTACCGTTAGACAAATACTGGCTAAATCTGACGAAAACAA
 CCAATCAACAATTGAATCCACTGGGAGGTATCTCTAATCCACTGACAACTTTGCTAAAACAAG
 AAAAAGTGGGGCCTCCGTTGCGGAGAAGACGTGCGCAGGCTTAAAAACACAAGAGAACACTTG
 GAAGTACCCCAGATTTTTAGCTTCCTACTATTCTGACACCCCTATTCAAGCACGACGGTGATT
 GATTCAATTCAATTTTGCTGCTCCAATGATAGGATAAACCCCTTTTGGACTTCAATCAGACCTCTG
 TCCTCCATAGCAATATAAATACCTTCTAGTTGCCCACTTCCTCTCTCCTGTACTGCCCCAATG
 AGTGACTTATTCAAGTTACTTTCTCTCTTTTCCTAACAATTAAACAAGAAGCTTTATTATAACA
 TTAATATACTATTTTATAACAGGATTGAAATTATTTATCTATCTAAAACATAAATTCAAA

P. pastoris transformants containing plasmids with CbAOD1, PmMOD1 and PmMOD2 promoters did not have any reporter protein fluorescence (Fig. 1). The CbFLD1 promoter exhibited repression on glucose and weak induction by methanol by approximately 10% of the PpAOX1 promoter. Both tested *H. polymorpha* promoters surprisingly retained their natural regulation profile from *H. polymorpha* and also in *Pichia pastoris* repression, derepression and methanol induction (Figs. 1 and 2). The HpFMD promoter surprisingly exceeded the constitutional PpGAP promoter under derepressed conditions and also achieved approximately 75% of the methanol-induced PpAOX1 promoter, even without feeding with additional carbon sources. The derepressed expression of the HpFMD promoter exceeded that of the reporter protein fluorescence of the strongest endogenous MUT promoter from *P. pastoris* (PpCAT1) by a factor of approximately 3.5. After methanol induction, the HpFMD promoter exceeded the PpAOX1 promoter by a factor of approximately 2. These results on a small scale (Fig. 1) have been confirmed by experiments in shaking flasks (Fig. 2), wherein glucose measurements also show clearly the derepressed regulation profile. A further increase in the technical advantages of the HpFMD promoter can be achieved by an optimized feeding rate in the bioreactor.

To investigate whether the unexpectedly strong expression of the HpFMD reporter can also be reproduced for other proteins in addition to GFP, the HpFMD promoter was cloned upstream from the coding sequences of other proteins: the secreted proteins horseradish peroxidase (HRP) and *Candida antarctica* lipase B (CalB)

and the intracellular hydroxynitrile lyase from manihot esculenta (cassava, MeHNL) (Figs. 3A to 3C).

With respect to the final yields of active protein in the culture supernatant in the shaking flask experiment, the derepressed expression of all proteins by the HpFMD promoter was equal to the constitutional expression by the GAP promoter and clearly exceeded the derepressed expression by the CAT1 promoter. Methanol-induced enzyme activities of the HpFMD promoter exceeded the AOX1 promoter activity by a factor of 2.5.

The strong expression the HpFMD promoter could also be observed with four different secreted reporter proteins as well as intracellular reporter proteins (eGFP, HRP, CalB, MeHNL). The orthologous HpFMD promoter even exceeded endogenous promoters in *P. pastoris*.

The orthologous promoters interestingly have very low or no sequence identities with promoters in *Pichia*. A BLAST search of the HpFMD promoter did not yield any significant hits in the *Pichia pastoris* genome; a direct alignment of the HpFMD promoter with the PpFDH1 promoter also did not yield any significant similarities (BLASTN 2.2.32+, Blast 2 sequences, setting for "somewhat similar sequences (blastn)"; molecule type: nucleic acid).

Such low sequence identity is a desirable property of promoters because these foreign sequences cannot recombine with the identical sequences in the genome of *Pichia* and therefore cannot be lost, for example, due to homologous recombination events with similar sequences already present in the genome.

Orthologous promoters may surprisingly be highly useful tools for protein expression, as demonstrated by the higher activities by a factor of as much as 2.5 due to the HpFMD promoter. Unexpectedly, the HpFMD promoter also retained its derepressed regulation profile from *H. polymorpha* in *P. pastoris* and thus constitutes the strongest derepressed promoter in *P. pastoris*. Therefore, efficient production processes free of toxic and highly inflammatory methanol can be made possible.

Example 2: FMD promoter variants

1. Cloning of promoters

The pPpT4mutZeoMlyI-intArg4-EGFP-P_FMD, containing the FMD promoter having SEQ ID No. 1 served as template for PCR amplifi-

cation of the promoter variants v01 to v22. Primers were designed in a way to introduce point mutations, insertions or different core promoters to the FMD promoter sequence. The promoter variants were amplified in two parts and then assembled with the backbone of the pPpT4mutZeoMlyI-intArg4-eGFP-P_FMD vector, which had been previously cut with the restriction endonuclease *SalI*. For the generation of the promoter variants v23 to v25 only one part was PCR amplified and the other part was ordered as synthetic DNA. In this case the two DNA fragments were assembled with the backbone of the pPpT4mutZeoMlyI-intArg4-eGFP-P_FMD vector, which had been previously cut with the restriction endonuclease *NheI*. For the assembly of the DNA fragments with the vector backbone assembly cloning based on sequence homology was used, resulting in a seamless transition from promoter to the reporter gene eGFP.

2. *P. pastoris* transformations and screening

For transformations of the vectors harboring the different promoter variants v01 to v25 into yeast the *P. pastoris* Bgl1 Δ KU70 strain was used. Compared to the wild type strain, this strain has two gene knock outs: First, the KU70 gene, which encodes for a protein involved in the non-homologous end joining machinery. By knocking out this gene, homologous recombination events are more likely to happen in *P. pastoris*. This facilitates targeting of the vectors into a defined locus, in this case the *ARG4* locus to avoid unexpected effects by different integration loci in the genome. The second knocked out gene is the *AOX1* gene (mutS/Bgl1 strain). By using this knock out strain higher yields of heterologous expressed proteins under the control of a methanol inducible promoter can be achieved (Krainer FW et al. Microb. Cell Fact. 11(2012)p. 22).

P. pastoris Bgl1 Δ KU70 was transformed with *BglIII* linearized plasmids according to the condensed protocol of Lin-Cereghino et al. (Biotechniques 38(2005):44-48). To have reference strains for the screening the same vector as for the promoter variants - but with the non modified FMD promoter of SEQ ID NR1 and the *AOX1* promoter instead - were transformed as well. About 500 ng, which is relatively low amounts of DNA were transformed to avoid multi copy integrations. For example, using 1 μ g of a linearized

pPpT4_S vector typically only yields single copy transformants (Vogl T et al. ACS Synth. Biol. 3(2014):188-191).

For 9 constructs 42 transformants were screened to show the uniformity of the expression landscapes. Since the landscape for all of those tested constructs proved to be uniform, only 16 transformants per construct were picked and cultivated on two different deep well plates (DWP) in the second screening round. DWP cultivations were adapted from the protocol reported by Weis et al. (Weis R et al. FEMS Yeast Res. 5 (2004):179-89). Single colonies were picked and used to inoculate BMD (250 µl) into 96 well DWPs and cultivated for 48 h. Then BMM2 (250 µl) was added to induce the cells for the first the time. The cells were induced another 3 times with BMM10 (50 µl) after 60, 72 and 84 hours of cultivation in the DWP. Samples were taken and measured after 48, 72 and 96 hours. Samples were taken as followed: 10 µl cell culture was mixed with 190 µl of deionized water in micro titer plates (Nunc MicroWell 96-Well Optical-Bottom Plates with Polymer Base, Black; Thermo Fisher Scientific). eGFP fluorescence measurements were performed using a FLUOstar® Omega plate reader (BMG LABTECH GmbH, Ortenberg, Germany). Fluorescence was measured at 488/507 nm (excitation/emission) and for data evaluation the resulting relative fluorescence units (RFU) were normalized to the OD600.

Table C: Primers and synthetic DNA for generation of FMD promoter variants

Name	Sequence	SEQ ID No.
intARG_fwd	GCCAATTCTCAATTTGCTAGAGACTCTG	60
P_FMD-v01_fwd	agaggcggcgAatcaagaaacacc	61
P_FMD-v01_rev	ggtgtttcttgatTcgccgcctct	62
P_FMD-v02_fwd	ctgccccgcGacgggaaaaagaatg	63
P_FMD-v02_rev	cattctttttcccgtCgcggggcag	64
P_FMD-v03_fwd	ggattttgcagaaaaaTagcaagttccggg	65
P_FMD-v03_rev	cccggaaacttgctAtttttctgcaaatcc	66
P_FMD-v04_fwd	gtctctcagagGggggaatggc	67
P_FMD-v04_rev	gccattccccCctctgagagac	68
P_FMD-v05_fwd	cactcgactaccaGccgggtctctc	69
P_FMD-v05_rev	gagagaccggCtggtagtcgagtg	70

P_FMD-06_fwd	CACTCGACTACCATCCGGGTCTCTCCGAGAGGG GAATGGCACTATAAATAC	71
P_FMD-07_fwd	CACTCGACTACCATCCGGGTCTCTCACAGAGGG GAATGGCACTATAAATAC	72
P_FMD-08_fwd	CACTCGACTACCATCCGGGTCTCTCAGCGAGGG GAATGGCACTATAAATAC	73
P_FMD-09_fwd	CACTCGACTACCATCCGGGTCTCTCAGACAGGG GAATGGCACTATAAATAC	74
P_FMD-10_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGCGGG GAATGGCACTATAAATAC	75
P_FMD-11_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGACGG GAATGGCACTATAAATAC	76
P_FMD-12_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGAGCG GAATGGCACTATAAATAC	77
P_FMD-13_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGAGGC GAATGGCACTATAAATAC	78
P_FMD-14_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGAGGG CAATGGCACTATAAATAC	79
P_FMD-15_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGAGGG GCATGGCACTATAAATAC	80
P_FMD-16_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGAGGG GACTGGCACTATAAATAC	81
P_FMD-17_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGAGGG GAACGGCACTATAAATAC	82
P_FMD-18_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGAGGG GAATCGCACTATAAATAC	83
P_FMD-19_fwd	CACTCGACTACCATCCGGGTCTCTCAGAGAGGG GAATGCCACTATAAATAC	84
P_FMD_rev	GAGAGACCCGGATGGTAGTCG	85
P_FMD-v20_fwd	ctcatactcaaactatattaaaaactacaacaAT GGCTAGCAAAGGAGAAGAAGAACTTTTCAC	86
P_FMD-v20_rev	tgttgtagttttaatatagtttgagtatgagat ggaactcagaacgaaggaattatcaccagttta tatagtgccattccccctctctgag	87
P_FMD-v21_fwd	gactcaccataaacaataatcaataaatATG GCTAGCAAAGGAGAAGAAGAACTTTTCAC	88
P_FMD-v21_rev	at tt t att g att at tt t g t t t at g g g t g a g t c t a g aaaaggacgcactcgtcttgat t t t at a g t g c c	89

	attccccTctctgag	
P_FMD-v22_fwd	acttgtcctctattccttcatcaatcacatcAT GGCTAGCAAAGGAGAAGAAGCTTTTCAC	90
P_FMD-v22_rev	gatgtgattgatgaaggaatagaggacaagtag gcagtatatttatagtgccattccccTctctgag	91
Pcore_FMD_v23 (synthetic DNA)	atcaagctgtctccgatacactcgactaccatc cgggtctctcagagAggggaatggcacCGATAG GGCAGAAATATATAAAGTAGGAGGTTGTATACC AAATATACCAACGCAGTACAAGCAACTCTTGGT TTAAACGGAAGAAACAATTCTTCGAACATTTAC AACAAAGAAGGTACCGTAACATTAATAATCGGA AGGGTATGGCTAGCAAAGGAGAAGAAGCTTTTCA CTGGAGTTGTCCCAATTCT	92
Pcore_FMD_v24 (synthetic DNA)	atcaagctgtctccgatacactcgactaccatc cgggtctctcagagAggggaatggcacGTAATC TTTCGGTCAATTGTGATCTCTCTTGTAGATATT TAATAGGACGGCCAAGGTAGAAAAAGATACATA ACTAGTTAGCAAACCTTCAATTGCTTAAGTTACA AGTGCAATCCATATCTTAAAGTTATTACATTAT TTATAATGGCTAGCAAAGGAGAAGAAGCTTTTCA CTGGAGTTGTCCCAATTCT	93
Pcore_FMD_v25 (synthetic DNA)	atcaagctgtctccgatacactcgactaccatc cgggtctctcagagAggggaatggcacCCTCCT CTAGGTTTATCTATAAAAGCTGAAGTCGTTAGA ATTTTTTCATTTAAAGCATAATCAAACATCTAGA TTCGAATCGATAAAAAGCAGATAGAAGTTATTA AGATTATAGGTTACATTCTAGAGTAGTATAGGA AGGTAATGGCTAGCAAAGGAGAAGAAGCTTTTCA CTGGAGTTGTCCCAATTCT	94

3. Results

The results of the reporter protein fluorescence of the HpFMD promoter (P_FMD) and the AOX1 promoter (P_AOX1) wild type sequence promoters tested are shown in Fig. 4.

a) FMD promoter variants - point mutations and single nucleotide insertion

Table D: Relative promoter activities of all promoter variants containing point mutations and single nucleotide insertions. Relative fluorescence values (RFU) of the eGFP reporter protein were measured and these values were normalized to the OD600. These RFU/OD600 values were normalized to the RFU/OD600 value of the parental HpFMD promoter variant (wt = SEQ ID No. 1) sequence resulting in relative promoter activities. The strains were cultivated in DWPs cultivation on BMD1 media (24 and 48 h) and subsequently induced with methanol (72 and 96 h).

24 h derepressed	48 h derepressed	72 h induced with methanol	96 h induced with methanol
wt 1,0 ± 0,53	v13 0,62 ± 0,058	v09 0,56 ± 0,031	v09 0,56 ± 0,031
v12 1,0 ± 0,51	v12 0,63 ± 0,071	v14 0,56 ± 0,073	v14 0,56 ± 0,073
v13 1,1 ± 0,58	v14 0,67 ± 0,088	v12 0,57 ± 0,028	v12 0,57 ± 0,028
v09 1,2 ± 0,47	v11 0,70 ± 0,062	v11 0,58 ± 0,028	v11 0,58 ± 0,028
v14 1,3 ± 0,52	v09 0,69 ± 0,088	v13 0,59 ± 0,029	v13 0,59 ± 0,029
v11 1,3 ± 0,37	v15 0,75 ± 0,062	v15 0,69 ± 0,051	v15 0,69 ± 0,051
v04 1,3 ± 0,49	v04 0,83 ± 0,083	v04 0,74 ± 0,036	v04 0,74 ± 0,036
v19 1,3 ± 0,48	v08 0,87 ± 0,047	v06 0,77 ± 0,049	v06 0,77 ± 0,049
v16 1,3 ± 0,41	v07 0,81 ± 0,071	v07 0,83 ± 0,076	v07 0,83 ± 0,076
v15 1,4 ± 0,14	v16 0,91 ± 0,082	v08 0,83 ± 0,056	v08 0,83 ± 0,056
v08 1,4 ± 0,46	v02 0,94 ± 0,10	v16 0,88 ± 0,024	v16 0,88 ± 0,024
v07 1,5 ± 0,49	v19 0,96 ± 0,053	v02 0,9 ± 0,066	v02 0,9 ± 0,066
v02 1,5 ± 0,60	wt 1,0 ± 0,13	v19 0,97 ± 0,079	v19 0,97 ± 0,079

v18	1,5 ± 0,59	v03	1,1 ± 0,11	v03	0,99 ± 0,080	v03	0,99 ± 0,08
v03	1,7 ± 0,62	v01	1,1 ± 0,12	wt	1,0 ± 0,088	wt	1,0 ± 0,088
v06	1,7 ± 0,73	v06	1,1 ± 0,069	v01	1,04 ± 0,066	v01	1,0 ± 0,066
v17	1,8 ± 0,63	v18	1,1 ± 0,11	v17	1,06 ± 0,056	v17	1,1 ± 0,056
v01	1,8 ± 0,66	v17	1,2 ± 0,15	v18	1,08 ± 0,12	v18	1,1 ± 0,12
v10	1,9 ± 0,65	v10	1,3 ± 0,16	v05	1,1 ± 0,061	v05	1,1 ± 0,061
v05	2,4 ± 0,64	v05	1,4 ± 0,17	v10	1,2 ± 0,066	v10	1,2 ± 0,066

b) FMD promoter variants - core promoter exchanges

Table E: Relative promoter activities of all promoter variants containing with an exchanged core promoter. Relative fluorescence values (RFU) of the eGFP reporter protein were measured and these values were normalized to the OD600. These RFU/OD600 values were normalized to the RFU/OD600 value of the parental HpFMD promoter variant (wt = SEQ ID No. 1) sequence resulting in relative promoter activities. The strains were cultivated in DWPs cultivation on BMD1 media (24 and 48 h) and subsequently induced with methanol (72 and 96 h).

24 h derepressed	48 h derepressed	72 h induced with methanol	96 h induced with methanol
0,36 ±	0,29 ±	0,24 ±	0,42 ±
v23 0,30	v25 0,067	v25 0,032	v25 0,032
0,53 ±	0,42 ±	0,41 ±	0,58 ±
v25 0,31	v24 0,056	v24 0,054	v24 0,022
0,59 ±	0,54 ±	0,50 ±	0,60 ±
v24 0,44	v23 0,070	v23 0,063	v23 0,074
1,0 ±	0,96 ±	0,76 ±	0,92 ±
wt 0,44	v22 0,097	v21 0,074	v21 0,06
v21 1,9 ±	v21 1,0 ±	v22 0,78 ±	v22 0,99 ±

	0,90		0,14		0,089		0,051
	2,8 ±		1,0 ±		1,0±		1,0 ±
v22	0,36	wt	0,098	wt	0,132	wt	0,134
	3,7 ±		1,6 ±		1,4 ±		1,5 ±
v20	0,65	v20	0,14	v20	0,173	v20	0,072