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(54) Title: ENGINEERED LOWER EUKARYOTIC HOST STRAINS FOR RECOMBINANT PROTEIN EXPRESSION

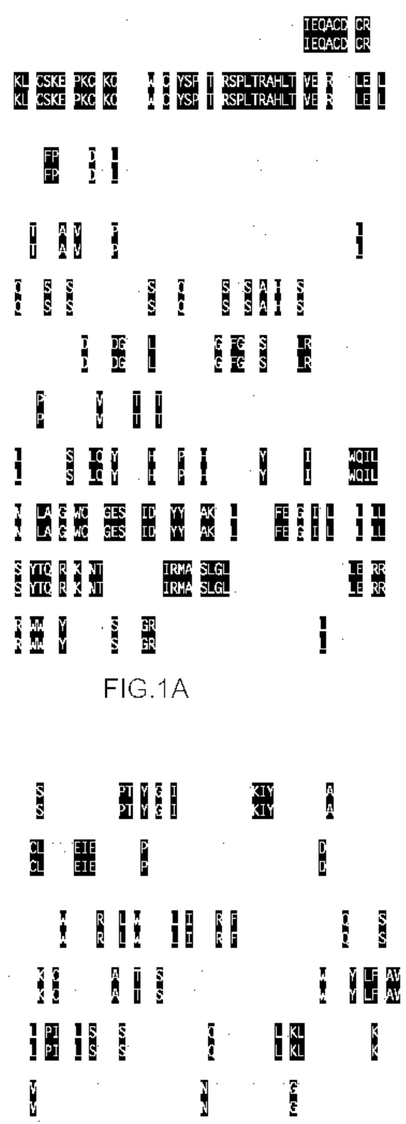

FIG.1A

FIG.1B

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The present invention relates to novel engineered lower eukaryotic host cells for expressing heterologous proteins and to methods of generating such strains.

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[Continued on next page]

(54) Title: ENGINEERED LOWER EUKARYOTIC HOST STRAINS FOR RECOMBINANT PROTEIN EXPRESSION

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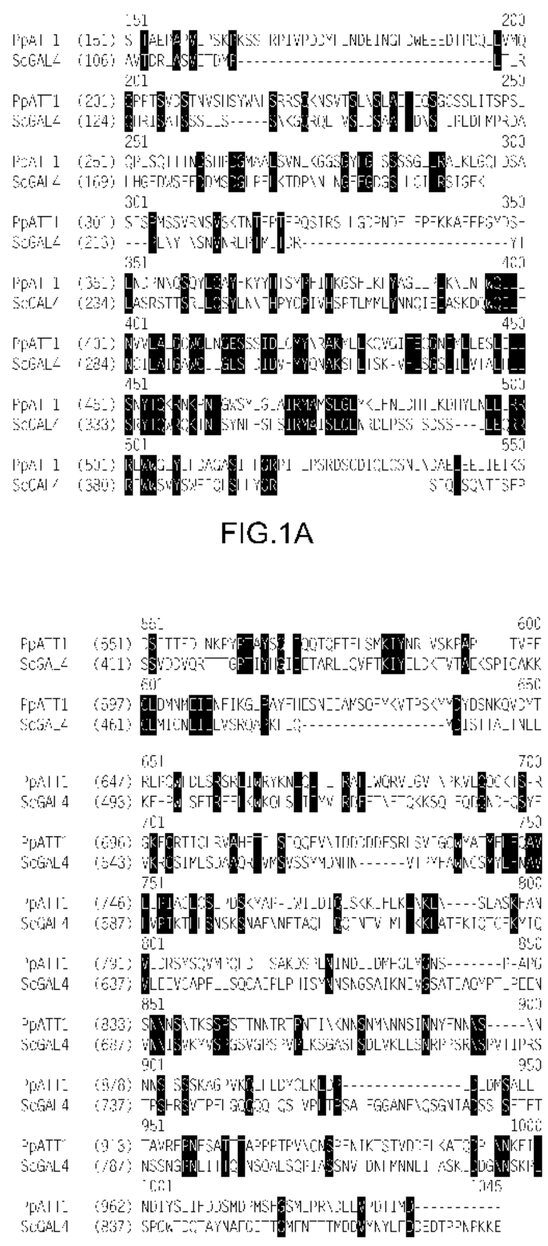

FIG.1A

FIG.1B

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ENGINEERED LOWER EUKARYOTIC HOST STRAINS FOR RECOMBINANT PROTEIN EXPRESSION

FIELD OF THE INVENTION

[0001] The present invention relates to novel engineered lower eukaryotic host cells for expressing heterologous proteins and to methods of generating such strains.

BACKGROUND OF THE INVENTION

[0002] Lower eukaryotic host cells can be engineered to produce heterologous proteins. Further, lower eukaryotic host cells can be glyco-engineered to produce glycoproteins where the N- or O-linked glycosylation are modified from their native forms.

[0003] Engineered *Pichia* strains have been utilized as an alternative host system for producing recombinant glycoproteins with human-like glycosylation. However, the extensive genetic modifications have also caused fundamental changes in cell wall structures in many glyco-engineered yeast strains, predisposing some of these strains to cell lysis and reduced cell robustness during fermentation. Certain glyco-engineered strains have substantial reductions in cell viability as well as a marked increase in intracellular protease leakage into the fermentation broth, resulting in a reduction in both recombinant product yield and quality.

[0004] Current strategies for identifying robust glyco-engineered production strains rely heavily on screening a large number of clones using various platforms such as 96-deep-well plates, 5ml mini-scale fermenters and 1L-scale bioreactors to empirically identify clones that are compatible for large-scale (40L and above) fermentation processes (Barnard et al. 2010). Despite the fact that high-throughput screening has been successfully used to identify several *Pichia* hosts capable of producing recombinant monoclonal antibodies with yields in excess of 1 g/L (Potgieter et al. 2009; Zhang et al. 2011), these large-scale screening approach is very resource-intensive and time-consuming, and often only identify clones with incremental increases in cell-robustness.

[0005] Therefore, lower eukaryotic host strains that have improved robustness and the ability to produce high quality proteins with human-like glycans would be of value and interest to the field. Here, we present engineered *Pichia* host strains having a deletion, truncation or nonsense mutation in a novel gene ATT1 (acquiring thermal tolerance) which under relevant bioprocess conditions exhibit improved viability, stability, and protein

production. Surprisingly, engineered *Pichia* host strains over-expressing ATT1 or fragments thereof under relevant bioprocess conditions also exhibit improved viability, stability, and protein production. These strains are especially useful for heterologous gene expression.

SUMMARY OF THE INVENTION

[0006] The invention relates to engineered lower eukaryotic host cells that have been modified to reduce or eliminate the activity of the ATT1 gene. The activity of the ATT1 gene can be reduced by any means. In one embodiment, the activity of the ATT1 gene is reduced or eliminated by reducing or eliminating the expression of the ATT1 gene (for example by using interfering RNA or antisense RNA). In another embodiment, the activity of the ATT1 gene is reduced or eliminated by mutating the ATT1 gene or its product. In another embodiment, the activity of the ATT1 gene is reduced or eliminated by degrading the ATT1 polypeptide. In another embodiment, the activity of the ATT1 gene is reduced or eliminated by using an inhibitor of ATT1, for example a small molecule inhibitor or an antibody inhibitor. The invention encompasses any means of inactivating the ATT1 gene or its protein including transcriptionally, translationally, or post-translationally means (for example, using repressible promoter, interfering RNA, antisense RNA, inducible protein degradation, and the like). In one embodiment, the lower eukaryotic cell is a glyco-engineered lower eukaryotic host cells. In one embodiment, the lower eukaryotic cell is a lower eukaryotic host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a fungal host cell. In one embodiment, the lower eukaryotic cell is a fungal host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a yeast host cell. In one embodiment, the lower eukaryotic cell is a yeast host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a *Pichia sp.* In one embodiment, the lower eukaryotic cell is a *Pichia sp.* host cell that lacks OCH1 activity. In another embodiment, the host cell is *Pichia pastoris* and the ATT1 gene encodes a polypeptide comprising the amino acid of SEQ ID NO:7 or a polymorph thereof. In another embodiment, the host cell is *Hansenula polymorpha* and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:23 or a polymorph thereof.

[0007] In other embodiments, the present invention relates to an engineered lower eukaryotic host cell that has been modified to express a mutated form of the ATT1 gene. The mutation could be a single nucleotide mutation, a frame-shift mutation, an insertion, a truncation or a deletion of one or more nucleotides. In one embodiment, said mutation is a deletion of the entire ATT1 gene. In another embodiment, said mutation is a deletion of a

fragment of the ATT1 gene. In one embodiment, the lower eukaryotic cell is a glyco-engineered lower eukaryotic host cell. In one embodiment, the lower eukaryotic cell is a lower eukaryotic host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a fungal host cell. In one embodiment, the lower eukaryotic cell is a fungal host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a yeast host cell. In one embodiment, the lower eukaryotic cell is a yeast host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a *Pichia sp.* In one embodiment, the lower eukaryotic cell is a *Pichia sp.* host cell that lacks OCH1 activity. In another embodiment, the host cell is *Pichia pastoris* and the ATT1 gene encodes a polypeptide comprising the amino acid of SEQ ID NO:7 or a polymorph thereof. In one embodiment, said mutated form of the ATT1 gene is a deletion of a fragment comprising amino acids 32-995 of SEQ ID NO:7. In one embodiment, said mutated form of the ATT1 gene is a deletion of fragment comprising amino acids 165-995 of SEQ ID NO:7. In another embodiment, said mutated form of the ATT1 gene is a deletion of fragment comprising amino acids 277-995 of SEQ ID NO:7. In another embodiment, said mutated form of the ATT1 gene is a deletion of fragment comprising amino acids 540-995 of SEQ ID NO:7. In another embodiment, said mutated form of the ATT1 gene is a deletion of fragment comprising amino acids 729-995 of SEQ ID NO:7. In another embodiment, said mutated form of the ATT1 gene is an insertion or a frameshift mutation in the nucleic acid encoding SEQ ID NO:7. In another embodiment, said mutated form of the ATT1 gene is a single nucleotide mutation in the nucleic acid sequence encoding SEQ ID NO:7. In another embodiment, said mutated form of the ATT1 gene results in a single amino acid change in SEQ ID NO:7. In another embodiment, the host cell is *Hansenula polymorpha* and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:23 or a polymorph thereof.

[0008] In some embodiments, the engineered lower eukaryotic host cell of the invention exhibits an increase in culture stability, thermal tolerance and/or improved fermentation robustness compared with an ATT1 naïve parental host cell under similar culture conditions. In one embodiment, said engineered host cell is capable of surviving in culture at 32°C for at least 80 hours of fermentation with minimal cell lysis. In one embodiment, said engineered host cell is capable of surviving in culture at 32°C for at least 80 hours of fermentation after induction (for example, methanol induction) with minimal cell lysis. In one embodiment, said engineered host cell is capable of surviving in culture at 32°C for at least 100 hours of fermentation with minimal cell lysis. In one embodiment, said

engineered host cell is capable of surviving in culture at 32°C for at least 100 hours of fermentation after induction with minimal cell lysis.

[0009] In some embodiments, the engineered lower eukaryotic host cell of the invention further comprises a mutation, disruption or deletion of one or more of functional gene products. In one embodiment, the host cell comprises a mutation, disruption or deletion of one or more genes encoding: protease activities, alpha-1,6-mannosyltransferase activities, alpha-1,2-mannosyltransferase activities, mannosylphosphate transferase activities, β-mannosyltransferase activities, O-mannosyltransferase (PMT) activities, and/or dolichol-P-Man dependent alpha(1-3) mannosyltransferase activities. In one embodiment, the host cell comprises a mutation, disruption or deletion in the OCH1 gene. In one embodiment, the host cell comprises a mutation, disruption or deletion in the BMT1, BMT2, BMT3, and BMT4 genes. In one embodiment, the host cell comprises a mutation, disruption or deletion in the PNO1, MNN4, and MNN4L1 genes. In one embodiment, the host cell comprises a mutation, disruption or deletion in the PEP4 and PRB1 genes. In another embodiment, the host cell comprises a mutation, disruption or deletion of the ALG3 gene (as described in US Patent Publication No. US2005/0170452). In one embodiment, the host cell further comprises a mutation, disruption or deletion of all of the following genes: OCH1, BMT1, BMT2, BMT3, BMT4, PNO1, MNN4, and MNN4L1. In one embodiment, the host cell further comprises a mutation, disruption or deletion of all of the following genes: OCH1, BMT1, BMT2, BMT3, BMT4, PNO1, MNN4, MNN4L1, PEP4 and PRB1. In one embodiment, the host cell further comprises a mutation, disruption or deletion of all of the following genes: OCH1, BMT1, BMT2, BMT3, BMT4, PNO1, MNN4, MNN4L1, ALG3, PEP4 and PRB1. In one embodiment, the engineered lower eukaryotic host cell of the invention further comprises a mutation, disruption or deletion of a gene selected from the group consisting of: SSK2, RRT12, SDS23, NOT5, DRS1, CRZ1, CTK1, RGD2, AVO2, YMR196W, PEX1, TYW1, POM152, YPR84W, MAK5, AZF1.

[0010] In yet additional embodiments, the engineered lower eukaryotic host cell of the invention further comprises one or more nucleic acid sequences of interest. In certain embodiments, the nucleic acid sequences of interest encode one or more glycosylation enzymes. In certain embodiments, the glycosylation enzymes are selected from the group consisting of glycosidases, mannosidases, phosphomannosidases, phosphatases, nucleotide sugar transporters, nucleotide sugar epimerases, mannosyltransferases, N-acetylglucosaminyltransferases, CMP-sialic acid synthases, N-acetylneuraminate-9-phosphate synthases, galactosyltransferases, sialyltransferases, and oligosaccharyltransferases.

In yet additional embodiments, the engineered lower eukaryotic host cell of the invention further comprises a nucleic acid sequences encoding one or more recombinant proteins. In one embodiment, the recombinant protein is a therapeutic protein. The therapeutic protein can contain or lack oligosaccharides. In certain embodiments, the therapeutic proteins are selected from the group consisting of antibodies (IgA, IgG, IgM or IgE), antibody fragments, kringle domains of the human plasminogen, erythropoietin, cytokines, coagulation factors, soluble IgE receptor α-chain, urokinase, chymase, urea trypsin inhibitor, IGF-binding protein, epidermal growth factor, growth hormone-releasing factor, annexin V fusion protein, angiostatin, vascular endothelial growth factor-2, myeloid progenitor inhibitory factor-1, osteoprotegerin, α-1 antitrypsin, DNase II, α-feto proteins, insulin, Fc-fusions, HSA-fusions, viral antigens and bacterial antigens. In one embodiment, the therapeutic protein is an antibody or a fragment thereof. In one embodiment, the therapeutic protein is an antibody or antibody fragment (Fc-containing polypeptide) comprising N-glycans. In one embodiment, the N-glycans comprise predominantly $NANA_{(1-4)}Gal_{(1-4)}Man_3GlcNAc_2$. In one embodiment, the N-glycans comprise predominantly $NANA_2Gal_2Man_3GlcNAc_2$.

[0011] The present invention further provides an engineered lower eukaryotic host cell comprising a disruption, deletion or mutation of the ATT1 gene in the genomic DNA, and further comprising a nucleic acid encoding an ATT1 polypeptide or a fragment thereof. In one embodiment, the lower eukaryotic cell is glyco-engineered. In one embodiment, the lower eukaryotic cell lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a fungal host cell. In one embodiment, the lower eukaryotic cell is a fungal host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a yeast host cell. In one embodiment, the lower eukaryotic cell is a yeast host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a *Pichia sp.* In one embodiment, the lower eukaryotic cell is a *Pichia sp.* host cell that lacks OCH1 activity. In one embodiment, the fragment is a "functional fragment" of ATT1 or a "dominant-negative fragment" of ATT1. As used herein, a "functional fragment" of an ATT1 gene or polypeptide, refers to a fragment that has ATT1 activity. A “dominant-negative fragment” of an ATT1 gene or polypeptide, refers to a fragment that negatively interferes with the function of the intact ATT1 gene or its polypeptide product, so that even in the presence of the endogenous naïve ATT1 gene, such a fragment is capable of increasing the cell culture stability, thermal tolerance and/or improved fermentation robustness of a host cell (for example, fragments 1-31aa and 1-164aa, in Example 11). In one embodiment, host cell is *Pichia pastoris* and the ATT1 polypeptide comprises the amino acid sequence of SEQ ID NO:7 or a polymorph

thereof. In one embodiment, the host cell is *Pichia pastoris* and the functional fragment comprises or consists of amino acids 1-296 of SEQ ID NO:7. In one embodiment, the host cell is *Pichia pastoris* and the dominant-negative fragment comprises or consists of amino acids 1-31 of SEQ ID NO:7. In one embodiment, the host cell is *Pichia pastoris* and the dominant-negative fragment comprises or consists of amino acids 1-164 of SEQ ID NO:7. In one embodiment, the host cell is *H. polymorpha* and the ATT1 polypeptide comprises the amino acid sequence of SEQ ID NO:23 or a polymorph thereof. In some embodiments, the engineered host cell comprises an over-expression cassette comprising a nucleic acid sequence encoding SEQ ID NO:7 or a natural variant (polymorph) of said polypeptide.

[0012] The invention also relates to an engineered lower eukaryotic host cell, wherein the host cell has been modified to increase expression of a nucleic acid sequence encoding an ATT1 polypeptide or a fragment thereof. In one embodiment, the lower eukaryotic host cell is glyco-engineered. In one embodiment, the lower eukaryotic cell lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a fungal host cell. In one embodiment, the lower eukaryotic cell is a fungal host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a yeast host cell. In one embodiment, the lower eukaryotic cell is a yeast host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a *Pichia sp.* In one embodiment, the lower eukaryotic cell is a *Pichia sp.* host cell that lacks OCH1 activity. In one embodiment, host cell is *Pichia pastoris* and the ATT1 polypeptide comprises the amino acid sequence of SEQ ID NO:7 or a polymorph thereof. In one embodiment, the host cell is *Pichia pastoris* and the functional fragment comprises or consists of amino acids 1-296 of SEQ ID NO:7. In one embodiment, the host cell is *Pichia pastoris* and the dominant-negative fragment comprises or consists of amino acids 1-31 of SEQ ID NO:7. In one embodiment, the host cell is *Pichia pastoris* and the dominant-negative fragment comprises or consists of amino acids 1-164 of SEQ ID NO:7. In one embodiment, the host cell is *H. polymorpha* and the ATT1 polypeptide comprises the amino acid sequence of SEQ ID NO:23 or a polymorph thereof. In some embodiments, the engineered host cell comprises an over-expression cassette comprising a nucleic acid sequence encoding SEQ ID NO:7 or a natural variant (polymorph) of said polypeptide.

[0013] In certain embodiments, the engineered lower eukaryotic host cell of the invention, which has been modified to increase expression of ATT1 or a fragment thereof, further comprises a mutation, disruption or deletion of one or more functional gene products. In one embodiment, the host cell comprises a mutation, disruption or deletion of one or more genes encoding protease activities, alpha-1,6-mannosyltransferase activities, alpha-1,2-

mannosyltransferase activities, mannosylphosphate transferase activities, β-mannosyltransferase activities, O-mannosyltransferase (PMT) activities, and/or dolichol-P-Man dependent alpha(1-3) mannosyltransferase activities. In one embodiment, the host cell comprises a mutation, disruption or deletion in the OCH1 gene. In one embodiment, the host cell comprises a mutation, disruption or deletion in the BMT1, BMT2, BMT3, and BMT4 genes. In one embodiment, the host cell comprises a mutation, disruption or deletion in the PNO1, MNN4, and MNN4L1 genes. In one embodiment, the host cell comprises a mutation, disruption or deletion in the PEP4 and PRB1 genes. In another embodiment, the host cell comprises a mutation, disruption or deletion of the ALG3 gene (as described in US Patent Publication No. US2005/0170452). In one embodiment, the host cell further comprises a mutation, disruption or deletion of all of the following genes: OCH1, BMT1, BMT2, BMT3, BMT4, PNO1, MNN4, and MNN4L1. In one embodiment, the host cell further comprises a mutation, disruption or deletion of all of the following genes: OCH1, BMT1, BMT2, BMT3, BMT4, PNO1, MNN4, MNN4L1, PEP4 and PRB1. In one embodiment, the host cell further comprises a mutation, disruption or deletion of all of the following genes: OCH1, BMT1, BMT2, BMT3, BMT4, PNO1, MNN4, MNN4L1, ALG3, PEP4 and PRB1. In one embodiment, the engineered lower eukaryotic host cell of the invention further comprises a mutation, disruption or deletion of a gene selected from the group consisting of: SSK2, RRT12, SDS23, NOT5, DRS1, CRZ1, CTK1, RGD2, AVO2, YMR196W, PEX1, TYW1, POM152, YPR84W, MAK5, AZF1. In yet additional embodiments, the engineered lower eukaryotic host cell of the invention, which has been modified to increase expression of ATT1 or a fragment thereof, further comprises one or more nucleic acid sequences of interest. In certain embodiments, the nucleic acid sequences of interest encode one or more glycosylation enzymes. In yet additional embodiments, the glycosylation enzymes are selected from the group consisting of glycosidases, mannosidases, phosphomannosidases, phosphatases, nucleotide sugar transporters, mannosyltransferases, N-acetylglucosaminyltransferases, UDP-N-acetylglucosamine transporters, galactosyltransferases, sialyltransferases, and oligosaccharyltransferases.

[0014] In yet additional embodiments, the nucleic acid sequences of interest encode one or more recombinant proteins. In one embodiment, the recombinant protein is a therapeutic protein. In certain embodiments, the therapeutic protein is selected from the group consisting of antibodies (IgA, IgG, IgM or IgE), antibody fragments, kringle domains of the human plasminogen, erythropoietin, cytokines, coagulation factors, soluble IgE receptor α-chain, urokinase, chymase, urea trypsin inhibitor, IGF-binding protein, epidermal

growth factor, growth hormone-releasing factor, annexin V fusion protein, angiostatin, vascular endothelial growth factor-2, myeloid progenitor inhibitory factor-1, osteoprotegerin, α-1 antitrypsin, DNase II, α-feto proteins, insulin, Fc-fusions, HSA-fusions, viral antigens and bacterial antigens. In one embodiment, the therapeutic protein is an antibody or a fragment thereof. In one embodiment, the therapeutic protein is an antibody or antibody fragment (Fc-containing polypeptide) comprising N-glycans. In one embodiment, the N-glycans comprise predominantly $NANA_{(1-4)}Gal_{(1-4)}Man_3GlcNAc_2$. In one embodiment, the N-glycans comprise predominantly $NANA_2Gal_2Man_3GlcNAc_2$.

[0015] In yet additional embodiments, the engineered lower eukaryotic host cell of the invention, which has been modified to increase expression of ATT1 or a fragment thereof, exhibits an increase in culture stability, thermal tolerance or improved fermentation robustness compared with the ATT1 naïve parental host cell under similar culture conditions. In one embodiment, said host cell is capable of surviving in culture at 32°C for at least 80 hours of fermentation with minimal cell lysis. In one embodiment, said host cell is capable of surviving in culture at 32°C for at least 80 hours of fermentation after induction (for example, methanol induction) with minimal cell lysis. In one embodiment, said host cell is capable of surviving in culture at 32°C for at least 100 hours of fermentation with minimal cell lysis. In one embodiment, said host cell is capable of surviving in culture in at 32°C for at least 100 hours of fermentation after induction with minimal cell lysis.

[0016] The invention also provides an engineered *H. polymorpha* host cell that has been modified to reduce or eliminate the activity of the ATT1 gene of SEQ ID NO:23. In one embodiment, the invention provides a *H. polymorpha* host cell that has been modified to reduce or eliminate expression of a nucleic acid encoding SEQ ID NO:23, a natural variant thereof or a fragment thereof. The invention also provides a *H. polymorpha* host cell which has been modified to express a mutated form of an ATT1 gene encoding SEQ ID NO:23.

[0017] The invention also provides an engineered *H. polymorpha* host cell, wherein the host cell has been modified to increase expression of a nucleic acid encoding SEQ ID NO:23, a natural variant thereof or a fragment thereof.

[0018] In certain embodiments, the invention also provides engineered lower eukaryotic host cells comprising a disruption, deletion or mutation (e.g., a single nucleotide mutation, insertion mutation, or deletion mutation) of a nucleic acid sequence selected from the group consisting of: the coding sequence of the ATT1 gene, the promoter region of the ATT1 gene, the 3' un-translated region (UTR) of ATT1, a nucleic acid sequence that is a

degenerate variant of the coding sequence of the *P. pastoris* ATT1 gene and related nucleic acid sequences and fragments, in which the host cells have an increase in culture stability, thermal tolerance or improved fermentation robustness compared to a host cell without the disruption, deletion or mutation.

[0019] The invention also relates to methods of using the engineered lower eukaryotic host cells of the invention for producing heterologous polypeptides and other metabolites. In one embodiment, the invention provides for methods for producing a heterologous polypeptide in any of the *Pichia sp.* host cells described above comprising culturing said host cell under conditions favorable to the expression of the heterologous polypeptide; and, optionally, isolating the heterologous polypeptide from the host cell.

[0020] The invention also comprises a method for producing a heterologous polypeptide in an engineered lower eukaryotic host cell, said method comprising: (a) introducing a polynucleotide encoding a heterologous polypeptide into an engineered host cell which has been modified to reduce or eliminate the activity of an ATT1 gene which is an ortholog to the *Pichia pastoris* ATT1 gene; (b) culturing said host cell under conditions favorable to the expression of the heterologous polypeptide; and, optionally, (c) isolating the heterologous polypeptide from the host cell. In one embodiment, the lower eukaryotic host cell is glyco-engineered. In one embodiment, the lower eukaryotic cell lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a fungal host cell. In one embodiment, the lower eukaryotic cell is a fungal host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a yeast host cell. In one embodiment, the lower eukaryotic cell is a yeast host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a *Pichia sp.* In one embodiment, the lower eukaryotic cell is a *Pichia sp.* host cell that lacks OCH1 activity. In another embodiment, the host cell is *Pichia pastoris* and the ATT1 gene encodes a polypeptide comprising the amino acid of SEQ ID NO:7 or a polymorph thereof. In another embodiment, the host cell is *Hansenula polymorpha* and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:23 or a polymorph thereof.

[0021] The invention also comprises a method for producing a heterologous polypeptide in an engineered lower eukaryotic host cell, said method comprising: (a) introducing a polynucleotide encoding a heterologous polypeptide into an engineered host cell which has been modified to overexpress an ATT1 gene which is an ortholog of the *Pichia pastoris* ATT1 gene of SEQ ID NO:7; (b) culturing said host cell under conditions

favorable to the expression of the heterologous polypeptide; and, optionally, (c) isolating the heterologous polypeptide from the host cell. In one embodiment, the heterologous polypeptide may be operably linked to a methanol inducible promoter, and the host cell is cultured under conditions favorable to expression of the heterologous polypeptide in the presence of methanol. In one embodiment, the lower eukaryotic host cell is glyco-engineered. In one embodiment, the lower eukaryotic cell is a lower eukaryotic host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a fungal host cell. In one embodiment, the lower eukaryotic cell is a fungal host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a yeast host cell. In one embodiment, the lower eukaryotic cell is a yeast host cell that lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a *Pichia sp.* In one embodiment, the lower eukaryotic cell is a *Pichia sp.* host cell that lacks OCH1 activity. In another embodiment, the host cell is *Pichia pastoris* and the ATT1 gene encodes a polypeptide comprising the amino acid of SEQ ID NO:7 or a polymorph thereof. In another embodiment, the host cell is *Hansenula polymorpha* and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:23 or a polymorph thereof.

[0022] The invention also provides a method for producing a heterologous polypeptide in an engineered *H. polymorpha* host cells, said method comprising: (a) introducing a polynucleotide encoding a heterologous polypeptide into an engineered *H. polymorpha* host cell that has been modified to reduce or eliminate expression of a nucleic acid encoding SEQ ID NO:23, a natural variant thereof or a fragment thereof; (b) culturing said cell under conditions favorable to the expression of the heterologous polypeptide; and, optionally, (c) isolating the heterologous polypeptide from the host cell.

[0023] The invention also provides a method for producing a heterologous polypeptide in an engineered *H. polymorpha* host cells, said method comprising: (a) introducing a polynucleotide encoding a heterologous polypeptide into an engineered *H. polymorpha* host cell that has been modified to increase expression of a nucleic acid encoding SEQ ID NO:23, a natural variant thereof or a fragment thereof; (b) culturing said cell under conditions favorable to the expression of the heterologous polypeptide; and, optionally, (c) isolating the heterologous polypeptide from the host cell.

[0024] The invention also provides a method for making any of the host cells of the invention, comprising introducing a heterologous polynucleotide into the cell which

homologously recombines with the endogenous ATT1 gene and partially or fully deletes the endogenous ATT1 gene or disrupts the endogenous ATT1 gene.

[0025] In addition, the invention provides methods for the genetic integration of a heterologous nucleic acid sequence into a host cell comprising a disruption, deletion or mutation of the ATT1 gene in the genomic DNA of the host cell. These methods comprise the step of introducing a sequence of interest into the host cell comprising a disrupted, deleted or mutated nucleic acid sequence derived from a sequence selected from the group consisting of the coding sequence of the *P. pastoris* ATT1 gene, a nucleic acid sequence that is a degenerate variant of the coding sequence of the *P. pastoris* ATT1 gene and related nucleic acid sequences and fragments.

[0026] The invention also provides isolated polynucleotides encoding the *P. pastoris* ATT1 gene, or a fragment of the *P. pastoris* ATT1 gene, or an ortholog or polymorph (natural variant) of the *P. pastoris* ATT1 gene. The invention also provides isolated polynucleotides encoding mutants of the ATT1 gene (single nucleotide mutations, frame-shift mutations, insertions, truncations or deletions). The invention also provides vectors and host cells comprising these isolated polynucleotides or fragments of these polynucleotides. The invention further provides isolated polypeptides comprising or consisting of the polypeptide sequence encoded by the *P. pastoris* ATT1 gene, by a fragment of the *P. pastoris* ATT1 gene, or an ortholog or polymorph of the *P. pastoris* ATT1 gene. Antibodies that specifically bind to the isolated polypeptides of the invention are also encompassed herein.

[0027] In one embodiment, the invention comprises an expression vector comprising a nucleic acid encoding a wild-type or mutated ATT1 gene selected from the group consisting of: a nucleotide sequence encoding SEQ ID NO:7 or a fragment thereof; a nucleotide sequence encoding SEQ ID NO:8 or a fragment thereof'; a nucleotide sequence encoding SEQ ID NO:9 or a fragment thereof ; a nucleotide sequence encoding SEQ ID NO:10 or a fragment thereof; and a nucleotide sequence encoding SEQ ID NO:23 or a fragment thereof. In one embodiment, the isolated nucleic acid encodes a polypeptide comprising or consisting essentially of residues 1-296, 1-31 or 1-164 of SEQ ID NO:7. In one embodiment, the invention comprises an expression vector comprising a nucleic acid encoding a wild-type or mutated ATT1 gene selected from the group consisting of: SEQ ID NO:24 or a fragment thereof, SEQ ID NO:25 or a fragment thereof, SEQ ID NO:26 or a fragment thereof, SEQ ID NO:27 or a fragment thereof, SEQ ID NO:28 or a or a fragment thereof, SEQ ID NO:29 or a fragment thereof, SEQ ID NO:30 or a fragment thereof, SEQ ID NO:31 or a fragment thereof,

and SEQ ID NO:32 or a fragment thereof. In one embodiment, an isolated host cell expressing said nucleic acid exhibits an increase in culture stability, thermal tolerance and/or improved fermentation robustness compared to an ATT1 naive parental host cell under similar conditions. The invention also comprises vectors and host cells comprising the nucleic acids of the invention, and the polypeptides encoded by these nucleic acids.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **Figures 1A-B** shows a sequence alignment between *Saccharyomyces cerevisiae GAL4* and *Pichia pastoris ATT1* amino acid sequences.

[0029] **Figures 2A-D** show N-glycan MALDI-TOF mass spectrometry traces using secreted and whole-cell proteins from four strains YGLY17108 (Fig. 2A), YGLY17177 (Fig. 2B), YGLY22835 (Fig. 2C), and YGLY17159 (Fig. 2D).

[0030] **Figures 3A-B** show plasmid maps of pGLY8045 and pGLY6391.

[0031] **Figures 4A-G** show plasmid maps of pGLY5933-5936 (Figs. 4A-D, respectively) and pGLY5947-pGLY5949 (Figs. 4E-G, respectively).

[0032] **Figure 5** shows the DNA constructs and integration strategy used to replace the endogenous *ATT1* gene in non-mutagenized *Pichia* strains to test temperature-resistance and fermentation robustness phenotypes.

[0033] **Figures 6A-C** show photographs (Fig. 6A-B) and correlating table (Fig. 6C) illustrating improved growth of *Pichia pastoris ATT1* mutants compared with growth of control host cells.

[0034] **Figure 7** shows strain lineages from yGLY6903 through yGLY17108.

[0035] **Figure 8** shows ATT1 mutant lineages from yGLY17108 (GFI5.0) background.

[0036] **Figure 9** shows strain lineages from yGLY6903 through yGLY22835 and yGLY17159.

[0037] **Figure 10** shows a plasmid map of pGLY8369.

[0038] **Figure 11** shows a plasmid map of pGLY9959.

[0039] **Figure 12** shows a plasmid map of pGLY5883.

[0040] **Figure 13** shows strain lineages from NRRL 11430 through yGLY13979.

[0041] **Figures 14A-H** show plasmid maps. Figs. A4A-D show plasmid maps of pGLY9955-pGLY9958, respectively; Figs.14E-H show plasmid maps of pGLY9960-pGLY9963, respectively.

[0042] **Figure 15** shows a graph illustrating reduced lysis for *Pichia* strains with ATT1 deletions.

[0043] **Figure 16** shows a graph illustrating protein titer for the *Pichia* ATT1 over-expression strain yGLY13979+ TEF-ATT1 1-655.

[0044] **Figure 17** shows a table indicating induction time, strain lysis, and protein titer for various ATT1 engineered *Pichia* host strains.

[0045] **Figure 18** shows an alignment of *S. cerevisiae* Gal4, *P. pastoris* ATT1 and *H. polymorpha* ATT1.

[0046] **Figures 19A and 19B** show the effects of different ATT1 truncations on cell robustness and product titers.

[0047] **Figure 20** shows the effect of different ATT1 modifications in cell lysis in 15L bioreactors.

[0048] **Figure 21** shows a restriction map of plasmid pGLY5952. The *E. coli/P. pastoris* shuttle vector is depicted circularly as it is maintained in *E. coli*. The plasmid contains the pUC19 sequence for bacterial maintenance as well as the 5' and 3' regions of the *P. pastoris* ATT1 gene flanking the *S. cerevisiae* ARR3 gene (driven by the *P. pastoris* RPL10 promoter) as a selectable marker. For introduction into *P. pastoris* the plasmid is digested with SfiI to linearize and remove the pUC19 region, and selected for on medium containing 1-3mM sodium arsenite, thus promoting replacement integration at the ATT1 locus with correct integrants (i.e. att1Δ strains) screened for by PCR.

[0049] **Figure 22** shows an SDS-PAGE of GFI2.0 ATT1 wild type and att1Δ fermentation supernatants. Fermentation samples of GFI2.0 ATT1 wild type and att1Δ strains were taken every ~24h and clarified supernatant was obtained by centrifugation at 13000 x g. Samples were separated on SDS-PAGE and the gel was stained by coomassie blue. MW marker, Biorad Broad Range SDS-PAGE standard.

[0050] **Figure 23** shows supernatant DNA Quantification of GFI2.0 ATT1 wild type and att1Δ Fermentation Supernatants. Fermentation samples of GFI2.0 ATT1 wild type and att1Δ strains were taken every ~24h and clarified supernatant was obtained by centrifugation at 13000 x g. Cell lysis was measured by determining supernatant DNA loads. Samples were analyzed using the Picogreen (Invitrogen) fluorescent DNA stain and quantified using a plate reader as previously reported (Barnard, 2010).

[0051] **Figure 24** shows an SDS-PAGE of GFI2.0 ATT1 wild type and att1Δ Fermentation Supernatants. Fermentation samples of GFI1.0 ATT1 wild type and att1Δ strains were taken every ~24h and clarified supernatant was obtained by centrifugation at

13000 x g. Samples were separated on SDS-PAGE and the gel was stained by coomassie blue. MW marker, Biorad Broad Range SDS-PAGE standard.

[0052] **Figure 25** shows supernatant DNA Quantification of GFI1.0 ATT1 wild type and att1Δ Fermentation Supernatants. Fermentation samples of GFI1.0 ATT1 wild type and att1Δ strains were taken every ~24h and clarified supernatant was obtained by centrifugation at 13000 x g. Cell lysis was measured by determining supernatant DNA loads. Samples were analyzed using the Picogreen (Invitrogen) fluorescent DNA stain and quantified using a plate reader as previously reported (Barnard, 2010).

DETAILED DESCRIPTION OF THE INVENTION

Molecular Biology

[0053] In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include the plural and plural terms shall include the singular. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art. The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. *See*, e.g., James M. Cregg (Editor), *Pichia* Protocols (Methods in Molecular Biology), Humana Press (2010), Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates (1992, and Supplements to 2002); Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990); Taylor and Drickamer, Introduction to Glycobiology, Oxford Univ. Press (2003); Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, N.J.; Handbook of Biochemistry: Section A Proteins, Vol I, CRC Press (1976); Handbook of Biochemistry: Section A Proteins, Vol II, CRC Press (1976); Essentials of Glycobiology, Cold Spring Harbor Laboratory Press (1999),

Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984).

[0054] A "polynucleotide" and "nucleic acid" includes DNA and RNA in single stranded form, double-stranded form or otherwise.

[0055] A "polynucleotide sequence" or "nucleotide sequence" is a series of nucleotide bases (also called "nucleotides") in a nucleic acid, such as DNA or RNA, and means a series of two or more nucleotides. Any polynucleotide comprising a nucleotide sequence set forth herein (*e.g.*, promoters of the present invention) forms part of the present invention.

[0056] A "coding sequence" or a sequence "encoding" an expression product, such as an RNA or polypeptide is a nucleotide sequence (*e.g.*, heterologous polynucleotide) that, when expressed, results in production of the product (*e.g.*, a polypeptide comprising SEQ ID NO:7 or a fragment of SEQ ID NO:7).

[0057] A "protein", "peptide" or "polypeptide" (*e.g.*, a heterologous polypeptide such SEQ ID NO:7 or as an immunoglobulin heavy chain and/or light chain) includes a contiguous string of two or more amino acids.

[0058] A "protein sequence", "peptide sequence" or "polypeptide sequence" or "amino acid sequence" refers to a series of two or more amino acids in a protein, peptide or polypeptide.

[0059] The term "isolated polynucleotide" or "isolated polypeptide" includes a polynucleotide or polypeptide, respectively, which is partially or fully separated from other components that are normally found in cells or in recombinant DNA expression systems or any other contaminant. These components include, but are not limited to, cell membranes, cell walls, ribosomes, polymerases, serum components and extraneous genomic sequences. The scope of the present invention includes the isolated polynucleotides set forth herein, *e.g.*, the promoters set forth herein; and methods related thereto, *e.g.*, as discussed herein.

[0060] An isolated polynucleotide or polypeptide will, preferably, be an essentially homogeneous composition of molecules but may contain some heterogeneity.

[0061] In general, a "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell (*e.g.*, directly or through other promoter-bound proteins or substances) and initiating transcription of a coding sequence to which it operably links.

[0062] A coding sequence (*e.g.*, of a heterologous polynucleotide, *e.g.*, reporter gene or immunoglobulin heavy and/or light chain) is "operably linked to", "under the control of", "functionally associated with" or "operably associated with" a transcriptional and

translational control sequence (*e.g.*, a promoter of the present invention) when the sequence directs RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be RNA spliced (if it contains introns) and, optionally, translated into a protein encoded by the coding sequence.

[0063] The present invention includes vectors or cassettes which comprise a nucleic acid encoding a wildtype ATT1 or a mutated ATT1 coding region (including single nucleotide mutations, frameshift mutations, insertions, truncations and deletions in the ATT1 gene). The present invention also includes vectors that lead to over-expression of ATT1 or a fragment of ATT1 which is able to increase culture stability, thermal tolerance, and/or improved fermentation robustness when overexpressed. The term "vector" includes a vehicle (*e.g.*, a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence. Suitable vectors for use herein include plasmids, integratable DNA fragments, and other vehicles that may facilitate introduction of the nucleic acids into the genome of a host cell (*e.g.*, *Pichia pastoris*). Plasmids are the most commonly used form of vector but all other forms of vectors which serve a similar function and which are, or become, known in the art are suitable for use herein. See, *e.g.*, Pouwels, *et al.*, Cloning Vectors: A Laboratory Manual, 1985 and Supplements, Elsevier, N.Y., and Rodriguez *et al.* (eds.), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Buttersworth, Boston, MA.

[0064] A polynucleotide (*e.g.*, a heterologous polynucleotide, *e.g.*, encoding an immunoglobulin heavy chain and/or light chain), operably linked to a promoter, may be expressed in an expression system. The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems include fungal host cells (*e.g.*, *Pichia pastoris*) and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors.

[0065] In general, "inducing conditions" refer to growth conditions which result in an enhanced expression of a polynucleotide (e.g. a heterologous polynucleotide) in a host cell. The term methanol-induction refers to increasing expression of a polynucleotide (*e.g.*, a heterologous polynucleotide) operably linked to a methanol-inducible promoter in a host cell of the present invention by exposing the host cells to methanol.

[0066] The following references regarding the BLAST algorithm are herein incorporated by reference: **BLAST ALGORITHMS:** Altschul, S.F., *et al.*, J. Mol. Biol. (1990) 215:403-410; Gish, W., *et al.*, Nature Genet. (1993) 3:266-272; Madden, T.L., *et al.*,

Meth. Enzymol. (1996) 266:131-141; Altschul, S.F., *et al.*, Nucleic Acids Res. (1997) 25:3389-3402; Zhang, J., *et al.*, Genome Res. (1997) 7:649-656; Wootton, J.C., *et al.*, Comput. Chem. (1993) 17:149-163; Hancock, J.M., *et al.*, Comput. Appl. Biosci. (1994) 10:67-70; **ALIGNMENT SCORING SYSTEMS**: Dayhoff, M.O., *et al.*, "A model of evolutionary change in proteins." in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 345-352, Natl. Biomed. Res. Found., Washington, DC; Schwartz, R.M., *et al.*, "Matrices for detecting distant relationships." in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3." M.O. Dayhoff (ed.), pp. 353-358, Natl. Biomed. Res. Found., Washington, DC; Altschul, S.F., J. Mol. Biol. (1991) 219:555-565; States, D.J., *et al.*, Methods (1991) 3:66-70; Henikoff, S., *et al.*, Proc. Natl. Acad. Sci. USA (1992)89:10915-10919; Altschul, S.F., *et al.*, J. Mol. Evol. (1993) 36:290-300; **ALIGNMENT STATISTICS**: Karlin, S., *et al.*, Proc. Natl. Acad. Sci. USA (1990) 87:2264-2268; Karlin, S., *et al.*, Proc. Natl. Acad. Sci. USA (1993) 90:5873-5877; Dembo, A., *et al.*, Ann. Prob. (1994) 22:2022-2039; and Altschul, S.F. "Evaluating the statistical significance of multiple distinct local alignments." in Theoretical and Computational Methods in Genome Research (S. Suhai, ed.), (1997) pp. 1-14, Plenum, New York.

Host Cells

[0067] The invention relates to engineered lower eukaryotic host cells that have been modified to reduce or eliminate the activity of the ATT1 gene. In one embodiment, the lower eukaryotic host cell is glyco-engineered. In one embodiment, the lower eukaryotic host cell lacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a fungal host cell. In one embodiment, the lower eukaryotic host cell is a fungal host cell that lacks OCH1 activity. In another embodiment, the lower eukaryotic host cell host cell is a yeast host cell. In another embodiment, the lower eukaryotic host cell host cell is a yeast host cell that clacks OCH1 activity. In one embodiment, the lower eukaryotic host cell is a *Pichia sp.* In one embodiment, lower eukaryotic host cell is a *Pichia sp.* that lacks OCH1 activity. In one embodiment, the fungal host cell is selected from the group consisting of: *Pichia pastoris*, *Pichia angusta (Hansenula polymorpha)*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia minuta* (*Ogataea minuta*, *Pichia lindneri*), *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Yarrowia Lipolytica*, *Kluyveromyces lactis*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii*, *Schwanniomyces occidentalis*, *Kluyveromyces marxianus*, *Aspergillus niger*, *Arxula adeninivorans*, *Aspergillus nidulans*,

Aspergillus wentii, Aspergillus aureus, Aspergillus flavus, Ashbya gossypii, Methylophilus methylotrophus, Schizosaccharomyces pombe, Candida boidinii, Candida utilis, Rhizopus oryzae, Debaromyces hansenii and *Saccharyomyces cerevisiae*. In another embodiment, the fungal host cell is *Pichia pastoris*. In another embodiment, the fungal host cell is *Hansenula polymorpha*.

[0068] As used herein, a host cell which has reduced ATT1 gene activity or lacks ATT1 gene activity refers to a cell that has an increase in culture stability, thermal tolerance and/or improved fermentation robustness compared with an ATT1 naïve parental host cell under similar culture conditions. In order to determine if a gene has ATT1 activity, the gene can be deleted in a glyco-engineered host cell (for example, an OCH1 minus lower eukaryotic host cell) and the ability of the cell (with the ATT1 gene deletion) to survive in culture at 32°C within a bioreactor is determined, if the cell has increased culture stability, thermal tolerance and/or improved robustness compared to an ATT1 naïve cell then the gene has ATT1 activity.

[0069] As used herein, an "ATT1 naïve host cell" refers to a host cell that comprises a wild-type ATT1 gene in its native genomic state. For example, in one embodiment, an ATT1 naïve host cell refers to a *Pichia pastoris* strain comprising in its native genomic state an ATT1 gene encoding the polypeptide of SEQ ID NO:7 or a natural variant (polymorphs) thereof.

[0070] As used herein, an "engineered cell" refers to cell that has been altered using genetic engineering techniques. As used herein, a "glyco-engineered" cell refers to cell that has been genetically engineered to produce glycoproteins where the N- or O-linked glycosylation are modified from their native form, either through inactivation or deletion of genes or through the heterologous expression of glycosyltransferases or glycosidases.

[0071] As used herein "thermal tolerance" refers to increase in temperature resistance (i.e. ability to grow in culture to temperatures of at least about 32°C).

[0072] As used herein, "improved fermentation robustness" refers to an increase in cell viability or decrease in cell lysis during fermentation.

[0073] The invention encompasses any engineered lower eukaryotic host cell which has been modified to: reduce or eliminate the activity of an ATT1 gene which is an ortholog of the *Pichia pastoris* ATT1 gene; wherein the cell exhibits an increase in culture stability, thermal tolerance, and/or improved fermentation robustness when compared to an ATT1 naïve parental host cell.

[0074] The invention also relates to an engineered lower eukaryotic host cell which has been modified to (i) reduce or eliminate expression of an ATT1 gene or polypeptide which is an ortholog of the *Pichia pastoris* ATT1 gene, (ii) express a mutated form of an ATT1 gene which is an ortholog of the *Pichia pastoris* ATT1 gene, or (iii) to over-express an ATT1 gene which is an ortholog of the *Pichia pastoris* ATT1 gene or a fragment of said gene; wherein said cell exhibits an increase in culture stability, thermal tolerance, and/or improved fermentation robustness when compared to an ATT1 naïve parental host cell. In one embodiment, the invention relates to an engineered lower eukaryotic host cell which has been modified to reduce or eliminate expression of an ATT1 gene or polypeptide which is an ortholog of the *Pichia pastoris* ATT1 gene or to express a mutated form of an ATT1 gene which is an ortholog of the *Pichia pastoris* ATT1 gene; wherein said cell exhibits an increase in culture stability, thermal tolerance, and/or improved fermentation robustness when compared to an ATT1 naïve parental host cell. In another embodiment, the invention relates to a lower eukaryotic host cell which has been modified to over-express an ATT1 gene which is an ortholog of the *Pichia pastoris* ATT1 gene, or a functional or dominant-negative fragment of said gene; wherein said cell exhibits an increase in culture stability, thermal tolerance, and/or improved fermentation robustness when compared to an ATT1 naïve parental host cell.

[0075] As used herein, an ortholog to the *Pichia pastoris* ATT1 gene, is a gene that has sequence similarity to the *Pichia pastoris* ATT1 gene and has ATT1 activity. In one embodiment, the sequence similarity will be at least 25%. A person of skill in the art would be able to identify such orthologs using only routine experimentation. For example, the *H. polymorpha* ATT1 ortholog has been identified as described in Example 9. Other fungal/yeast orthologs could be similarly identified, for example by the use of reciprocal BLAST analysis. The following genes have been identified as potential orthologs of the *Pichia pastoris* ATT1 gene:

Fungal host cell	Sequence/GenBank Accession No.
Pichia stipitis	SEQ ID NO:24 (XP_001385092.2)
Pichia guilliermondii	SEQ ID NO:25 (XP_001482364.1)
Kluyveromyces lactis	SEQ ID NO:26 (XP_453627.1)
Aspergillus niger	SEQ ID NO:27 (EHA19999.1)
Aspergillus nidulans	SEQ ID NO:28 (CBF76786.1)
Aspergillus flavus	SEQ ID NO:29 (XP_002378619.1)
Debaryomyces hansenii	SEQ ID NO:30 (XP_458171.2)

Zygosaccharomyces rouxii SEQ ID NO:31 (XP_002499285.1)

Sacchromyces cerevisiae SEQ ID NO:32 (CAA97969.1)

[0076] The host cells of the invention could be in haploid, diploid, or polyploid state. Further, the invention encompasses a diploid cell wherein only one endogenous chromosomal ATT1 gene has been mutated, disrupted, truncated or deleted.

[0077] In one embodiment, the engineered lower eukaryotic host cell of the invention is selected from the group consisting of: *Pichia pastoris*, *Pichia angusta (Hansenula polymorpha)*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia minuta* (*Ogataea minuta*, *Pichia lindneri*), *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Yarrowia Lipolytica*, *Kluyveromyces lactis*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii*, *Schwanniomyces occidentalis*, *Kluyveromyces marxianus*, *Aspergillus niger*, *Arxula adeninivorans*, *Aspergillus nidulans*, *Aspergillus wentii*, *Aspergillus aureus*, *Aspergillus flavus*, *Ashbya gossypii*, *Methylophilus methylotrophus*, *Schizosaccharomyces pombe*, *Candida boidinii*, *Candida utilis*, *Rhizopus oryzae* and *Debaromyces hansenii*. In an embodiment of the invention, the host cell is selected from the group consisting of any *Pichia* cell, such as *Pichia pastoris*, *Pichia angusta (Hansenula polymorpha)*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia minuta* (*Ogataea minuta*, *Pichia lindneri*), *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, and *Pichia methanolica*. In one embodiment, the host cell is an engineered *Pichia pastoris* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:7 or a natural variant of said polypeptide. In another embodiment, the host cell is an engineered *H. polymorpha* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:23 or a natural variant of said polypeptide. In one embodiment, the host cell is an engineered *Pichia stipitis* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:24 or a natural variant of said polypeptide. In another embodiment, the host cell is an engineered *Pichia guilliermondii* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:25 or a natural variant of said polypeptide. In another embodiment, the host cell is an engineered *Kluyveromyces lactis* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:26 or a natural variant of said polypeptide. In another embodiment, the host cell is an engineered *Aspergillus niger* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of

SEQ ID NO:27 or a natural variant of said polypeptide. In another embodiment, the host cell is an engineered *Aspergillus nidulans* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:28 or a natural variant of said polypeptide. In another embodiment, the host cell is an engineered *Aspergillus flavus* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:29 or a natural variant of said polypeptide. In another embodiment, the host cell is an engineered *Debaryomyces hansenii* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:30 or a natural variant of said polypeptide. In another embodiment, the host cell is an engineered *Zygosaccharomyces rouxii* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:31 or a natural variant of said polypeptide. In another embodiment, the host cell is an engineered *Sacchromyces cerevisiae* host cell and the ATT1 gene encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:32 or a natural variant of said polypeptide.

[0078] In one embodiment, the engineered lower eukaryotic host cells of the invention further comprise a mutation, disruption or deletion of one or more of genes. In one embodiment, the engineered lower eukaryotic host cell of the invention comprises a mutation, disruption or deletion of one or more genes encoding protease activities, alpha-1,6-mannosyltransferase activities, alpha-1,2-mannosyltransferase activities mannosylphosphate transferase activities, β-mannosyltransferase activities, O-mannosyltransferase (PMT) activities, and/or dolichol-P-Man dependent alpha(1-3) mannosyltransferase activities. In one embodiment, an engineered lower eukaryotic host cell of the invention comprises a mutation, disruption or deletion in the OCH1 gene. In one embodiment, an engineered lower eukaryotic host cell of the invention comprises a mutation, disruption or deletion in the BMT1, BMT2, BMT3, and BMT4 genes. In one embodiment, an engineered lower eukaryotic host cell of the invention comprises a mutation, disruption or deletion in the PNO1, MNN4, and MNN4L1 genes. In one embodiment, an engineered lower eukaryotic host cell of the invention comprises a mutation, disruption or deletion in the PEP4 and PRB1 genes. In another embodiment, an engineered lower eukaryotic host cell of the invention comprises a mutation, disruption or deletion of the ALG3 gene (as described in US Patent Publication No. US2005/0170452). In one embodiment, an engineered lower eukaryotic host cell of the invention comprises a mutation, disruption or deletion of all of the following genes: OCH1, BMT1, BMT2, BMT3, BMT4, PNO1, MNN4, and MNN4L1. In one embodiment, an engineered lower eukaryotic host cell of the invention comprises a mutation, disruption or

deletion of all of the following genes: OCH1, BMT1, BMT2, BMT3, BMT4, PNO1, MNN4, MNN4L1, PEP4 and PRB1. In one embodiment, an engineered lower eukaryotic host cell of the invention comprises a mutation, disruption or deletion of all of the following genes: OCH1, BMT1, BMT2, BMT3, BMT4, PNO1, MNN4, MNN4L1, ALG3, PEP4 and PRB1. In some embodiments, the host cell of the invention can be cultivated in a medium that includes one or more Pmtp inhibitors. Pmtp inhibitors include but are not limited to a benzylidene thiazolidinedione. Examples of benzylidene thiazolidinediones are 5-[[3,4bis(phenylmethoxy) phenyl]methylene]-4-oxo-2-thioxo-3-thiazolidineacetic Acid; 5-[[3-(1-25 Phenylethoxy)-4-(2-phenylethoxy)]phenyl]methylene]-4-oxo-2-thioxo-3-thiazolidineacetic Acid; and 5-[[3-(l-Phenyl-2-hydroxy)ethoxy)-4-(2-phenylethoxy)]phenyl]methylene]-4-oxo-2-thioxo3-thiazolidineacetic acid.

[0079] In one embodiment, an engineered lower eukaryotic host cell of the invention lacks OCH1 activity. In one embodiment, the invention comprises a lower eukaryotic host cell (*e.g.*, *Pichia sp.*) that has been modified to: (i) reduce or eliminate expression of an ATT1 gene or polypeptide, (ii) express a mutated form of an ATT1 gene, or (iii) over-express an ATT1 gene or a fragment of the ATT1, wherein the cell lacks OCH1 activity. Lower eukaryotic cells lacking OCH1 activity have been described in the art and have been shown to be temperature sensitive. *See, e.g.,* Choi et al., 2003; Bates et al., *J. Biol. Chem.* 281(1):90-98 (2006); Woog Kim et al., *J. Biol. Chem.* 281(10):6261-6272 (2006); Yoko-o et al., *FEBS Letters* 489(1):75-80 (2001); and Nakayama et al., *EMBO J* 11(7):2511-2519 (1992). Accordingly, it is desirable to modify cells that lack OCH1 activity to render them thermotolerant.

[0080] In an embodiment of the invention, an engineered lower eukaryotic host cell of the invention is further genetically engineered to include a nucleic acid that encodes an alpha-1,2-mannosidase that has a signal peptide that directs it for secretion. For example, in an embodiment of the invention, the host cell of the invention is engineered to express an exogenous alpha-1,2-mannosidase enzyme having an optimal pH between 5.1 and 8.0, preferably between 5.9 and 7.5. In an embodiment of the invention, the exogenous enzyme is targeted to the endoplasmic reticulum or Golgi apparatus of the host cell, where it trims N-glycans such as $Man_8GlcNAc_2$ to yield $Man_5GlcNAc_2$. See U.S. Patent No. 7,029,872. Lower eukaryotic host cells expressing such alpha-1,2-mannosidase activity have been described in the art, see, e.g., Choi et al., 2003. In one embodiment, the glyco-engineered lower eukaryotic host cell of the invention lacks OCH1 activity and comprises an alpha1,2 mannosidase.

[0081] In another embodiment, engineered lower eukaryotic host cells (*e.g.*, *Pichia sp.*) of the invention that have been modified to: (i) reduce or eliminate expression of an ATT1 gene or polypeptide, (ii) express a mutated form of an ATT1 gene, or (iii) over-express an ATT1 gene or a fragment of the ATT1, are further genetically engineered to eliminate glycoproteins having alpha-mannosidase-resistant N-glycans by deleting or disrupting one or more of the beta-mannosyltransferase genes (*e.g.*, *BMT1*, *BMT2*, *BMT3*, and *BMT4*) (See, U.S. Patent No. 7,465,577) or abrogating translation of RNAs encoding one or more of the beta-mannosyltransferases using interfering RNA, antisense RNA, or the like.

[0082] In some embodiments, engineered lower eukaryotic host cells (*e.g.*, *Pichia sp.*) of the present invention that have been modified to: (i) reduce or eliminate expression of an ATT1 gene or polypeptide, (ii) express a mutated form of an ATT1 gene, or (iii) over-express an ATT1 gene or a fragment of the ATT1, are further genetically engineered to eliminate glycoproteins having phosphomannose residues, *e.g.*, by deleting or disrupting one or more of the phosphomannosyl transferase genes (i.e., *PNO1, MNN4* and *MNN4L1* (see e.g., U.S. Patent Nos. 7,198,921 and 7,259,007)), or by abrogating translation of RNAs encoding one or more of the phosphomannosyltransferases using interfering RNA, antisense RNA, or the like.

[0083] Additionally, engineered lower eukaryotic host cells (*e.g.*, *Pichia sp.*) of the invention that have been modified to: (i) reduce or eliminate expression of an ATT1 gene or polypeptide, (ii) express a mutated form of an ATT1 gene, or (iii) over-express an ATT1 gene or a fragment of the ATT1, may be further genetically engineered to include a nucleic acid that encodes the *Leishmania sp.* single-subunit oligosaccharyltransferase *STT3A* protein, *STT3B* protein, *STT3C* protein, *STT3D* protein, or combinations thereof such as those described in WO2011/06389.

[0084] In some embodiments, the engineered lower eukaryotic host cell of the invention further comprises a promoter operably linked to a polynucleotide encoding a heterologous polypeptide (*e.g.*, a reporter or immunoglobulin heavy and/or light chain). The invention further comprises methods of using the host cells of the invention, *e.g.*, methods for expressing the heterologous polypeptide in the host cell. The engineered lower eukaryotic host cell of the invention may be also genetically engineered so as to express particular glycosylation patterns on polypeptides that are expressed in such cells. For example, host cells of the present invention may be modified to produce polypeptides comprising N-glycans. In one embodiment, the host cells of the invention may be engineered to produce high mannose, hybrid or complex-type N-glycans.

[0085] As used herein, the terms "N-glycan" and "glycoform" are used interchangeably and refer to an N-linked oligosaccharide, *e.g.*, one that is attached by an asparagine-N-acetylglucosamine linkage to an asparagine residue of a polypeptide. N-linked glycoproteins contain an N-acetylglucosamine residue linked to the amide nitrogen of an asparagine residue in the protein. Predominant sugars found on glycoproteins are glucose, galactose, mannose, fucose, N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc) and sialic acid (*e.g.*, N-acetyl-neuraminic acid (NANA)).

[0086] N-glycans have a common pentasaccharide core of $Man_3GlcNAc_2$ ("Man" refers to mannose; "Glc" refers to glucose; and "NAc" refers to N-acetyl; GlcNAc refers to N-acetylglucosamine). N-glycans differ with respect to the number of branches (antennae) comprising peripheral sugars (*e.g.*, GlcNAc, galactose, fucose and sialic acid) that are added to the $Man_3GlcNAc_2$ ("Man_3") core structure which is also referred to as the "trimannose core", the "pentasaccharide core" or the "paucimannose core". N-glycans are classified according to their branched constituents (*e.g.*, high mannose, complex or hybrid). A "high mannose" type N-glycan has five or more mannose residues. A "complex" type N-glycan typically has at least one GlcNAc attached to the 1,3 mannose arm and at least one GlcNAc attached to the 1,6 mannose arm of a "trimannose" core. Complex N-glycans may also have galactose ("Gal") or N- acetylgalactosamine ("GalNAc") residues that are optionally modified with sialic acid or derivatives (*e.g.*, "NANA" or "NeuAc", where "Neu" refers to neuraminic acid and "Ac" refers to acetyl). Complex N-glycans may also have intrachain substitutions comprising "bisecting" GlcNAc and core fucose ("Fuc"). Complex N-glycans may also have multiple antennae on the "trimannose core," often referred to as "multiple antennary glycans." A "hybrid" N-glycan has at least one GlcNAc on the terminal of the 1,3 mannose arm of the trimannose core and zero or more mannoses on the 1,6 mannose arm of the trimannose core. The various N-glycans are also referred to as "glycoforms". "PNGase" or "glycanase" refers to peptide N-glycosidase F (EC 3.2.2.18).

[0087] In an embodiment of the invention, engineered lower eukaryotic host cells (*e.g.*, *Pichia sp.*) of the invention that have been modified to: (i) reduce or eliminate expression of an ATT1 gene or polypeptide, (ii) express a mutated form of an ATT1 gene, or (iii) over-express an ATT1 gene or a fragment of the ATT1, are further genetically engineered to produce glycoproteins that have predominantly an N-glycan selected from the group consisting of complex N-glycans, hybrid N-glycans, and high mannose N-glycans. In one embodiment, the high mannose N-glycans are selected from the group consisting of $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_9GlcNAc_2$. In one embodiment, the

host cell of the invention is engineered to produce glycoproteins that have predominantly $Man_{8-10}GlcNAc_2$ N-glycans (Example 13). In one embodiment, the N-glycans are selected from the group consisting of $Man_5GlcNAc_2$ (Example 13), $GlcNAcMan_5GlcNAc_2$, $GalGlcNAcMan_5GlcNAc_2$, and $NANAGalGlcNAcMan_5GlcNAc_2$. In one embodiment, the N-glycans are selected from the group consisting of $Man_3GlcNAc_2$, $GlcNAC_{(1-4)}Man_3GlcNAc_2$, $NANA_{(1-4)}GlcNAc_{(1-4)}Man_3GlcNAc_2$, and $NANA_{(1-4)}Gal_{(1-4)}Man_3GlcNAc_]$. In one embodiment, the N-glycans comprise predominantly a $Man_3GlcNAc_2$ structure. In one embodiment, the N-glycans comprise predominantly $NANA_{(1-4)}Gal_{(1-4)}Man_3GlcNAc_2$. In one embodiment, the N-glycans comprise predominantly $NANA_2Gal_2Man_3GlcNAc_2$. In one embodiment, the host cell of the invention is engineered to produce glycoproteins that have galactosylated N-glycans (Example 1). In one embodiment, the host cell of the invention is engineered to produce glycoproteins that have sialylated N-glycans (Example 2 and WO2011/149999).

CHARACTERIZATION OF *PICHIA PASTORIS* ATT1

[0088] Mutations within a novel *Pichia pastoris* gene ATT1 (SEQ ID NO:1, acquiring thermal tolerance, which is orthologous to *S. cerevisiae* GAL4 transcription factor) resulted in premature truncations of the ATT1 protein product and were identified in a set of *Pichia* mutants that exhibited increased thermal tolerance. These mutations led to a significant enhancement in temperature resistance (*i.e.* stability in culture to temperatures of at least about 35°C) and improved fermentation robustness for those *Pichia* host strains harboring these mutations (*i.e.* ATT1 mutant *Pichia* strains exhibited decreased lysis, extended induction/production phase, and produced heterologous protein products with decreased proteolytic degradation as well as desired glycosylation patterns).

[0089] To broadly improve strain quality, several temperature-resistant *P. pastoris* mutant strains with significantly improved fermentation robustness were identified from a set of temperature-resistant mutants. While non-mutagenized glyco-engineered parental strains typically display a temperature-sensitive phenotype when grown on Petri dishes (Choi et al. 2003) and generally display a high level of cell lysis within 24 hours of MeOH induction at 32°C when cultured within a bioreactor, the ATT1 mutant strains described herein are viable for more than 100 hours after induction at 32°C when cultured within a bioreactor, without showing obvious signs of cell-lysis. This extended induction period allows for significantly increased yield and quality of multiple recombinant proteins, desirable traits for production of heterologous proteins such as antibody and non-antibody therapeutics.

[0090] Such mutations in ATT1 when engineered into any yeast host strain could serve to improve fermentation robustness, improve recombinant protein yield, and reduce protein product proteolytic degradation.

Experimental Methods

[0091] Fed-batch fermentations, IgG purifications, N- glycan characterizations, as well as all other analytical assays, were performed as previously described (Barnard *et al.* 2010; Jiang *et al.* 2011; Potgieter *et al.* 2009; Winston F 2008). Except otherwise specified, all 1L Bioreactor fermentation runs described in this application are scheduled to end after 100-120 hours of MeOH induction. However, a fermentation run will be terminated prematurely if excess cell lysis is observed. Cell lysis is determined either by microscopic examination, or by measuring the amount of nuclear DNA released into the supernatant (Barnard, 2010). For this application, excess cell lysis is defined by either greater than 80% cells were lysed by microscopic examination, or greater than 30 microgram/ml DNA concentration in the supernatant determined by Picogreen assay. UV mutagenesis was performed as described by Winston (Winston 2008). Briefly, *Pichia* strains were grown in 40 ml YSD liquid medium overnight at 24°C. Upon reaching an OD_{600} of 5, an aliquot of 10^6 to 10^7 cells was transferred onto the surface of a 100 mm YSD agar Petri dish, and treated, with the lid off, with 5 mJ/cm^2 of UV irradiation. After the UV treatment, the Petri dish was immediately covered with aluminum foil (to prevent photo-induced DNA repair) and the mutagenized cells were allowed to recover at 24°C for 18 hours in the dark. Then, these recovered cells were transferred to 35°C incubator to select for temperature-resistant mutants. After 7-10 days incubation at 35°C, colonies were picked and re-streaked onto fresh YSD

plates and incubated at 35°C, and only the clones displaying the temperature-resistant phenotype upon restreak were retained as temperature-resistant mutants.

EXAMPLE 1

Temperature-Resistant Mutants Displayed Substantially Enhanced Fermentation Robustness and Productivity

[0092] Two temperature-sensitive glyco-engineered empty host strains, YGLY17108 and YGLY22835 were subjected to UV mutagenesis as described herein, to identify *Pichia* host strains with increased fermentation robustness. Host strain YGL17108 has been engineered to produce galactosylated glycoproteins having predominantly Gal2GlcNAc2Man3GlcNAc2 N-glycans (Bobrowicz *et al.* 2004, and U.S. Patent No. 7,795,002). Host strain YGL22835 has been engineered to produce sialylated glycoproteins having predominantly NANA2Gal2GlcNAc2Man3GlcNAc2 N-glycans (Hamilton *et al.* 2006 and wo2012/115904). The glyco-engineered host strain YGLY17108 was derived originally from the NRRL-Y11430 strain through a series of modifications as described in WO2011/06389, to arrive at strain YGLY7965. The genetic background and strain lineage for additionally modified strains from YGLY6903 and ending with host strain YGLY17108 is shown in Figure 7. The genetic background and strain lineages from YGLY6903 through YGLY22835 and yGLY17159 are shown in Figure 9. An overview of the strains obtained from the UV mutagenesis experiments is shown in Figure 8.

[0093] Temperature-resistant colonies were selected and fermented using standard MeOH fed-batch runs in 1.0L DasGip Bioreactors to evaluate strain robustness during the fermentation process (Hopkins *et al.* 2011). After an extensive fermentation screening, four mutants YGLY17172, YGLY17177, YGLY17178, and YGLY17159 were identified displaying dramatically enhanced fermentation robustness (Table 1). Both of the non-mutagenized parental strains YGLY17108 and YGLY22835 suffered heavy lysis and a major loss of cell viability within 24 hours of induction at 32°C (indicated in Table 1 as a lysis score of 5, which indicates a large amount of cell lysis). In contrast, the four temperature-resistant mutants (YGLY17172, YGLY17177, YGLY17178, and YGLY17159) all displayed dramatically improved fermentation robustness and were viable for more than 100 hours following induction at 32°C, with little cell-lysis observed (with lysis scores < 2 out of 5, as shown in Table 1). A lysis score of 0.5-5.0 was assigned based on microcopic examination. A lysis score of 0.5 indicates minimal lysis (more than 95% intact cells), and a lysis score of 5 indicates high lysis (less than 10% intact cells).

Table 1: Isolated Mutants Displayed Improved Fermentation Robustness

1L Bioreactor MeOH Induction Phase		Lysis score at 32°C			
		Day 1	Day 2	Day 3	Day 4
YGLY17108	GFI5.0 control	5*	Harvest		
YGLY17172	GFI5.0 mutant	0.5	0.5	1/1.5	2/2.5
YGLY17177	GFI5.0 mutant	0.5	0.5	1	1
YGLY17178	GFI5.0 mutant	0.5	1	1	2
YGLY22835	GFI6.0 control	5*	Harvest		
YGLY17159	GFI6.0 mutant	0.5	0.5	0.5/1	1

* lysis score:0-5, with 5 indicating the greatest amount of lysis

EXAMPLE 2

Genome Sequencing to Identify the Causative Mutation(s) Responsible for the Enhanced Thermal-tolerance and Fermentation Robustness

[0094] Genome-sequencing was performed on the four independently isolated mutants and the parental strains to identify and characterize the mutations responsible for the increased thermal tolerance and fermentation robustness. After genome-wide comparisons between the mutants and their corresponding parents, from 1 to 9 non-synonymous nucleotide alterations (indicated by a "+" in Table 2) were identified in each of these 4 mutants. Most of these non-synonymous mutations are caused by single-nucleotide variants with one exception of a 5 bp insertion found in the YGLY17159 mutant. One mutant, YGLY17178, contained only one non-synonymous SNV, which is a single non-sense mutation within an uncharacterized transcription factor, which was named ATT1 (acquiring thermal tolerance). Non-sense or frame-shift mutations in the ATT1 gene were also identified in the other three mutants, indicating that mutations of this transcription factor are associated with the temperature resistance and fermentation robustness phenotypes.

Table 2: Non-Synonymous Nucleotide Alterations Identified by Genome Sequencing

Chromosome	position	YGLY17108	YGLY17172	YGLY17177	YGLY17178	YGLY22835	YGLY17159	reference allele	mutanted allele	SNV type	gene-id	reference condon	mutanted condon	reference AA	mutanted AA	Gene Symbol
chr1	160070	-	-	-	-	-	+	T	TGAATC	insertion	Pp01g00680				frame-shift	ATT1
chr1	160305	-	-	-	+	-	-	C	T	nonsyn	Pp01g00680	CAG	TAG	Q	Stop	ATT1
chr1	160476	-	-	+	-	-	-	A	T	nonsyn	Pp01g00680	AAA	TAA	K	Stop	ATT1
chr1	161949	-	+	-	-	-	-	C	T	nonsyn	Pp01g00680	CGA	TGA	R	Stop	ATT1
chr1	279682	-	-	-	-	-	+	T	A	nonsyn	Pp01g01360	ATC	TTC	I	F	AIP1
chr1	981612	-	-	-	-	-	-	A	G	nonsyn	Pp01g05170	TAC	TGC	Y	C	SSK2
chr1	1029402	-	-	+	-	-	-	C	T	nonsyn	Pp01g05460	AGA	AAA	R	K	RRT12
chr1	1084421	-	+	-	-	-	-	A	G	nonsyn	Pp01g05760	AAG	GAG	K	E	SDS23
chr1	1203497	-	-	+	-	-	-	A	G	nonsyn	Pp01g06460	AAA	GAA	K	E	NOT5
chr1	1308858	-	+	-	-	-	-	A	G	nonsyn	Pp01g07000	TTA	TCA	L	S	DPS1
chr2	386047	-	-	-	-	-	+	T	C	nonsyn	Pp02g02120	TTC	TCC	F	S	CRZ1
chr2	1391195	-	-	+	-	-	-	A	G	nonsyn	Pp02g07550	TTG	TCG	L	S	CTK1
chr2	1401784	-	+	-	-	-	-	T	A	nonsyn	Pp02g07600	TAT	TTT	Y	F	RGD2
chr2	1941747	-	-	+	-	-	-	T	C	nonsyn	Pp02g10670	TCA	CCA	S	P	AVO2
chr3	139943	-	-	-	-	-	+	A	T	nonsyn	Pp03g00790	AAA	TAA	K	Stop	YMR196W
chr3	230758	-	+	-	-	-	-	G	A	nonsyn	Pp03g01260	GAA	AAA	E	K	PEX1
chr3	640255	-	-	-	-	-	+	C	T	nonsyn	Pp03g03550	TCT	TTT	S	F	TYW1
chr3	875268	-	-	+	-	-	-	A	G	nonsyn	Pp03g04770	CTG	CCG	L	P	POM152
chr3	973319	-	-	+	-	-	-	C	T	nonsyn	Pp03g05310	CCT	CTT	P	L	YPR084W
chr3	1586501	-	-	-	-	-	+	A	G	nonsyn	Pp03g08800	TAT	CAT	Y	H	
chr3	2123889	-	-	+	-	-	-	A	G	nonsyn	Pp03g11890	TTA	TCA	L	S	
chr4	704482	-	-	+	-	-	-	T	A	nonsyn	Pp05g04100	AAT	ATT	N	I	MAK5
chr4	1374513	-	-	-	-	-	+	A	G	nonsyn	Pp05g07960	CTA	CCA	L	P	AZF1

[0095] All four mutations (three non-sense mutations and one frame-shift insertion) resulted in premature gene-product truncations after amino acid 31, 107, 164, or 655 (Figures 1A-B). Figures 1A-B show the sequence homology between Sc*GAL4* and Pp*ATT1*. The symbol "#" at amino acid 31 indicates the position of the 5 bp insertion found in YGLY17159, and the symbol "@" signs indicate the stop-codon mutations identified from YGL17178 (at amino acid 107), YGL17177 (amino acid 164) and YGLY17172 (amino acid 655). Sc*GAL4* is a key transcription factor involved in the regulation of galactose metabolic enzymes in baker's yeast (Traven *et al.* 2006). *Pichia pastoris* does not metabolize galactose, so the biological function of this *ATT1* gene in *Pichia* was at the time that the application was filed still unknown.

[0096] The *Pichia* ATT1 gene (SEQ ID NO:1) codes for a 995 amino acid protein product SEQ ID NO:7, which contains an N-terminal domain (amino acid residues 39 to 114) highly homologous to the *S. cerevisiae* GAL4 DNA-binding domain, a conserved fungal specific transcription factor domain at amino acid residues 351-519, and a C-terminus region

sharing a low level of conservation to the C-terminus of the *S. cerevisiae* GAL4 gene product, as shown in the alignment in Figures 1A-B.

[0097] Three of the mutants, YGL17172, YGL17177, and YGL17178, are derived from the parent host strain YGL17108 which has been engineered to produce complex N-glycan forms with terminal galactose (Bobrowicz *et al.* 2004, and U.S. Patent No. 7,795,002). The fourth mutant, YGL17159, is derived from host strain YGL22835, which is capable of producing fully sialylated N-glycans (Hamilton *et al.* 2006, and U.S. Serial No. 61/446,853). Comparison of the N-glycan profiles obtained from the isolated mutants with those of their respective parents showed that N-glycan compositions isolated from the mutants are virtually indistinguishable to the N-glycan profiles obtained from their non-mutagenized parent strains, confirming that all four mutants retained their capability to make complex human glycans. Representative N-glycan MALDI traces of YGL17108, YGL17177, YGLY22835, and YGLY17159 are shown in Figures 2A-D. The N-glycan profiles of mutants YGLY17172 and YGLY17178 are indistinguishable from that of mutant YGL17177.

EXAMPLE 3

Recombinant Protein Expression in mutant YGLY17159

[0098] To illustrate that the increased fermentation robustness phenotype would be retained during recombinant protein production (*e.g.* heterologous protein production), two different recombinant proteins (an IgG4 mAb and an Fc-fusion protein) were transformed into mutant strain YGLY17159. The mAb-expressing strain was constructed by integrating the plasmid pGLY8045 (Figure 3A), with both the 1F11 IgG4 heavy chain and kappa light chain under the control of the AOX1-promtor, into the TRP2 locus of YGLY17159 by electoporation. Similarly, the Fc-fusion expressing strain was generated by integrating the plasmid pGLY6391 (Figure 3B), which harbored the AOX1p-driven TNFR-Fc fusion gene, into the THR1 locus of YGL17159. After fermentation under standard platform conditions at 24 °C and 32 °C in 1L DasGip bioreactors (Hopkins *et al.*, 2011), the recombinant protein products secreted from both the parent and the mutant were purified and evaluated.

Table 3: Recombinant Product Expressions in the Mutant YGLY17159

1F11 IgG4 transformants in 1L Bioreactor			Lysis during induction				Broth titer @ harvest (mg/liter)
			day 1	day 2	day 3	day 4	
D104125	24C	y22835 parent	0.5*	3.5/4	harvest		894
D104129		y17159 mutant	0.5/1	0.5	0.5/1	1.5	1813
D104134	32C	y17159 mutant	0.5	1	2.5	h	411
TNFR Fc-fusion transformants in 1L Bioreactor			Lysis during induction				Broth titer @ harvest (mg/liter)
			day 1	day 2	day 3	day 4	
D104503	24C	y22835 parent	0.5	3*	harvest		234
D104501		y17159 mutant	0.5	0.5/1	1	1.5/2	405
D104511	32C	y22835 parent	5*	harvest			
D104509		y17159 mutant	0.5	1	1	2	188

* lysis score: 0 - 5, with 5 indicating the greatest amount of lysis (i.e. worst lysis)

[0099] The results in Table 3 illustrate that the recombinant protein expressing mutants retained superior robustness and extended strain stability during the induction period, which translated into significant product yield improvements, compared with these characteristics in the parent strain. At 24°C, the mutant strains exhibited about 100% titer increase for the IgG4 antibody, and about 70% titer increase for the Fc-fusion in the mutant strains compared to expression in the parental control cells under similar conditions.

EXAMPLE 4

Confirmation of Phenotype by Directed Strain Engineering

[00100] Independent mutations in the same gene in each of the mutants strongly indicated that truncations of this *ATT1* transcription factor are responsible for the observed temperature-resistance and fermentation robustness phenotypes. To confirm this conclusion, the ATT1 ORF was either completely deleted, or the endogenous ATT1 gene was replaced with the truncated versions shown in Figure 5, in YGLY21203, which is a non-mutagenized ura5 auxtroph *Pichia* strain derived from YGLY17108 by 5FOA counterselection.

[00101] Plasmid pGLY5933 (Figure 4A) was constructed by cloning a 2 kb genomic DNA fragment immediately upstream of the ATT1 ORF in front of the ALG3 terminator, followed by the lacZ-URA5-lacZ URAblaster, and then connected to a 1.9kb genomic DNA

fragment containing the last 285 bp of the ATT1 ORF (SEQ ID NO:1) plus 1.6kb of the downstream region. After SfiI digestion, this ATT1-upstream-URAblaster-ATT1-downstream DNA fragment was transformed into a non-mutagenized host strain (*e.g.* YGLY17108). By homologous recombination at both the ATT1 upstream and downstream regions, this URAblaster-cassette replaced the endogenous ATT1 gene, deleting 90% of ATT1's coding region, thus generating a complete ATT1 knock-out mutant. To confirm the correct replacement of the ATT1 ORF, genomic DNA polymerase chain reaction (PCR) assays were conducted using the following oligos as PCR primers: "TTTCGAAAGTGGCTTGGAAT" (SEQ ID NO:12, 2370 bp upstream of ATT1 start) and "TGGGGAGAAGGTACCGAAG" (SEQ ID NO:13, within the ALG3 terminator) to confirm the 5' junction of the gene-replacement; "CACTACGCGTACTGTGAGCC" (SEQ ID NO:14, within the lacZ) and "GCTTGGTACGGTAGCCTCAA" (SEQ ID NO:15, 2014 bp downstream of the ATT1 stop codon) to confirm the 3' junction of the gene-replacement; plus "AGTCTGCGCTTTCCATGTCT" (SEQ ID NO:16, 365 bp upstream of the ATT1 start) and "GGCCTGGAGATATTGGGATT" (SEQ ID NO:17, within the ATT1 ORF, 1070 bp after the start) to confirm the absence of the wild-type ATT1 ORF.

[00102] Plasmid pGLY5947 (Figure 4E) was constructed by cloning a 2.3 kb DNA fragment (2.0 kb upstream region, the 1st 321 bp of the ATT1 ORF, plus 2 stop codons) in front of the the ALG3 terminator sequence, followed by the lacZ-URA5-lacZ URAblaster, and then connected to a 1.9kb genomic DNA fragment containing the last 285 bp of the ATT1 ORF plus 1.6kb of the downstream region.

[00103] Homologous recombination-mediated double-crossovers between the SfiI-fragment of pGLY5947 (Figure 4E) and the chromosomal ATT1 region replaced the endogenous ATT1 ORF with a truncated version of ATT1 with only the first 107 amino acid residues. Similarly, plasmid pGLY5948 (Figure 4F) and pGLY5949 (Figure 4G) contain the first 492 bp or 1965 bp of the ATT1 ORF (SEQ ID NO:1) respectively, and homologous recombination-mediated double-crossovers would replace the endogenous ATT1 ORF with a truncated region of ATT1 encoding the truncations 1-164 (SEQ ID NO:10) or 1-655 amino acids (SEQ ID NO: 11).

[00104] Plasmid pGLY5934 (Figure 4B) is almost identical to pGLY5947, except that it also contains a 5 bp (TGAATC, SEQ ID NO:18) frame-shift insertion after the 31st amino acid residue of the ATT1 ORF; and plasmid pGLY5935 (Figure 4C) is almost identical to pGL5948 (Figure 4F), except that it contains the same 5 bp (TGAATC, SEQ ID NO:18)

frame-shift insertion after the 31st amino acid of ATT1 (SEQ ID NO:7), coding for the ATT1 fragment SEQ ID NO:8.

[00105] After confirming the DNA constructs precisely replaced the endogenous *ATT1* gene with the corresponding deletion or truncations, their abilities to grow at 35°C was examined. It was confirmed that these *ATT1*Δ truncation and deletion mutants displayed temperature-resistant phenotypes very similar to those observed from the original mutants isolated by UV mutagenesis (Figures 6A-C).

[00106] Next, the truncation and deletion mutants were subjected to standard DasGip MeOH fed-batch fermentation runs (Hopkins *et al.*, 2011) to determine whether they would also display increased fermentation robustness. In order to determine the fermentation robustness of these strains, their lysis scores were measured after determined periods of induction at 32 °C. A lysis score of 0.5-5.0 was assigned based on microcopic examination. A lysis score of 0.5 indicates minimal lysis (more than 95% intact cells), and a lysis score of 5 indicates high lysis (less than 10% intact cells). As observed previously, the YGLY17108 control strain displayed heavy lysis and was not viable within 24 hours of MeOH induction at 32 °C. In contrast, the strains harboring the complete deletion and various truncations of the *ATT1* gene showed a remarkable increase in fermentation robustness and successfully completed 4 days of MeOH induction (Table 4). The ATT1 (1-164aa, SEQ ID NO:10) and ATT1 (1-655aa, SEQ ID NO:11) truncation mutants displayed slightly higher degrees of cell lysis (lysis score of 2.5 or 3) during the later stages of the induction phase (see Table 3). Strains containing the complete deletion and the ATT1 frame-shift (5bp insertion at 31aa) mutant displayed the highest level of fermentation robustness, with lysis scores of 0.5 throughout the 4 day induction phase. The finding that the strain harboring the 5bp insertion at 31aa exhibited the same robustness as the *ATT1* deletion mutant indicated that the 5bp frame-shift mutation likely results in the complete disruption of the function of *ATT1* gene product. On the other hand, the ATT1 (1-164aa, SEQ ID NO:10, encoded by SEQ ID NO:4) and ATT1 (1-655aa, SEQ ID NO:11, encoded by SEQ ID NO:5) truncated forms of *ATT1* might still retain some residual levels of transcriptional activity, because mutants harboring these truncations displayed an intermediate phenotype: dramatically more robust than the parental control, but not as strong as that of the deletion mutant. The phenotypes exhibited by these directed gene-replacement strains closely resembled those displayed by the corresponding UV-induced mutants (for example, compare YGL27601 with YGL17159; YGLY27608 with YGL17177; YGLY27610 with YGLY17172 in Table 4 and Figures 6A-C),

illustrating that the mutations within the *ATT1* gene were responsible for the improved thermal tolerance and fermentation robustness observed from the UV-induced mutants.

[00107] Table 4: Lysis scores of parental controls compared to Pp*ATT1* deletion and truncation strains fermented at 32°C.

1L Bioreactor MeOH Induction Phase		Lysis @ 32C			
		Day 1	Day 2	Day 3	Day 4
YGLY17108	empty parent control	5*		harvest	
YGLY27611	ATT1 complete KO	0.5	0.5	0.5	0.5
YGLY27601	ATT1 w/ 5bp insertion @ 31 aa	0.5	0.5	0.5	0.5
YGLY27624	ATT1 w/ 5bp insertion @ 31 aa	0.5	1	1/1.5	1/1.5
YGLY27630	ATT1 (1-164aa) fragment	0.5	0.5	0.5	0.5
YGLY27608	ATT1 (1-164aa) fragment	0.5/1	1	1/1.5	2
YGLY27610	ATT1 (1-655aa) fragment	0.5/1	1/1.5	2/2.5	3
YGLY27633	ATT1 (1-655aa) fragment	0.5	1.5	1.5/2	3

* lysis score: 0 - 5, with 5 indicating the greatest amount of lysis (i.e. worst lysis)

EXAMPLE 5

Construction of plasmids to overexpress the *P. pastoris* ATT1 ORF and truncated alleles

[00108] The *P. pastoris* TEF promoter (SEQ ID NO: 6) was synthesized using oligonucleotides and manufacturer's methods as described by Genscript (Piscataway, NJ) and cloned into the plasmid, pGLY8369 (Figure 10) using the restriction sites BglII/EcoRI to generate plasmid pGLY9959 (Figure 11). Plasmid pGLY8369 is a roll-in *Escherichia coli*/*P. pastoris* shuttle plasmid that contains the *P. pastoris* AOX1 promoter and the *S. cerevisiae* ARR3 gene as a selectable marker, which confers resistance to arsenite. The *P. pastoris* wild type ATT1 open reading frame (Pp01g00680, SEQ ID NO:1) was synthesized using oligonucleotides by Genscript and manufacturer's methods, and inserted into pGLY8369 (Figure 10) and pGLY9959 (Figure 11) using restriction sites EcoRI/FseI to generate plasmids pGLY9955 (Fig. 14A) and pGLY9960 (Fig. 14E), respectively. Truncated versions or fragments of ATT1 encoding the first 107 amino acids (1-107 aa, SEQ ID NO:3) of SEQ ID NO:7, the first 164 amino acids (1-164 aa, SEQ ID NO:4) of SEQ ID NO:7 and the first 655 amino acids (1-655 aa, SEQ ID NO:5) of SEQ ID NO:7 were subsequently generated from pGLY9955 (Fig. 14A) to yield the AOX1-driven ATT1 truncated allele containing plasmids, named pGLY9956 (Fig. 14B), pGLY9957 (Fig. 14C), pGLY9958 (Fig. 14D), respectively, and from pGLY9960 (Fig. 14E) to yield the TEF-driven ATT1 truncated allele

containing plasmids, named pGLY9961 (Fig. 14F), pGLY9962 (Fig. 14G), pGLY9963 (Fig. 14H), respectively.

EXAMPLE 6

Generation of an Anti-Her2 Monoclonal Antibody in Glyco-engineered *P. pastoris* Strains

[00109] Plasmid pGLY5883 was generated by fusing DNA sequences encoding the κ and γ chains of the Trastuzumab anti-HER2 monoclonal antibody (Carter, 1992) individually to the *P. pastoris* AOX1 promoter and is depicted in Figure 12. DNA from this plasmid was digested with SpeI to linearize the DNA and was transformed by standard electroporation methods (*Pichia* kit, Invitrogen, Carlsbad, CA) into the *P. pastoris* glyco-engineered strain YGLY8316, which has been modified to produce complex-type human N-glycans with terminal β-1,4-galactose (described as GFI5.0; Davidson U.S. Patent No. 7,795,002) (strain lineage shown in Figure 13). Clones were selected on medium containing Zeocin and further screened by standard cultivation in 96 deep well plates and 0.5L Sixfors multifermentation fermenters (ATR Biotech, Laurel, MD; Barnard, 2010). One positive expression clone was picked and named YGLY13979 (strain lineage shown in Figure 13).

EXAMPLE 7

Over-expression of the *P. pastoris* ATT1 Open Reading Frame and Truncated Alleles Leads to Improved Strain Survival and Protein Titer

[00110] Plasmids pGLY9955, pGLY9956, pGLY9957, pGLY9958, pGLY9960, pGLY9961, pGLY9962, and pGLY9963 (Figures14A-H, respectively) were linearized with SpeI and transformed by electroporation (*Pichia* kit, Invitrogen, Carlsbad, CA) into strain YGLY13979 and clones were selected on YSD medium containing 1 mM and 3 mM sodium arsenite. Clones that overexpress the full length and various truncations of ATT1 were further isolated on YSD media and then confirmed to contain the desired ATT1 expression constructs by PCR using primers RCD1019 (SEQ ID NO:19 5'-CAGATCTTCCAACATTCGTACACG-3') and AOX1seq (SEQ ID NO:20 5'-GCTTACTTTCATAATTGCGACTGGTTCC-3') for the AOX1-driven constructs and RCD1019 and TEFseq (SEQ ID NO:21 5'-CGCAGTCCCACACGCACTCGTACATG-3') for the TEF-driven constructs.

[00111] PCR positive clones were selected and cultivated in a modified version of an Applikon (Foster City, CA) micro24 5ml mini-fermenter apparatus, along with the parental

strain YGLY13979 (Figure 13, also expressing the same anti-HER2 mAb). Seed cultures were prepared by inoculating strains from YSD plates to a Whatman 24-well Uniplate (10 ml, natural polypropylene) containing 3.5 ml of 4% BMGY medium (Invitrogen, Carlsbad, CA) buffered to pH 6.0 with potassium phosphate buffer. The seed cultures were grown for approximately 65-72 hours in a temperature controlled shaker at 24°C and 650 rpm agitation. One millilter of the 24 well plate grown seed culture and 4.0ml of 4% BMGY medium was then used to inoculate each well of a Micro24 plate (Type : REG2). Thirty microliters of Antifoam 204 (1:25 dilution, Sigma Aldrich) was added to each well. The Micro24 was operated in Microaerobic1 mode and the fermentations were controlled at 200% dissolved oxygen, pH at 6.5, temperature at 24°C and agitation at 800rpm. The induction phase was initiated upon observance of a dissolved oxygen (DO) spike after the growth phase by adding bolus shots of methanol feed solution (100% [w/w] methanol, 5 mg/l biotin and 12.5 ml/l PTM2 salts), 50µl in the morning and 125µl in the afternoon. After approximately 72 hours of methanol induction, the cell-free culture supernatant was harvested by centrifugation at 2500 x g in a Beckman swinging bucket centrifuge and subjected to protein A purification by standard methods (Jiang, 2011). Antibody was quantified by reverse phase HPLC and calculated on a per liter basis. Supernatant was also subjected to the Picogreen (Invitrogen, Carlsbad, Ca) double stranded DNA quantification assay as a method to quantitatively measure cell breakage or lysis.

[00112] In an experiment comparing over expression of the wild-type full length ATT1 vs. the 1-655aa ATT1 truncation, six clones of each were evaluated, including TEF and AOX1 promoter versions of the 1-655 ATT1 truncation. Two of the six wild-type ATT1 over-expression strains produced significantly more antibody than the YGLY13979 control strains (Fig. 16). The control strains produced 587.5 +/- 70 mg/L of antibody whereas two AOX1-ATT1 full length over-expression strains produced 806 and 934 mg/L, respectively (Fig. 16). Similarly two of the AOX1-ATT1 1-655 over-expression strains also produced significantly more secreted mAb, with 906 and 1098 mg/L, respectively. Moreover, in all of the AOX1-ATT1 full length and AOX1-ATT1 1-655 over-expression strains, the lysis, as determined by picogreen assay, was reduced compared to the control strains (Figure 15). Finally, when the 1-655 ATT1 truncation was expressed under control of the TEF promoter, lysis was unaffected or potentially increased (Figure 15), while all of the clones still produced increased levels of secreted mAb (Figures 16-17).

EXAMPLE 8

Impact of ATT1 Full-Length Over-expression on Protein Productivity and Lysis is Scalable

[00113] The impact of over-expression of the ATT1 full length ORF on antibody productivity can be demonstrated clearly in microfermentation models. However, these models differ from full scale fermentation cultivation in several key aspects, including vessel size, shear and oxidative stress on the cells, and method of carbon source feed (bolus versus limiting or excess feed). To demonstrate the scalability of the ATT1 over-expression, six *Pichia* strains over-expressing ATT1 full length ORF (denoted as FL): (YGLY27927, YGLY27928, YGLY27929, YGLY27930, YGLY27931, and YGLY27932) along with the parental (YGLY13979) and ATT1 knockout (YGLY27638) control anti-HER2 expressing strains were cultured in 1L Fedbatch Pro fermenters (DASGIP Biotools, Shrewsbury, MA) using a glycerol feedbatch followed by limiting-methanol feed induction process at 24°C as previously described (Hopkins *et al.*, 2011). Methanol induction was continued until lysis was too severe to continue for each strain (up to 113h), after which the cell-free culture supernatant was harvested by centrifugation at 2500 x g in a Beckman swinging bucket centrifuge and subjected to protein A purification by standard methods (Jiang, 2011). Antibody was quantified by reverse phase HPLC and calculated on a per liter basis. The YGLY13979 parental control strain produced 671 mg/L of secreted mAb but with very high lysis after 66h of induction (Figure 17).

[00114] In contrast, the ATT1 knockout strain produced 1256 mg/L in 106h of induction with minimal lysis (Figure 17), consistent with previous results. Similarly, and consistent with smaller scale micro24 fermentation results, two AOX1-ATT1 over-expressing clones produced 1974 and 1960 mg/L, respectively, in 112h of induction, surprisingly demonstrating that over-expression of the ATT1 ORF can positively affect robustness and productivity in extended induction at larger scale (Figure17). Additionally, three of the six ATT1 over-expressing clones displayed reduced lysis compared to the YGLY13979 control while another clone only exhibited lysis after more than 100h of induction (Figure 17). These results demonstrate that the impact of the ATT1 over-expression on mAb productivity in recombinant protein-producing *Pichia* strains is independent of scale and is not affected by standard variations in culture and induction protocols.

[00115] To further illustrate the impact of the AOX-ATT1 over-expression on glyco-engineered strain robustness, the two high mAb expression strains, YGLY27929 and YGLY27930 were cultivated again in the same 1L Fedbatch Pro fermenters using the same

protocol but at 32°C instead of 24°C. Based on previous experiments, the parental strain, YGLY13979, is not capable of fermentation at elevated temperature, with a complete loss due to foaming and severe cell lysis typically occurring 24-48h into methanol induction. After 50h of methanol induction at 32°C the two over-expression strains YGLY27929 and YGLY27930 produced 473 and 458 mg/L of anti-HER2 mAb compared to 842 mg/L for the ATT1 knockout strain (in 73 hours of induction). Lysis of the over-expression strains was slightly elevated compared to the ATT1 knockout strain, but survival to 50h of induction with moderate lysis represents a significant improvement over the parental glyco-engineered anti-HER2 expressing strain, and demonstrates the utility of over-expression of ATT1 in addition to deletion/truncation of this gene.

[00116] Strains described in Examples 6 to 8 all have the chromosomal copy of ATT1 still intact. Over-expression of full-length ATT1 or ATT1 truncations (both AOX1- and TEF- promoter driven) in strains with their endogenous ATT1 ORF deleted are expected to produce similar improvements in cell robustness and productivity as shown in Examples 6 to 8.

EXAMPLE 9

Hansenula polymorpha* ATT1 Ortholog Functionally Complements the ATT1 null mutation in *Pichia pastoris

[00117] The *H. polymorpha* ATT1 ortholog (GenBank EFW98022.1) was identified by performing BLAST sequence similarity search against the GenBank non-redundant protein database. Figure 18 shows an alignment of *S. cerevisiae* Gal4, *P. pastoris* ATT1 and *H. polymorpha* ATT1.

[00118] *H. polymorpha*, also as known as *Ogataea parapolymorpha* or *Pichia angusta*, is a methylotrophic yeast commonly used for expressing heterologous recombinant proteins. Similar to *P. pastoris*, *H. polymorpha* also has lost its ability to utilize galactose as a carbon source because it has lost most of the genes involved in galactose metabolism. When we searched the GenBank non-redundant protein database by BLAST, we identified a *H. polymorpha* ATT1 ortholog (GenBank EFW98022.1) that displays 41% amino acid sequence identity over the entire length of the proteins. To evaluate if the *H. polymorpha* ATT1 ortholog (HpATT1) would perform similar functions as PpATT1, we cloned the HpATT1 open reading frame (ORF) downstream of the native PpATT1 promoter, and stably integrated this DNA construct at the URA6 locus of YGLY30547, which has its endogenous ATT1 already deleted. For comparison, we similarly integrated the *S. cerevisiae* ATT1 ortholog

(ScGAL4) ORF at the URA6 locus of YGL30547 as well. To test what effects these ATT1 orthologs had on the temperature-resistant phenotype caused by the PpATT1 gene deletion, we randomly picked independent transformants containing either HpATT1 or ScGAL4 genes, patched them onto regular YPD agar plates, and monitored their growth at the permissive 24C and non-permissive 35C.

[00119] All ScGAL4-containing clones remained to be temperature resistant, just as the parent YGLY27611 does. These results indicated that, under the testing condition used in this experiment, the ScGAL4 could not substitute PpATT1's function in *P. pastoris*. In contrast, the HpATT1-containing transformants failed to grow at 35C, thus reverting the temperature-resistant phenotype of YGLY30547 back to temperature-sensitivity. These results demonstrated that the HpATT1 was able to functionally substitute PpATT1's activity *in vivo*.

EXAMPLE 10

PpATT1 Truncations Screening for Improved Fermentation Robustness

[00120] To further explore the effects of ATT1 truncations on cell robustness, we constructed a series of new strains, in which the endogenous ATT1 ORF was deleted and replaced by DNA cassettes encoding varies length of the N-terminal portion of the ATT1 gene. The DNA cassettes were constructed very similarly to pGLY5948 or pGLY5949 described in Example 4, except that they contain, in their 5' cross-over region, DNA fragments encoding for the N-terminal 1-143aa, 1-196aa, 1-216aa, 1-276aa, 1-296aa, 1-341aa, 1-539aa, 1-622aa, 1-655aa, 1-728aa, 1-802aa, or 1-828aa of the ATT1 protein of SEQ ID NO:7. For comparisons, we also tested: a complete ATT1 deletion strain, as well as strains harboring the N-terminal 1-31aa, 1-164aa fragments of ATT1 which were described in Example 4.

[00121] In order to also examine how these truncated ATT1 fragments influence product titers, we transformed these DNA constructs into YGLY19315, a URA5-minus derivative of the YGLY13979 strain described above which expresses anti-HER2 mAb. These strains harboring the ATT1 deletions or truncations were cultivated in 1L bioreactors at 32°C using methanol-limited fedbatch fermentation process as described in Example 4. Cell lysis was monitored by microscopy every day, and fermentation was terminated when excess lysis was observed. If cell lysis levels were low, the fermentations were run up to between 100 to 120 hours of methanol induction. At the end of each fermentation run, cell-

free supernatants were collected and mAb titers were determined using a protein A based HPLC method as described in Example 7.

[00122] The results of these experiments are shown in Figs. 19A and 19B. The control strain, which contained the full-length ATT1 gene, only survived ~ 40 hours of methanol induction at 32°C (Figure 19A). The ATT1 deletion strains lasted more than 80 hours induction, demonstrating that inactivating ATT1 activity dramatically improved fermentation robustness during fermentation. Surprisingly, the ATT1 (1-296 aa) fragment did not provide any robustness protection during fermentation. All other ATT1 truncations clearly improved fermentation robustness to different extents, with the 1-216aa, 1-341aa, 1-655aa, 1-802aa, and 1-828aa fragments providing modest levels of improvements, and 1-31aa, 1-143aa, 164aa, 1-196aa, 1-276aa, 1-539aa, 1-622aa and 1-728aa fragments providing strong improvements. Among all the truncations, the strain containing ATT1 (1-164aa) fragment appeared to display the highest level of robustness during these fermentation runs. As for product titers (Figure 19B), only the 1-828aa ATT1 fragment significantly reduced to mAb titer to ~ 100 mg/liter, whereas strains containing other ATT1 fragments yielded between ~ 600 to 1200 mg/liter mAb, which are not significantly different than the ~ 1000 mg/liter titer observed from strains without any ATT1-modicifations.

EXAMPLE 11

ATT1 (1-31aa) and (1-164aa) Fragments are Dominant in the Presence of Full-Length ATT1 Gene

[00123] As shown in Example 10, the strain containing ATT1 (1-164aa) fragment exhibited the most robust phenotype. Because the 1-164aa fragment contains the complete DNA-binding and dimerization regions, but is devoid of any transcriptional activation regions of the protein, the 1-164aa fragment could bind to the promoters of the target genes as non-active dimers, and prevent other transcriptional factors that might activate these target genes from binding, thus resulting in transcriptional repressions. If this is the case, one could predict that the 1-164aa ATT1 fragment should also compete with the full length ATT1 protein for promoter-binding and interfere with the ATT1 normal transcriptional activities. To verify this hypothesis, we introduced an ATT1 (1-164aa)-containing DNA construct into strain YGLY13979, which harbors the full-length ATT1 gene, and examined its effect on cell robustness during fermentation using 1L bioreactor. As a control, we also transformed an ATT1 (1-31aa)-containing DNA construct into YGLY13979. Because the 1-31aa ATT1 fragment is upstream of the DNA-binding and dimerization domain (and is absence from the

ScGAL4 protein), we expected that it would not interfere with the DNA-binding and transcriptional functions of the full-length ATT1 gene present in the YGLY13979 host. As shown in Figs. 19A and 19B (bottom two bars), strains containing (1-164aa fragment + ATT1 full length) and (1-31aa fragment + ATT1 full length) lasted more than 100 hours of methanol induction at 32°C, demonstrating that both 1-164aa and 1-31aa fragments provided cell robustness protection in a dominant-fashion (i.e. in the presence of the wild type full length ATT1). The dominant-nature of the 1-164aa ATT1 fragment is consistent with the hypothesis described above. However, the observation that the 1-31aa fragment also exhibited a dominant-negative effect over the wild type ATT1 full-length gene was surprising.

EXAMPLE 12

ATT1 deletion and truncations improved strain robustness in 15L bioreactors

[00124] For all previous examples, fermentation robustness was all evaluated using 1 liter lab-scale bioreactors. To see if ATT1-modification would provide the same robustness improvement in a larger bioreactor, we selected several strains containing either complete ATT1 deletion or distinct ATT1 truncations and performed methanol-limited fed-batch fermentations in 15L bioreactors at 24° as described by Potgieter et al. 2009. Cell lysis, cell growth, and product titer were monitored throughout the fermentation process, and the fermentation runs were terminated once the cell growth stopped and cell lysis became excessive.

[00125] As shown in Figure 20, the control strain (YGLY13979), which contains the wild type ATT1 gene intact, normally lasted around 120 hours of induction, at which time point the cell lysis became too high to continue.

[00126] For YGLY30539, which contains the ATT1 (1-164aa) fragment and the full-length ATT1 gene simultaneously, it became significantly more robust than the control strain and was able to survive more than 150 hours of induction. This finding confirmed that the 1-164aa fragment was capable of improving cell robustness even in the presence of the full length ATT1 gene.

[00127] The strain containing the full-length ATT1 overexpression cassette also displayed approximately the same level of robustness improvement, and was viable for about 170 hours after induction.

[00128] The strain containing the 1-655aa ATT1 fragment had a more pronounced fermentation robustness, and was viable for more than 200 hours after induction

[00129] Strains harboring the complete ATT1 deletion or the 1-31aa fragment provided even further robustness protection, and both survived more than 250 hours of induction.

[00130] Remarkably, the 1-164aa fragment containing strain displayed the highest level of cell robustness, and finished more than 340 hours of induction at 24°C in the 15L bioreactor.

[00131] Collectively, these results demonstrated that ATT1-deletions, truncations, and selected form of overexpressions as described above, can dramatically improve the cell robustness during fermentations runs, not only in 1L small-scale bioreactors, but in 15L-scale bioreactors as well. These findings strongly suggested the effects of these ATT1-modifications on cell robustness were easily scalable, and most likely would remain the same at larger fermentation scales (i.e. pilot-plant or production-scale) as well.

EXAMPLE 13

ATT1 deletion also improves cell robustness in other Pichia strains

[00132] *P. pastoris* glycoengineered strain YGLY23506 is a GFI2.0 strain, which secretes proteins with predominantly $Man_5GlcNAc_2$ N-glycans. (The construction of other *Pichia* strains, which are able to secrete $Man_5GlcNAc_2$ has been previously described in Choi et al., 2003.) This host strain lineage has been sequentially modified by knockout of the yeast OCH1 gene as well as several other members of the mannosyl transferase family, including the four BMT β-mannosyl transferase genes and phosphomannosyl transferases. This strain engineering also includes expression of an alpha-1,2-mannosidase, which results in trimming of the core $Man_8GlcNAc_2$ N-glycan to a uniform $Man_5GlcNAc_2$ form form. This particular strain has also been engineered to secrete an N-terminal H9G3 histidine-tagged soluble form of HIV gp120 from strain JR-FL (Varadarajan, 2005; Pang, 1991; Genbank AAB05604.1) containing amino acids 33-556 fused to the *S. cerevisiae* alpha factor prepro signal peptide.

[00133] Strain YGLY23506 was then transformed with plasmid pGLY5952, the Ppatt1::ArsR knockout plasmid (Figure 21) using standard electroporation and clones were selected on YSD medium containing 1mM sodium arsenite. Positive knockout clones were identified by PCR as described above. PCR confirmed strains YGLY30447, YGLY30448, YGLY30449, and YGLY30450 were saved as GFI2.0 att1Δ clones derived from YGLY23506.

[00134] *P. pastoris* glycoengineered strain YGLY23512 is a GFI1.0 strain, which secretes proteins with predominantly $Man_{8-10}GlcNAc_2$ N-glycans. (The construction of other

Pichia strains, which are able to secrete $Man_{8-10}GlcNAc_2$ has been previously described in Choi et al., 2003.) This host strain lineage has been modified by knockout of the yeast N-glycosylation machinery similar to YGLY23506 but this strain does not express an alpha-mannosidase. This particular strain has also been engineered to secrete an N-terminal H9G3 histidine-tagged soluble form of HIV gp120 similar to strain YGLY23506, in this case fused to the *S. cerevisiae* alpha factor pre signal peptide. Strain YGLY23512 was transformed with plasmid pGLY5952 (Figure 21), the Ppatt1::ArsR knockout plasmid using standard electroporation and clones were selected on YSD medium containing 1mM sodium arsenite. Positive knockout clones were identified by PCR as described above. PCR confirmed strains YGLY30451, YGLY30452, YGLY30453, and YGLY30454 were saved as GFI1.0 att1Δ clones of YGLY23512.

[00135] Strains YGLY23506, YGLY23512 and att1Δ versions of these strains were cultivated in 1L fermentation to assess the robustness of these GFI2.0 and GFI1.0 glycoengineered strains.

[00136] Fermentation runs were carried out in a 1L (0.75L working volume) bioreactor from Sartorius. The 1L system was controlled by Sartorius Biostat Q controllers with closed loop control of pH, temperature, and dissolved oxygen concentration. Shake flask cultures were grown in 4% BSGY medium in 1.0L baffled flask at 24°C while shaking for 48h. Shake flask culture was then aseptically transferred into a 1.0L Sartorius vessel containing 0.75L of BSGY media at intial OD600 to 2. A standard glycerol-to-methanol fed-batch process was performed, including standard Pichia media components, as previously described (Potgieter et al, 2008). At the end of the transition phase a dose of 1.33 mL/L of methanol containing 2.5mg/mL Pepstatin A and 1.59mg/mL Chymostatin, 0.64 mg/ml PMTi4 was added prior to methanol induction. The culture temperature was measured by the pt100 sensor and controlled at 24 ± 0.5 °C. The pH was controlled at 6.0 ± 0.1 during batch and fed-batch and 5.0 ± 0.1 during induction phase using 30% NH4OH. No acid addition was done and the pH was allowed to reach the set point before inoculation using 30% NH4OH. The airflow was controlled at 0.7 vvm for 1L. The DO set point of 20% (of saturation with air at atmospheric conditions, 1.7mg/L) was maintained throughout the run by agitation speed (450 - 1200 rpm) cascaded onto O2 addition to the fixed airflow rate. Foaming was controlled by the addition of antifoam (Antifoam 204, Sigma-Aldrich) in the initial batch medium. An initial charge of 0.128 g/L of antifoam was added to the media before inoculation. A constant feed of 100% Methanol containing PTM2 and Biotin was initiated after the transition phase at constant feed rate (1.5 g/l/hr). The pH was gradually decreased to 5 from 6 in 120 min (line increment to

6.5 in 60 min whereas to 7 in further 60 min). Temp was maintained at 24°C. Every 24 hours of induction, 1.33 mL/L of methanol containing 2.5 mg/mL Pepstatin A and 1.59 mg/mL of Chymostatin was added. At the end of induction, all controls and pumps were stopped. Harvest was performed at room temperature. Primary clarification was then performed by centrifugation. The whole cell broth was transferred into 800ml centrifuge bottles and centrifuged at 4°C for 30 minutes at 13,000 x g (8500RPM).

[00137] Samples were taken from each fermenter approximately every 24h starting prior to induction by removing 5ml of broth, centrifuging in a tabletop centrifuge and removing the supernatant for analysis.

[00138] Fermentation cultured supernatants from strain YGLY23506, parental ATT1 WT GFI2.0 expressing secreted HIV gp120, and strains YGLY30447, YGLY30448, YGLY30449, and YGLY30450, att1Δ clones of YGLY23506, were separated on SDS-PAGE and Coomassie stained by standard means. The stained SDS-PAGE gel is shown in Figure 22. The supernatant protein visible in the att1 knockout strains is significantly reduced compared to the parental GFI2.0 strain, particularly after methanol induction was initiated when the parental strain started extensively lysing, as apparent from the heavy burden of supernatant protein after 18h of induction. This fermentation was ended due to extensive lysis after 66h of induction. Conversely, the att1Δ strains still showed only a modest supernatant protein burden after more than 80h of induction compared to the YGLY23506 parental strain at 18h (Figure 22).

[00139] In addition to SDS-PAGE, fermentation supernatants were analyzed by quantification of supernatant DNA using the Picogreen reagent (Invitrogen) as previously described (Barnard, 2010). Similar to the SDS-PAGE results, the supernatant DNA assay revealed that the YGLY23506 accumulated significantly more DNA at similar timepoints compared to the att1Δ clones. Moreover, the att1Δ clones maintained moderate supernatant DNA loads after 192h of fermentation (159h of induction) indicating at the GFI2.0 att1Δ clones are significantly more robust during standard Pichia fermentation process than the parental ATT1 wild type GFI2.0 comparator (Figure 23).

[00140] Similarly to the GFI2.0 strains above, fermentation cultured supernatants from strain YGLY23512, parental ATT1 WT GFI1.0 expressing secreted HIV gp120, and YGLY30451, YGLY30452, YGLY30453, and YGLY30454, att1Δ clones of YGLY23512, were separated on SDS-PAGE and Coomassie stained by standard means. The stained SDS-PAGE gel is shown in Figure 24. The supernatant protein visible in the att1 knockout strains is significantly reduced compared to the parental GFI1.0 strain, particularly after methanol

induction was initiated when the parental strain started extensively lysing, as apparent from the heavy burden of supernatant protein. This fermentation was ended due to extensive lysis after 49h of methanol induction. Conversely, the att1Δ strains continued to show after than 90h of induction a very low to modest supernatant protein burden compared to the YGLY23512 parental strain at very early induction timepoints (Figure 24).

[00141] As with the GFI2.0 strains, in addition to SDS-PAGE, fermentation supernatants were analyzed by quantification of supernatant DNA. The picogreen assay revealed that the parental ATT1 wild type strain YGLY23512 accumulated significantly more supernatant DNA at similar timepoints compared to the att1Δ clones (Figure 25). Moreover, the att1Δ clones maintained moderate supernatant DNA loads after 193h of fermentation (165h of induction) indicating at the GFI1.0 att1Δ clones are significantly more robust during a standard Pichia fermentation process than the parental ATT1 wild type GFI1.0 comparator (Figure 25).

SUMMARY

[00142] Deletion or truncations in *ATT1* in naïve *Pichia* temperature-sensitive strains resulted in significant enhancements in both thermal-tolerance and fermentation robustness. The results described in Examples 1-4 have illustrated that the loss of function of the *Pichia* *ATT1* gene is the genetic basis for the improved thermal-tolerance and fermentation robustness in these glyco-engineered *Pichia* strains. The *ATT1* gene can be deleted or truncated in any *Pichia* strain and render the recipient strain highly robust during the fermentation induction phase, providing broad utility for any heterologous protein-expressing *Pichia* host strain where desired attributes include increased strain robustness and viability during fermentation, improved product yield, or reduced proteolytic degradation of the recombinant product.

[00143] Surprisingly, Examples 5-8, and 11 illustrate that engineered *Pichia* host strains overexpressing ATT1 under relevant bioprocess conditions also exhibit improved viability, stability, and protein production (Figures 15-17).

[00144] Currently, most of the marketed biologic therapeutics are produced using mammalian cell hosts. Over the past 30 years, through the collective efforts of the biotech and biopharmaceutical industry, the specific productivity of mammalian cell cultures has been improved for more than 100 fold, from ~ 50 mg per liter to ~ 5 g per liter range for mAb

production. Contributing factors for such yield improvements have been attributed to the advancements of growing mammalian cell cultures to very high cell densities (from 10^5 to 10^7 cells per ml), and, more importantly, the ability to maintain high levels of cell viability for an extended time-period (from ~ 100 hours to ~ 400 hours) (Wurm 2004). Improving cell robustness and viability in *Pichia* host strains by engineering *ATT1* overexpression, deletions or truncations enable higher production yield for recombinant protein therapeutics and facilitate the use of glyco-engineered *Pichia* as a recombinant-protein production platform.

[00145] **Glossary**

ScSUC2:	*S. cerevisiae* invertase
OCH1:	Alpha-1,6-mannosyltransferase
KlMNN2-2:	K. lactis UDP-GlcNAc transporter
BMT1:	Beta-mannose-transfer (beta-mannose elimination)
BMT2:	Beta-mannose-transfer (beta-mannose elimination)
BMT3:	Beta-mannose-transfer (beta-mannose elimination)
BMT4:	Beta-mannose-transfer (beta-mannose elimination)
MNN4L1:	MNN4-like 1 (charge elimination)
MmSLC35A3:	Mouse orthologue of UDP-GlcNAc transporter
PNO1:	Phosphomannosylation of N-linked oligosaccharides (charge elimination)
MNN4:	Mannosyltransferase (charge elimination)
ScGAL10:	UDP-glucose 4-epimerase
XB33:	Truncated HsGalT1 fused to ScKRE2 leader
DmUGT:	UDP-Galactose transporter
KD53:	Truncated DmMNSII fused to ScMNN2 leader
TC54:	Truncated RnGNTII fused to ScMNN2 leader
NA10:	Truncated HsGNTI fused to PpSEC12 leader
FB8:	Truncated MmMNS1A fused to ScSEC12 leader
CiMNS1:	Secreted Coccidioides immitis mannosidase I
STE13	Golgi dipeptidyl aminopeptidase
DAP2	Vacuolar dipeptidyl aminopeptidase
ALG3	dolichol-P-Man dependent alpha(1-3) mannosyltransferase
POMGNT1	protein O-mannose beta-1,2-N-acetylglucosaminyltransferase
LmSTT3D:	*Leishmania* major oligosaccharyl transferase subunit D

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Table 5	**List of Sequences and Brief Description**
SEQ ID NO:1	*P. pastoris* wild-type *ATT1* ORF
SEQ ID NO:2	mutant *P. pastoris* ATT1 with 5 bp insertion at position 92 produces truncation at amino acid 31
SEQ ID NO:3	mutant *P. pastoris* ATT1 with mis-sense mutation at position 322 produces truncation at amino acid 107
SEQ ID NO:4	mutant *P. pastoris* ATT1 with mis-sense mutation at position 493 produces truncation at position 164aa
SEQ ID NO:5	mutant *P. pastoris ATT1* with mis-sense mutation at position 1966 produces truncation at amino acid 655aa
SEQ ID NO:6	*P. pastoris* TEF promoter
SEQ ID NO:7	*P. pastoris* wild-type ATT1 amino acid sequence
SEQ ID NO:8	*P. pastoris* truncation mutant att1 resulting from the 5bp insertion (amino acids 1-31 att1 + mutant insert amino acids)
SEQ ID NO:9	*P. pastoris* truncation mutant att1p 1-107
SEQ ID NO:10	*P. pastoris* truncation mutant att1p 1-164
SEQ ID NO:11	*P. pastoris* truncation mutant att1p 1-655

SEQ ID NO:12	*P. pastoris* primer located 2370 bp upstream of ATT1 start
SEQ ID NO:13	*P. pastoris* primer within the ALG3 terminator to confirm the 5' junction of the gene-replacement
SEQ ID NO:14	Primer within lacZ
SEQ ID NO:15	*P. pastoris* primer located 2014 bp downstream of the ATT1 stop codon to confirm the 3' junction of the gene-replacement
SEQ ID NO:16	*P. pastoris* primer located 365 bp upstream of the ATT1 start
SEQ ID NO:17	*P. pastoris* primer within the ATT1 ORF, 1070 bp after the start to confirm the absence of the wild-type ATT1 ORF
SEQ ID NO:18	*P. pastoris* frame-shift insertion after the 31st amino acid residue of the ATT1 ORF
SEQ ID NO:19	*P. pastoris* primer located 1081bp downstream of the ATG start codon of ATT1 ORF
SEQ ID NO:20	*P. pastoris* primer located within the AOX1 promoter
SEQ ID NO:21	*P. pastoris* primer located within the TEF promoter
SEQ ID NO:22	*P. pastoris* AOX1 promoter
SEQ ID NO:23	*H. polymorpha ATT1* polypeptide
SEQ ID NO:24	*Pichia stipitis ATT1* polypeptide
SEQ ID NO:25	*Pichia guilliermondii ATT1* polypeptide
SEQ ID NO:26	*Kluyveromyces lactis ATT1* polypeptide
SEQ ID NO:27	*Aspergillus niger ATT1* polypeptide
SEQ ID NO:28	*Aspergillus nidulans ATT1* polypeptide
SEQ ID NO:29	*Aspergillus flavus ATT1* polypeptide
SEQ ID NO:30	*Debaryomyces hansenii ATT1* polypeptide
SEQ ID NO:31	*Zygosaccharomyces rouxii ATT1* polypeptide
SEQ ID NO:32	*Sachharomyces cerevisiae* ATT1 polypeptide

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in

their entireties for all purposes. All references cited herein are incorporated by reference to the same extent as if each individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, was specifically and individually indicated to be incorporated by reference. This statement of incorporation by reference is intended by Applicants, pursuant to 37 C.F.R. §1.57(b)(1), to relate to each and every individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, each of which is clearly identified in compliance with 37 C.F.R. §1.57(b)(2), even if such citation is not immediately adjacent to a dedicated statement of incorporation by reference. The inclusion of dedicated statements of incorporation by reference, if any, within the specification does not in any way weaken this general statement of incorporation by reference. Citation of the references herein is not intended as an admission that the reference is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

The present invention is not to be limited in scope by the specific embodiments described herein; the embodiments specifically set forth herein are not necessarily intended to be exhaustive. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An engineered lower eukaryotic host cell that has been modified to reduce or eliminate the activity of an ATT1 gene.

2. The host cell of claim 1, wherein the activity of the ATT1 gene is reduced or eliminated by: (i) reducing or eliminating the expression of an ATT1 gene or polypeptide, or (ii) expressing a mutated form of an ATT1 gene.

3. The host cell of claim 1 or 2, further comprising a mutation, disruption or deletion of one or more genes encoding protease activities, alpha-1,6-mannosyltransferase activities, alpha-1,2-mannosyltransferase activities, mannosylphosphate transferase activities, β-mannosyltransferase activities, O-mannosyltransferase (PMT) activities, and/or dolichol-P-Man dependent alpha(1-3) mannosyltransferase activities.

4. The host cell of any one of claims 1-3, further comprising one or more nucleic acids encoding one or more glycosylation enzymes selected from the group consisting of: glycosidases, mannosidases, phosphomannosidases, phosphatases, nucleotide sugar transporters, nucleotide sugar epimerases, mannosyltransferases, N-acetylglucosaminyltransferases, CMP-sialic acid synthases, N-acetylneuraminate-9-phosphate synthases, galactosyltransferases, sialyltransferases, and oligosaccharyltransferases.

5. The host cell of any one of claims 1-4, further comprising a nucleic acid encoding a recombinant protein.

6. The host cell of claim 5, wherein the recombinant protein is selected from the group consisting of: an antibody (IgA, IgG, IgM or IgE), an antibody fragment, kringle domains of the human plasminogen, erythropoietin, cytokines, coagulation factors, soluble IgE receptor α-chain, urokinase, chymase, urea trypsin inhibitor, IGF-binding protein, epidermal growth factor, growth hormone-releasing factor, annexin V fusion protein, angiostatin, vascular endothelial growth factor-2, myeloid progenitor inhibitory factor-1, osteoprotegerin, α-1 antitrypsin, DNase II, α-feto proteins, insulin, Fc-fusions, and HSA-fusions.

7. The host cell of any one of claims 1-6, wherein the cell exhibits an increase in culture stability, thermal tolerance and/or improved fermentation robustness compared with an ATT1 naïve parental host cell under similar culture conditions.

8. The host cell of claim 7, wherein the cell is capable of surviving in culture at 32°C for at least 80 hours of fermentation with minimal cell lysis.

9. The host cell of any one of the above claims, wherein the host cell is glyco-engineered.

10. The host cell of any one of the above claims, wherein the host cell lacks OCH1 activity.

11. The host cell of any one of the above claims, wherein the host cell is a fungal host cell.

12. The host cell of any one of the above claims, wherein the host cell is a yeast host cell.

13. The host cell of any one of the above claims, wherein the host cell is a *Pichia sp.* host cell.

14. The host cell of claim 13, wherein the host cell is *Pichia pastoris*.

15. The host cell of claim 14, wherein the ATT1 gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:7 or a natural variant (polymorph) of said polypeptide.

16. The host cell of claim 13, wherein the host cell is *Hansenula polymorpha*.

17. The host cell of claim 16, wherein the ATT1 gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:23 or a natural variant (polymorph) of said polypeptide.

18. The host cell of any one of the above claims, wherein the host cell has been further modified to express a nucleic acid sequence encoding an ATT1 polypeptide or a fragment thereof.

19. An engineered lower eukaryotic host cell host cell, wherein the cell has a wild-type ATT1 gene in its native genomic state and has been modified to increase expression of an ATT1 polypeptide or a fragment thereof.

20. The host cell of any one of claims 19, further comprising a mutation, disruption or deletion of one or more genes encoding protease activities, alpha-1,6-mannosyltransferase activities, alpha-1,2-mannosyltransferase activities, mannosylphosphate transferase activities, β-mannosyltransferase activities, O-mannosyltransferase (PMT) activities, and/or dolichol-P-Man dependent alpha(1-3) mannosyltransferase activities.

21. The host cell of any one of claims 19 or 20, further comprising one or more nucleic acids encoding one or more glycosylation enzymes selected from the group consisting of:

glycosidases, mannosidases, phosphomannosidases, phosphatases, nucleotide sugar transporters, mannosyltransferases, N-acetylglucosaminyltransferases, UDP-N-acetylglucosamine transporters, galactosyltransferases, sialyltransferases, andoligosaccharyltransferases.

22. The host cell of any one of claims 19-21, further comprising a nucleic acid encoding a recombinant protein.

23. The host cell of claim 22, wherein the recombinant protein is selected from the group consisting of: an antibody (IgA, IgG, IgM or IgE), an antibody fragment, kringle domains of the human plasminogen, erythropoietin, cytokines, coagulation factors, soluble IgE receptor α-chain, urokinase, chymase, urea trypsin inhibitor, IGF-binding protein, epidermal growth factor, growth hormone-releasing factor, annexin V fusion protein, angiostatin, vascular endothelial growth factor-2, myeloid progenitor inhibitory factor-1, osteoprotegerin, α-1 antitrypsin, DNase II, α-feto proteins, insulin, Fc-fusions, and HSA-fusions.

24. The host cell of any one of claims claim 19-23, wherein the cell exhibits increase in culture stability, thermal tolerance and/or improved fermentation robustness compared with an ATT1 naïve parental host cell under similar culture conditions.

25. The host cell of claim 24, wherein the cell is capable of surviving in culture at 32°C for at least 80 hours of fermentation with minimal cell lysis.

26. The host cell of any one of claims 19-25, wherein the host cell is glyco-engineered.

27. The host cell of any one of claims 19-26, wherein the host cell lacks OCH1 activity.

28. The host cell of any one of the claims 19-27, wherein the host cell is a yeast host cell.

29. The host cell of any one of claims 19-28, wherein the host cell is a *Pichia sp.* host cell.

30. The host cell of claim 29, wherein the host cell is *Pichia pastoris*.

31. The host cell of claim 30, wherein the ATT1 gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:7 or a natural variant (polymorph) of said polypeptide or a fragment of said polypeptide.

32. The host cell of claim 30, wherein said fragment is selected from the group consisting of: amino acids 1-31 and 1-164 of SEQ ID NO:7.

33. The host cell of claim 29, wherein the host cell is *Hansenula polymorpha*.

34. The host cell of claim 32, wherein the ATT1 gene encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:23 or a natural variant (polymorph) of said polypeptide.

35. A method for producing a heterologous polypeptide in an engineered lower eukaryotic host cell, said method comprising: (a) introducing a polynucleotide encoding a heterologous polypeptide into the host cell of any one of claims 1-34; (b) culturing said host cell under conditions favorable to the expression of the heterologous polypeptide; and, optionally, (c) isolating the heterologous polypeptide from the host cell.

36. An isolated nucleic acid encoding a wild-type or mutated ATT1 gene or fragment thereof.

37. The isolated nucleic acid of claim 36, wherein an isolated host cell expressing said nucleic acid exhibits an increase in culture stability, thermal tolerance and/or improved fermentation robustness compared to an ATT1 naive parental host cell under similar conditions.

38. The nucleic acid of claim 36 or 37, selected from the group consisting of:

a. a nucleotide sequence encoding SEQ ID NO:7 or a fragment thereof,

b. a nucleotide sequence encoding SEQ ID NO:8 or a fragment thereof,

c. a nucleotide sequence encoding SEQ ID NO:9 or a fragment thereof ,

d. a nucleotide sequence encoding SEQ ID NO:10 or a fragment thereof, and

e. a nucleotide sequence encoding SEQ ID NO:23 or a fragment thereof.

39. The nucleic acid of clam 37, wherein said fragment comprises residues 1-31 or 1-164 of SEQ ID NO:7.

40. An isolated vector comprising the nucleic acid of any one of claims 36-39.

```
                1                                                  50
PpATT1     (1)  MHHKERLIDHISSESNFSLSTSSMPSFSHESNQSPNPMLIEQACDSCRKR
ScGAL4     (1)  ----------------------------------MKLLSSIEQACDICRLK
                51                                                 100
PpATT1    (51)  KLRCSKEYPKCSKCVTHKWSCVYSPRTVRSPLTRAHLTKVENRVRMLEDL
ScGAL4    (18)  KLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVESRLERLEQL

               101                                                 150
PpATT1   (101)  LERVFPTQSVDQLLEKRTSLSGNSTGHSPSYPNSNSVSPQNSSPKVSDSS
ScGAL4    (68)  FLLIFPREDLDMILKMDSLQDIKALLTGLFVQDN------------VNKD

               151                                                 200
PpATT1   (151)  STTAEPAPVLPSKPKSSFRPIVPDDYFLNDEINGFDWEEEDTPDQLLVMQ
ScGAL4   (106)  AVTDRLASVETDMP--------------------------------LTLR
               201                                                 250
PpATT1   (201)  QPPTSVDSTNVSHSYWNHSRRSQKNSVTSLNSLAEHEQSGCSSLITSPSL
ScGAL4   (124)  QHRISATSSEES-----SNKGQRQLTVSIDSAAHHDNSTIPLDFMPRDA
               251                                                 300
PpATT1   (251)  QPLSQTTTNDSHPDGMAALSVNLKGGSGYFGFSSSSGLLRALKLGQFDSA
ScGAL4   (169)  LHGFDWSEEDDMSDGLPFLKTDPNN-NGFFGDGSLLCILRSIGFK-----
               301                                                 350
PpATT1   (301)  SISPMSSVRNSVSKTNTEPTEPQSIRSLLGDPNDFLEPEKKAEFPGYDSH
ScGAL4   (213)  ---PENYTNSNVNRLPTMITDR--------------------------YT
               351                                                 400
PpATT1   (351)  LNDPNNQSQYLQAYFKYYHTSYPFIHKGSFLKHYAGELPIKNENHWQILL
ScGAL4   (234)  LASRSTTSRLLQSYLNNFHPYCPIVHSPTLMMLYNNQIEIASKDQWQILF
               401                                                 450
PpATT1   (401)  NVVLALGCWCLNGESSSIDLCYYNRAKMLLKQVGIFECGNIMLLESLILL
ScGAL4   (284)  NCILAIGAWCIEGESTDIDVFYYQNAKSHLTSK-VFESGSIILVTALHLL
               451                                                 500
PpATT1   (451)  SNYTQKRNKPNTGWSYLGIAIRMAMSLGLYKEFNLDHTEKDHYLNLEIRR
ScGAL4   (333)  SRYTQWRQKTNTSYNFHSFSIRMAISLGLNRDLPSSFSDSS---ILEQRR
               501                                                 550
PpATT1   (501)  RLWWGLYIFDAGASITFGRPITLPSRDSCDIQLCSNINDAELEELIEIKS
ScGAL4   (380)  RIWWSVYSWEIQLSLLYGR--------------------SIQLSQNTISFP
```

FIG.1A

```
                551                                                   600
PpATT1    (551) DSITTEDLNKPYPTAYSGLIQQTQFTELSMKIYNRLVSKPAP----TVEE
ScGAL4    (411) SSVDDVQRTTTGPTIYHGIIETARLLQVFTKIYELDKTVTAEKSPICAKK
                601                                                   650
PpATT1    (597) CLDMNMEIENFIKGLPAYFHESNEIAMSQFYKVTPSKYYDYDSNKQVDYT
ScGAL4    (461) CLMICNEIEEVSRQAPKFLQ------------------MDISTTALTNLL

                651                                                   700
PpATT1    (647) RLPQWFDLSRSRLIWRYKNLQITLFRAFIWQRVIGVTNPKVLQQCKTS-R
ScGAL4    (493) KEHPWLSFTRFELKWKQLSLIIYVLRDFFTNFTQKKSQLEQDQNDHQSYE
                701                                                   750
PpATT1    (696) GKECRTICLRVAHETILSIQQFVNIDDDDDFSRLSVIGCWYATYFLFQAV
ScGAL4    (543) VKRCSIMLSDAAQRTVMSVSSYMDNHN------VTPYFAWNCSYYLFNAV
                751                                                   800
PpATT1    (746) LIPIACLCSEPDSKYAP-IWIEDIQISKKIFLKLNKLN----SLASKFAN
ScGAL4    (587) LVPIKTLLSNSKSNAENNETAQLLQQINTVLMLLKKLATFKIQTCEKYIQ
                801                                                   850
PpATT1    (791) VIDRSMSQVMPQFDTTSAKDSPLNINDLIDMHGLMGNS-------P-APG
ScGAL4    (637) VLEEVCAPFLLSQCAIPLPHISYNNSNGSAIKNIVGSATIAQYPTLPEEN
                851                                                   900
PpATT1    (833) SNNNSNTKSSPSTTNNTRTPNTINKNNSNMNNNSINNYFNNNS-----NN
ScGAL4    (687) VNNISVKYVSPGSVGPSPVPLKSGASFSDLVKLLSNRPPSRNSPVTIPRS
                901                                                   950
PpATT1    (878) NNSFSSSKAGPVKQEFEDYCLKLDP----------------EDEDMSALEF
ScGAL4    (737) TPSHRSVTPFLGQQQQLQSLVPLTPSALFGGANFNQSGNIADSSLSFTFT
                951                                                  1000
PpATT1    (913) TAVRFPNFSATTTAPPPTPVNCNSPENIKTSTVDDFLKATQDP-NNKEIL
ScGAL4    (787) NSSNGPNLITTQTNSQALSQPIASSNVHDNFMNNEITASKIDDGNNSKPL
                1001                                                 1045
PpATT1    (962) NDIYSLIFDDSMDPMSFGSMEPRNDLEVPDTIMD----------
ScGAL4    (837) SPGWTDQTAYNAFGITTGMFNTTTMDDVYNYLFDDEDTPPNPKKE
```

FIG.1B

N-glycan MALDI traces of parent strains YGLY17108 and YGLY22835 compared with mutant strains YGLY17177 and YGLY17159

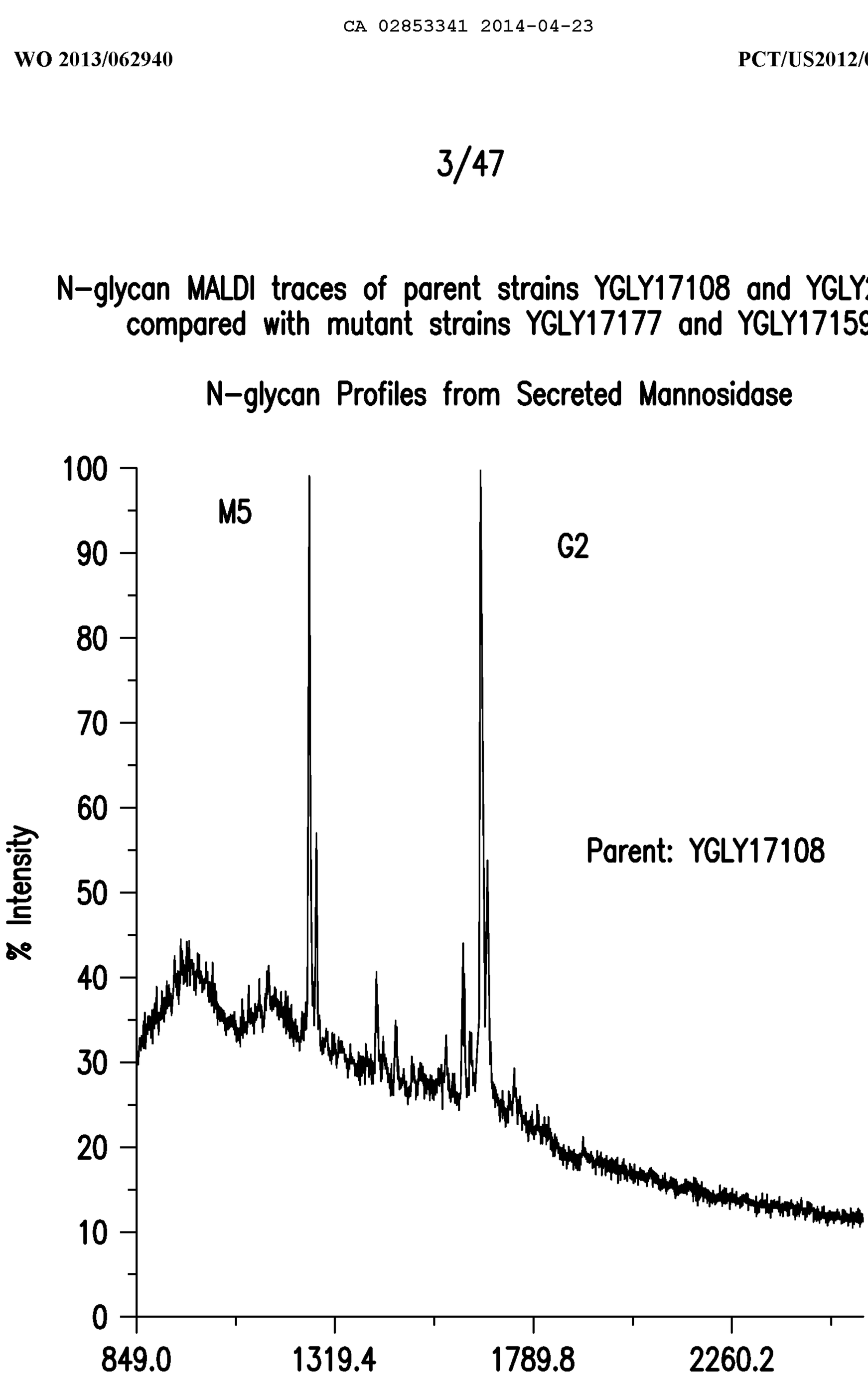

FIG.2A

N-glycan MALDI traces of parent strains YGLY17108 and YGLY22835 compared with mutant strains YGLY17177 and YGLY17159

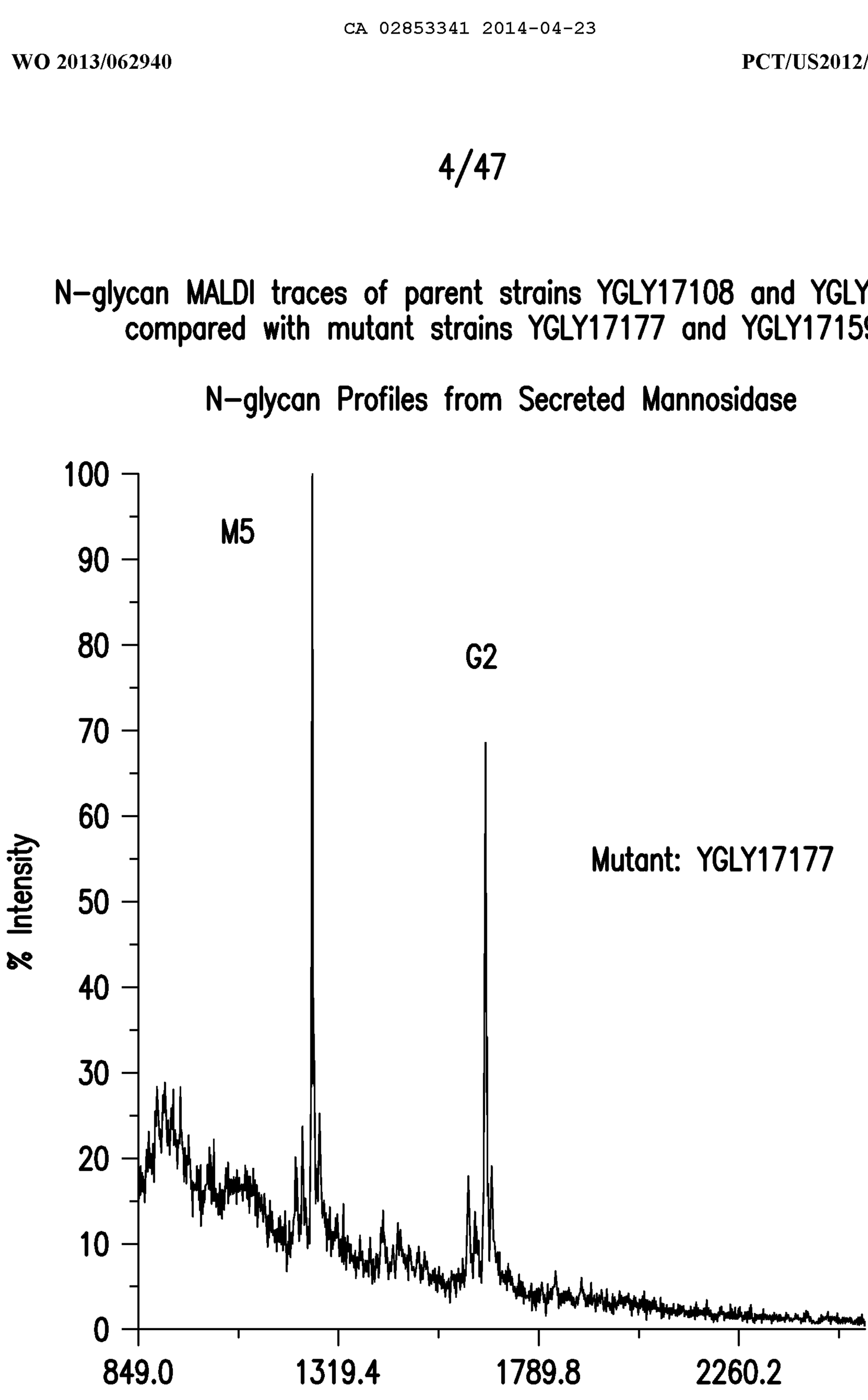

FIG.2B

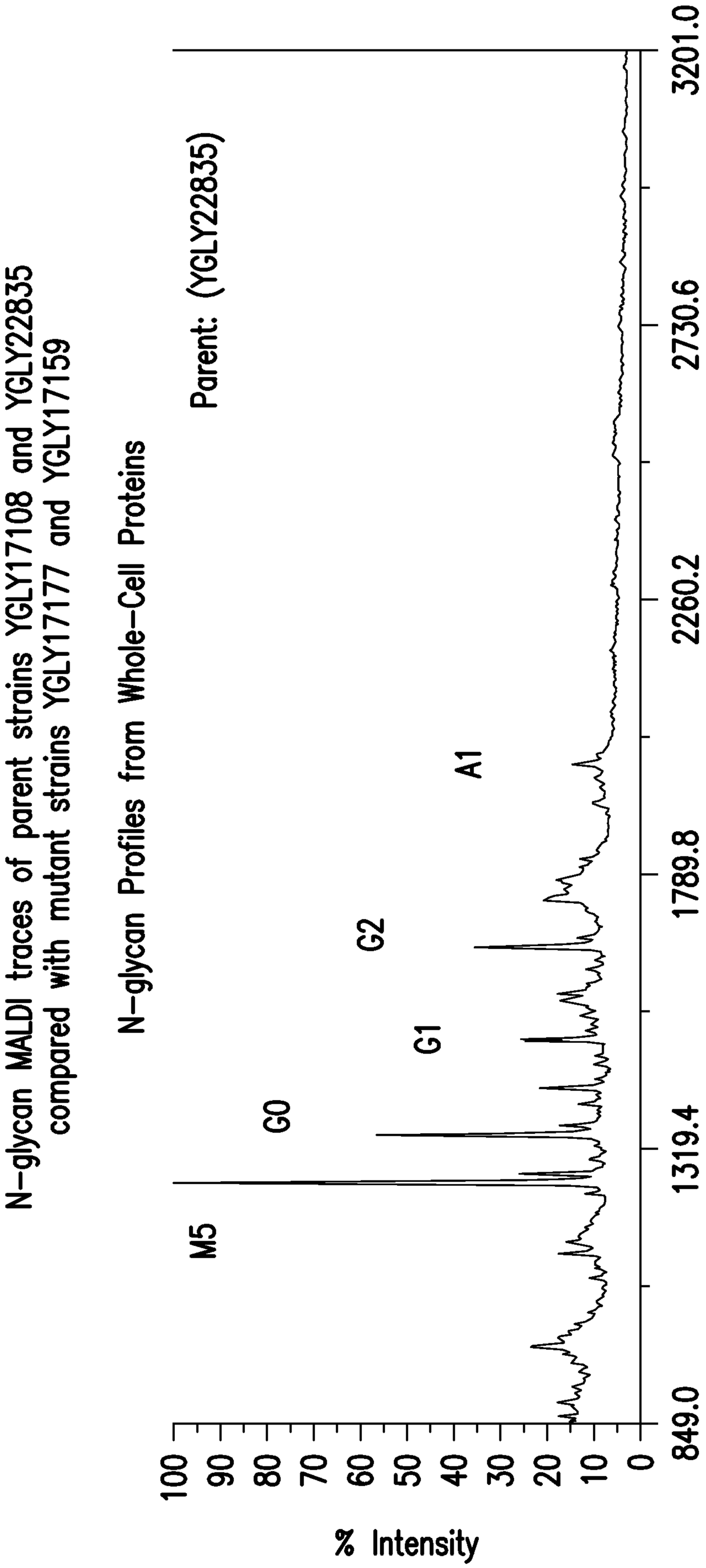
N-glycan MALDI traces of parent strains YGLY17108 and YGLY22835
compared with mutant strains YGLY17177 and YGLY17159
N-glycan Profiles from Whole-Cell Proteins
Parent: (YGLY22835)
M5
G0
G1
G2
A1
100
90
80
70
60
50
40
30
20
10
0
% Intensity
1319.4
1789.8
2260.2
2730.6
3201.0
849.0
Mass (m/v)

FIG.2C

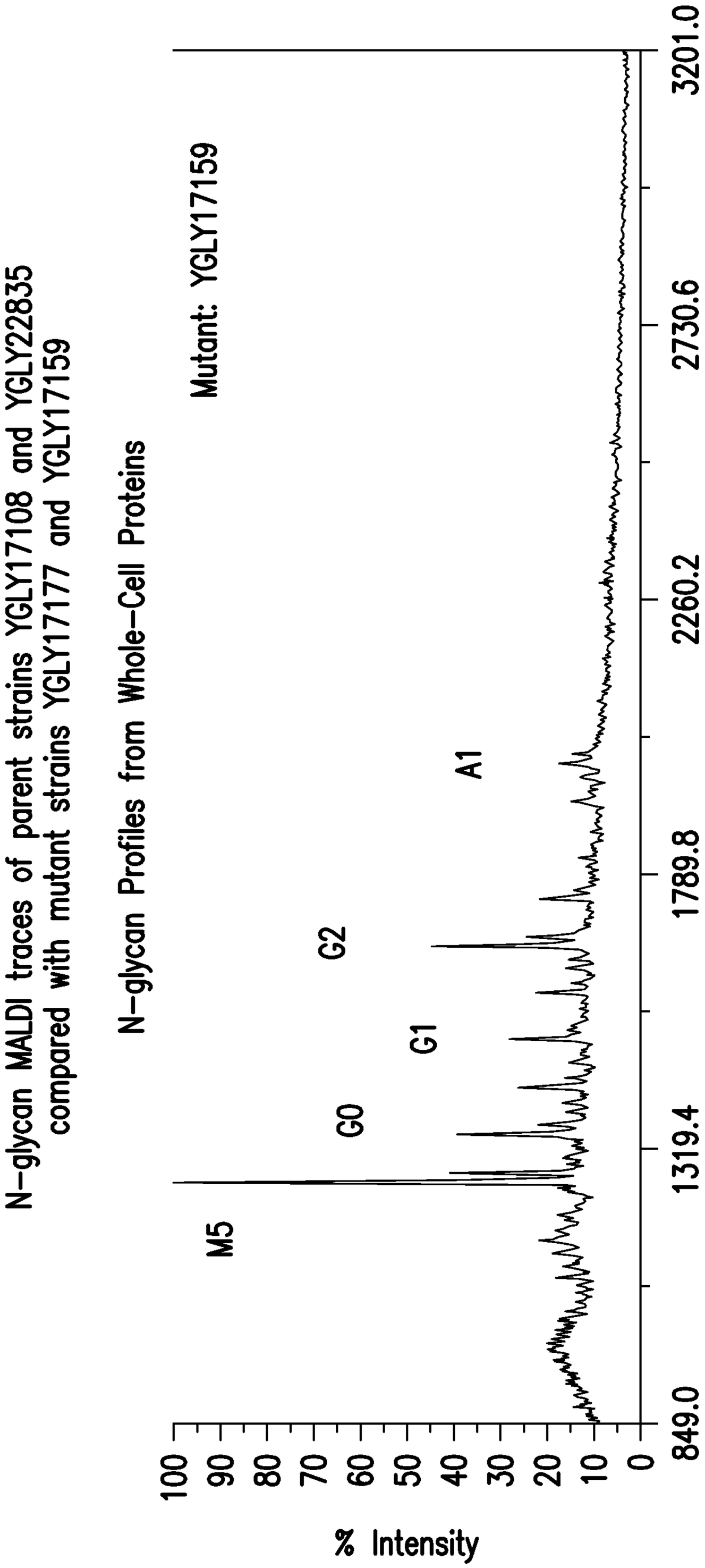
N-glycan MALDI traces of parent strains YGLY17108 and YGLY22835
compared with mutant strains YGLY17177 and YGLY17159
N-glycan Profiles from Whole-Cell Proteins
Mutant: YGLY17159
M5
G0
G2
G1
A1
100
90
80
70
60
50
40
30
20
10
0
% Intensity
1319.4
1789.8
2260.2
2730.6
3201.0
849.0
Mass (m/v)

FIG.2D

Plasmid maps of pGLY8045 and pGLY6391

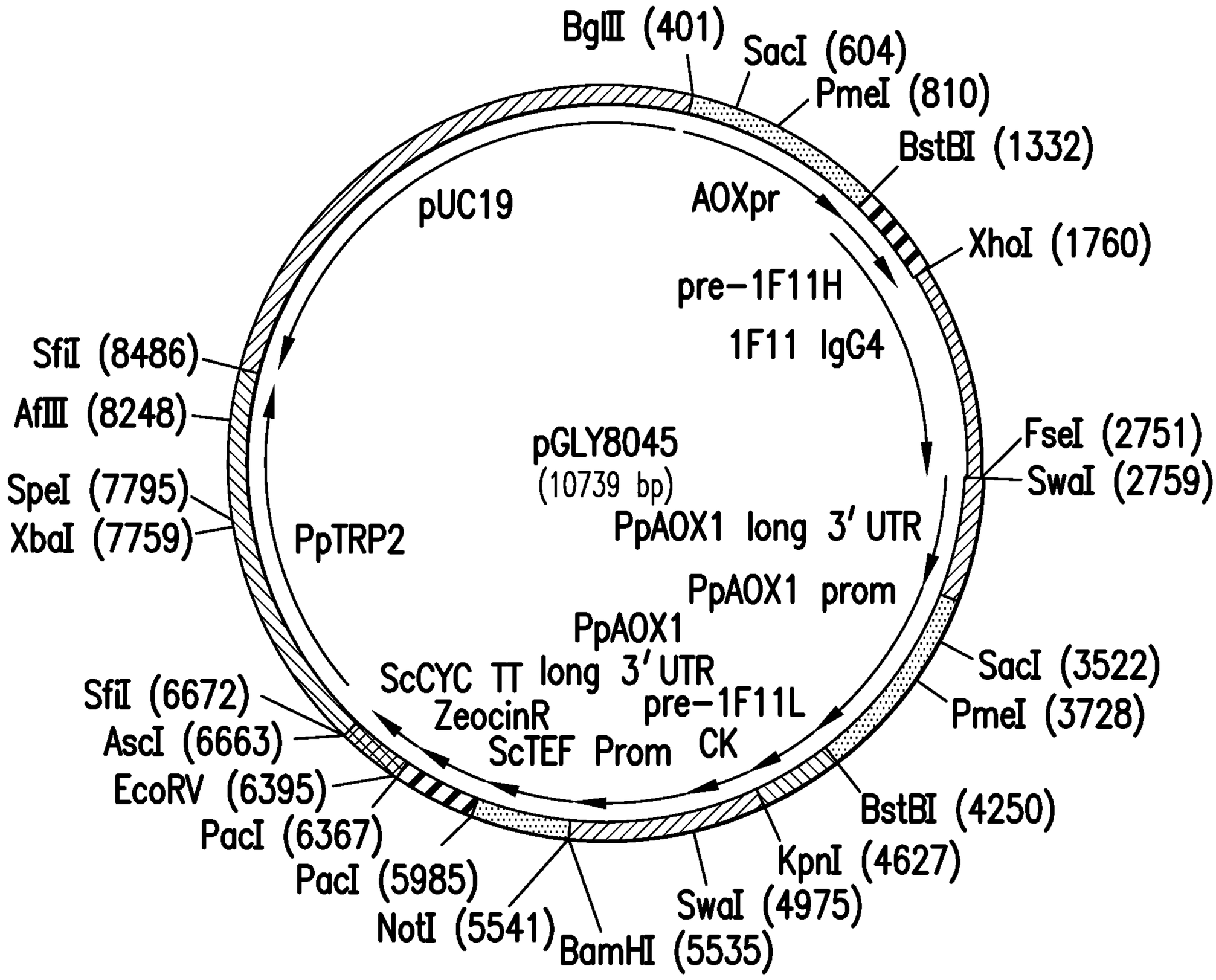

FIG.3A

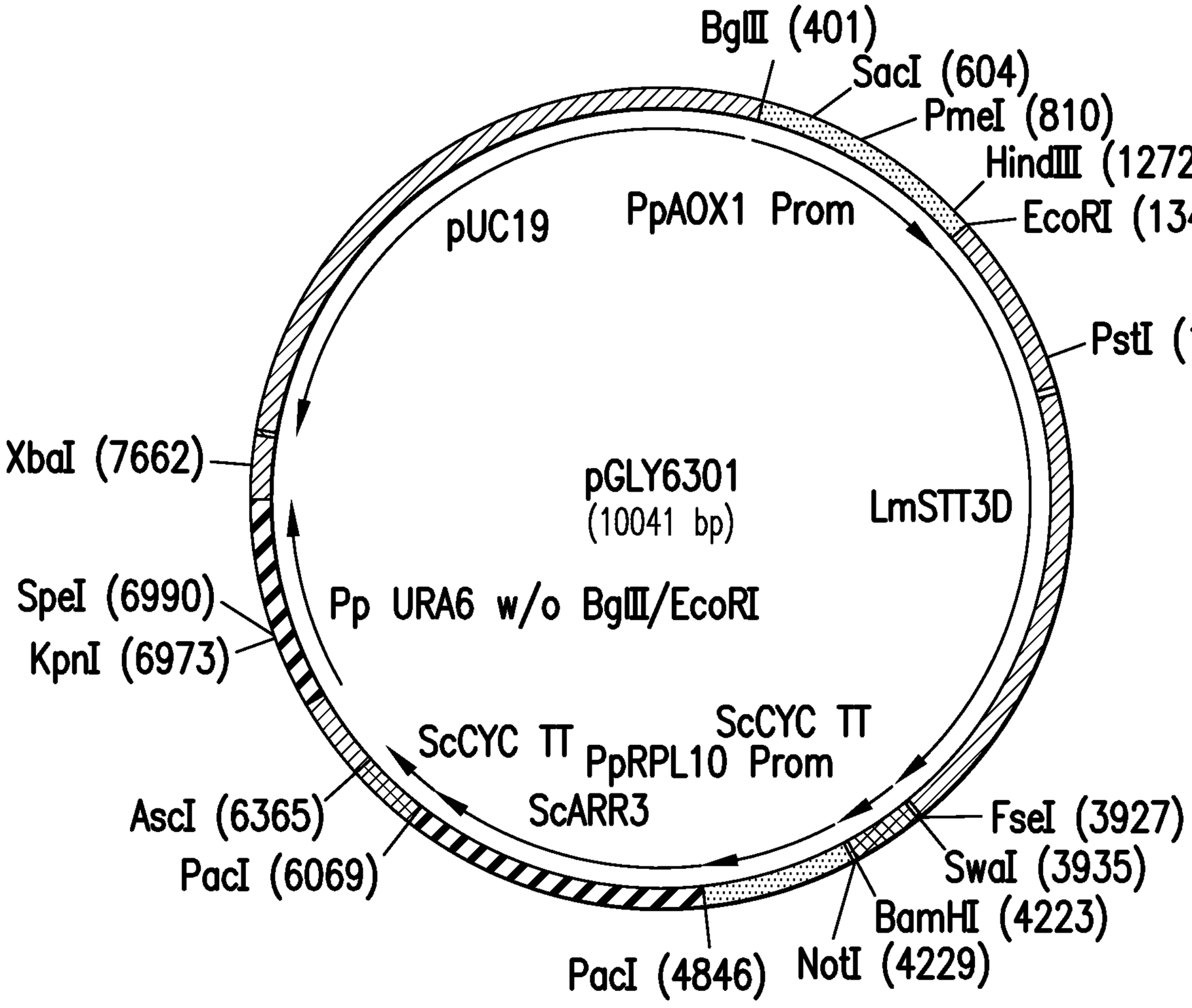
BglII (401)
SacI (604)
PmeI (810)
HindIII (1272)
EcoRI (1341)
PstI (1951)
pUC19
PpAOX1 Prom
pGLY6301
(10041 bp)
LmSTT3D
Pp URA6 w/o BglII/EcoRI
XbaI (7662)
SpeI (6990)
KpnI (6973)
ScCYC TT
ScCYC TT
PpRPL10 Prom
ScARR3
AscI (6365)
PacI (6069)
FseI (3927)
SwaI (3935)
BamHI (4223)
NotI (4229)
PacI (4846)

FIG.3B

Plasmid maps of pGLY5933–5936

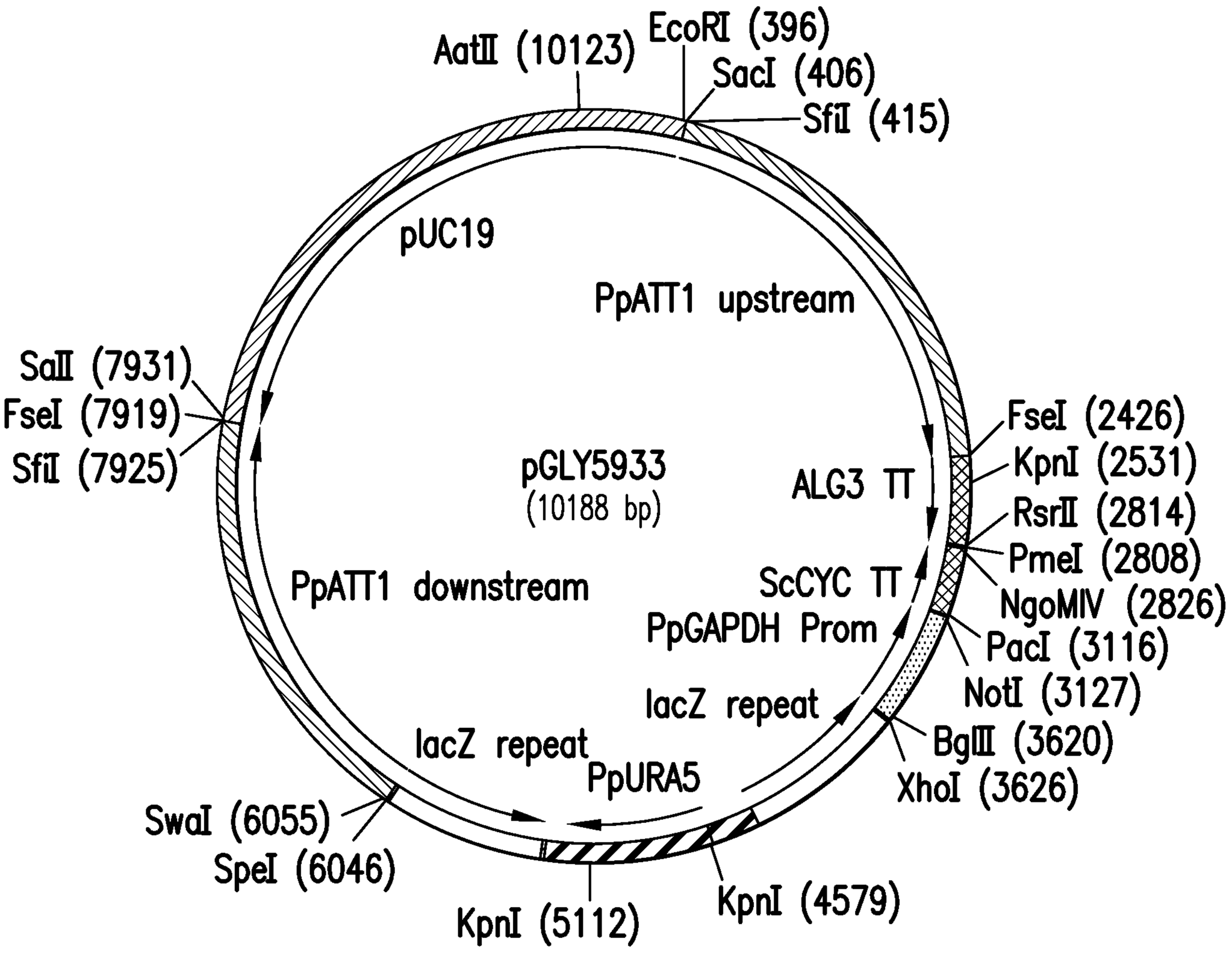

FIG.4A

Plasmid maps of pGLY5933-5936

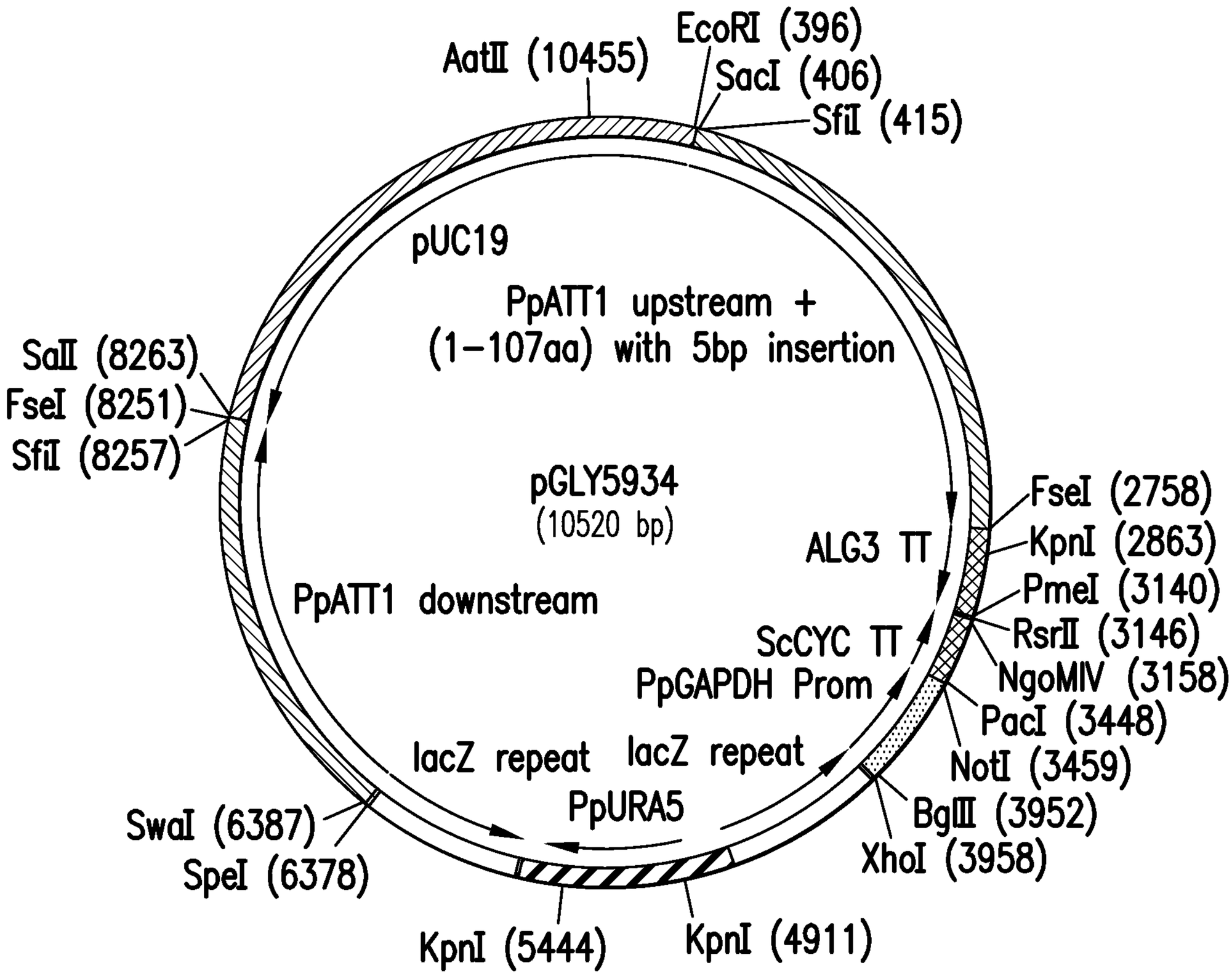

FIG.4B

Plasmid maps of pGLY5933–5936

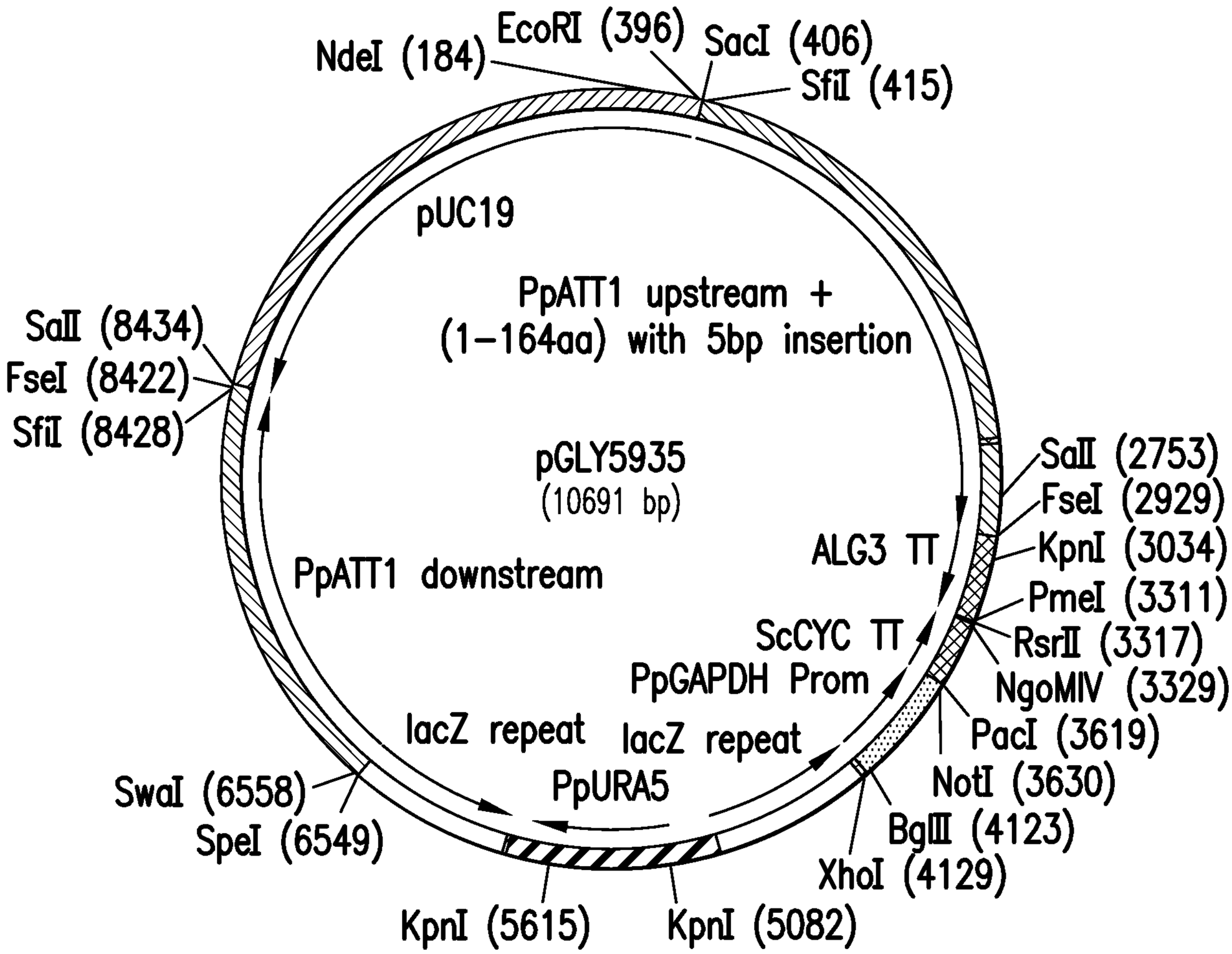

FIG.4C

Plasmid maps of pGLY5933–5936

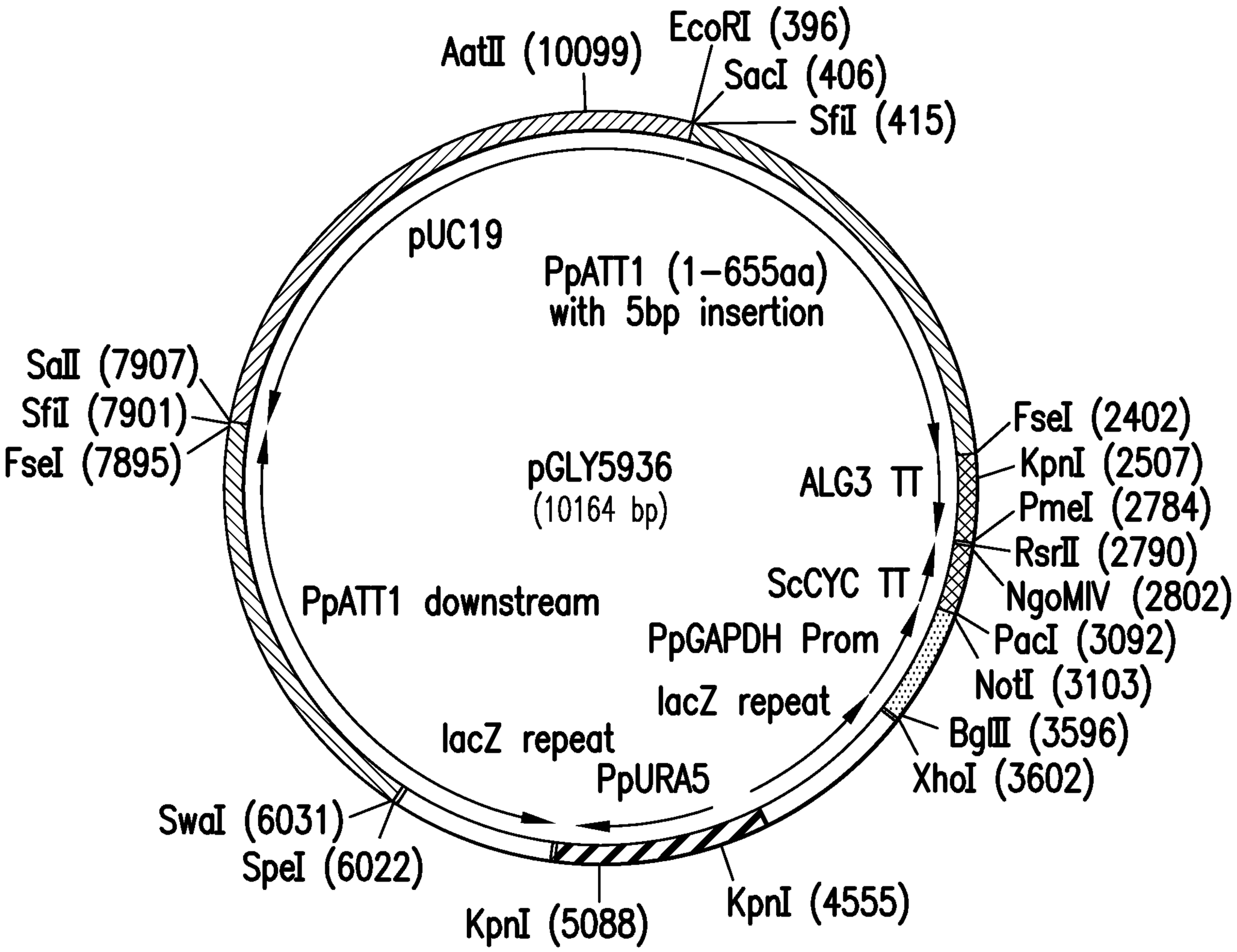

FIG.4D

Plasmid maps of pGLY5947–5949

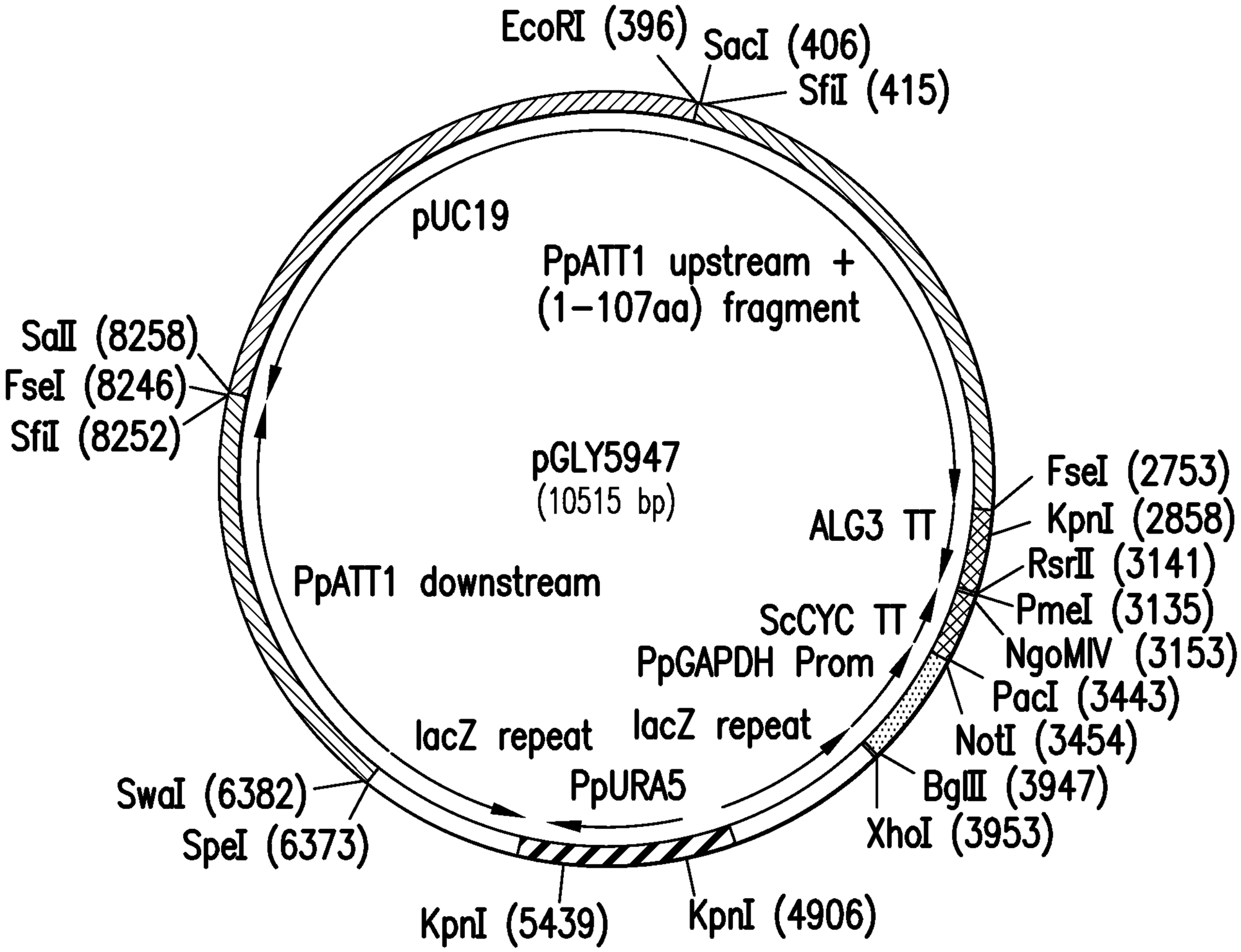

FIG.4E

Plasmid maps of pGLY5947-5949

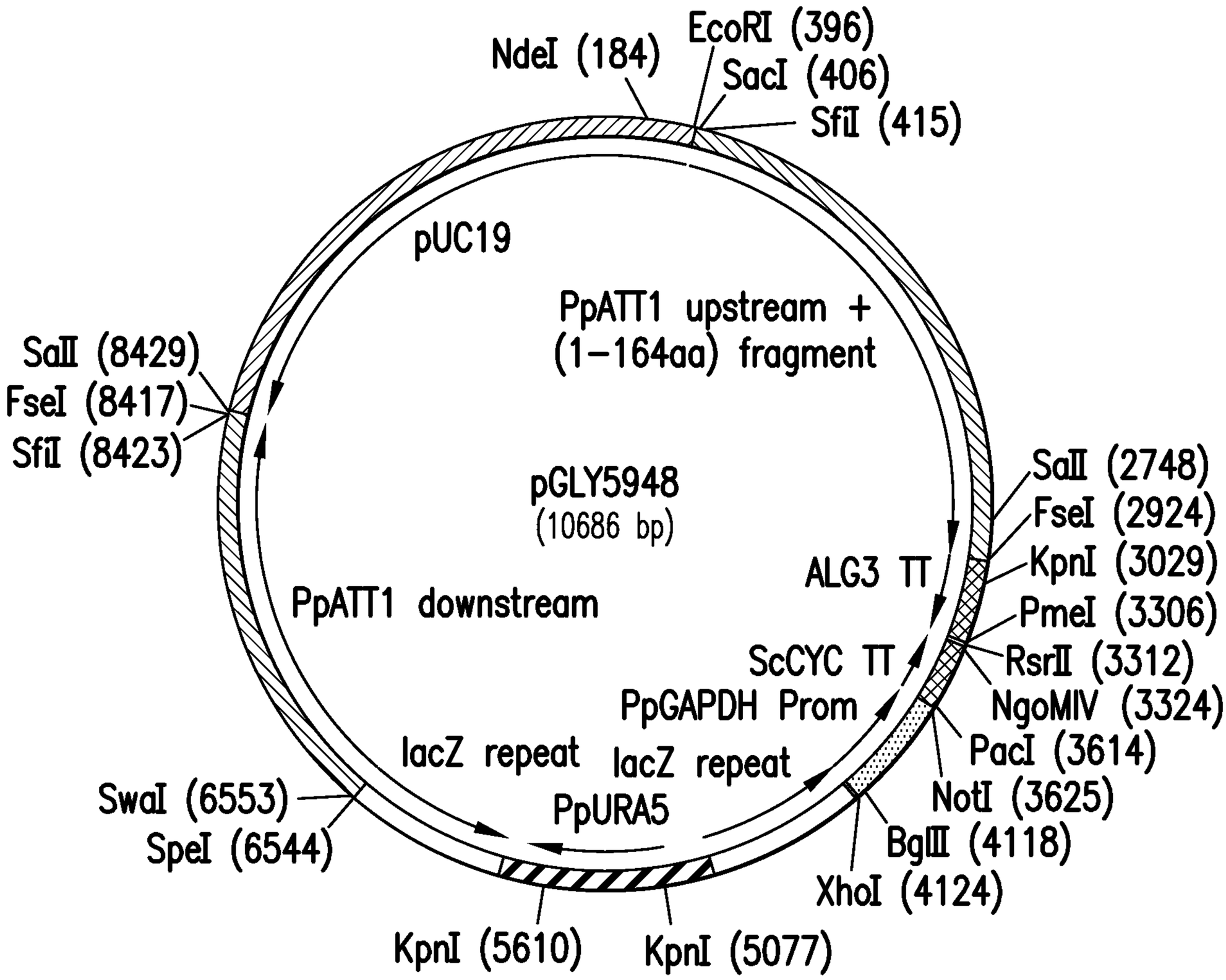

FIG.4F

Plasmid maps of pGLY5947–5949

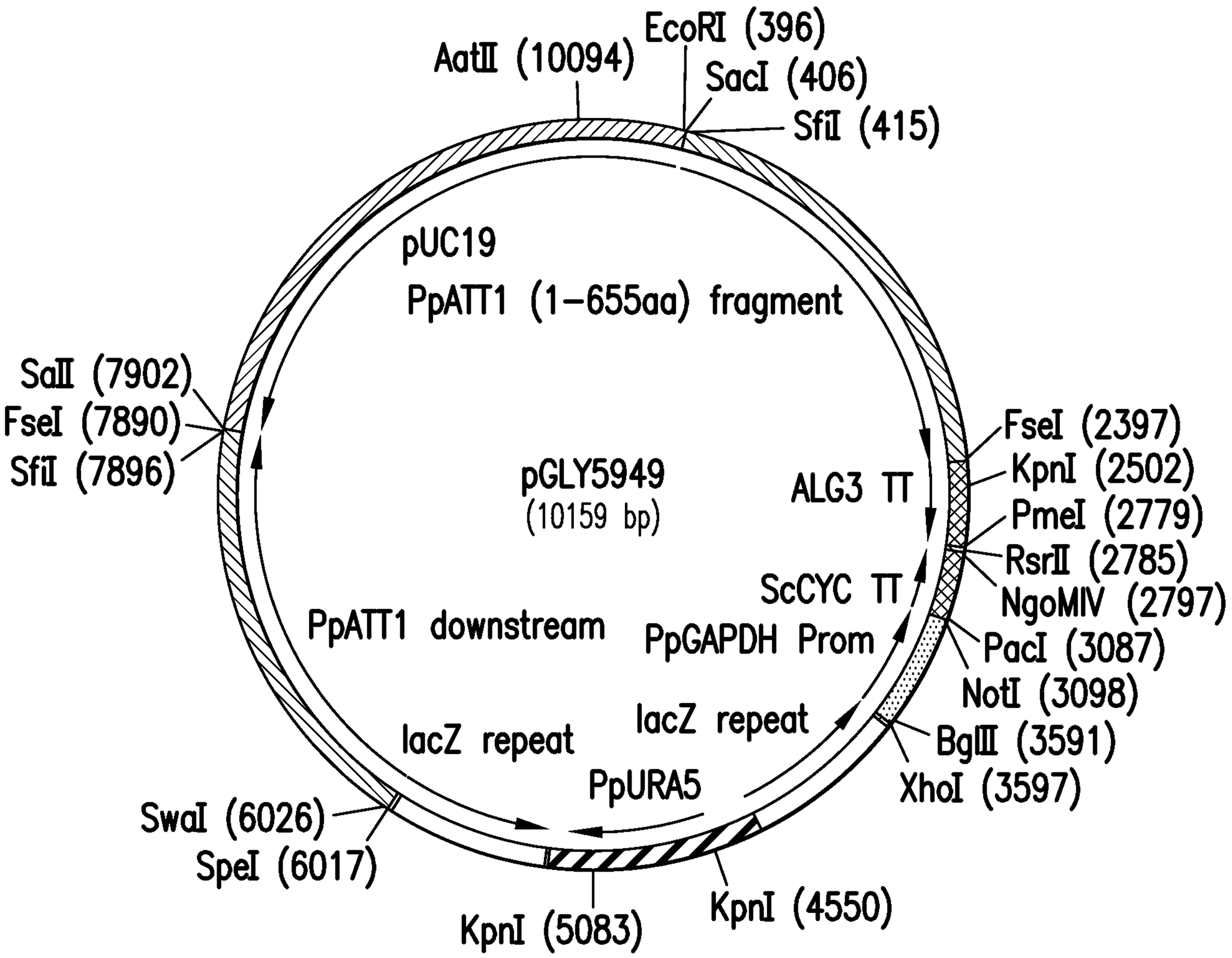

FIG.4G

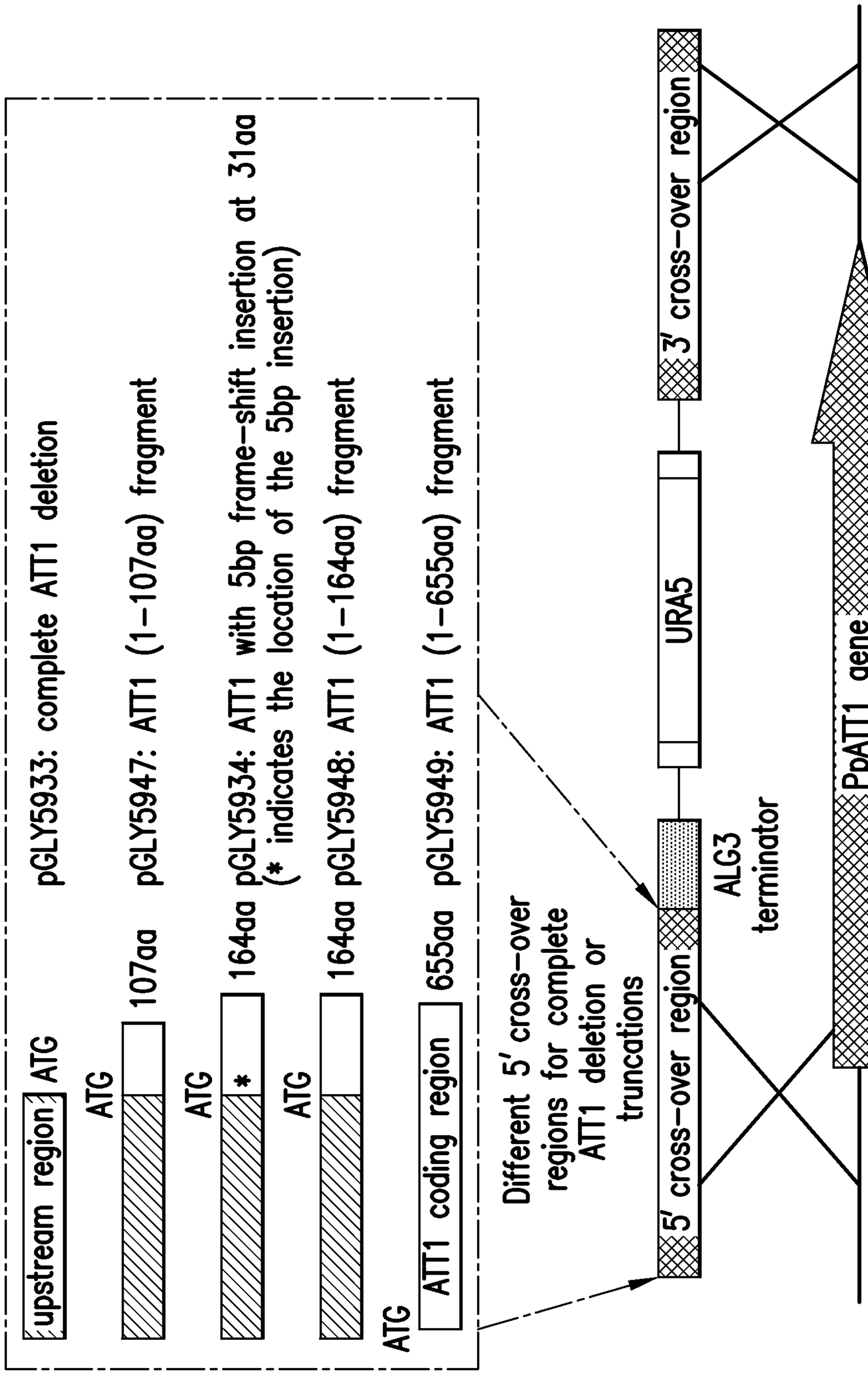
DNA Constructs for PpATT1 Deletion and Truncations
upstream region
ATG
pGLY5933: complete ATT1 deletion
ATG
107aa
pGLY5947: ATT1 (1–107aa) fragment
ATG
164aa
pGLY5934: ATT1 with 5bp frame–shift insertion at 3
(* indicates the location of the 5bp insertion)
ATG
164aa
pGLY5948: ATT1 (1–164aa) fragment
ATG
ATT1 coding region
655aa
pGLY5949: ATT1 (1–655aa) fragment
Different 5′ cross–over regions for complete ATT1 deletion or truncations
5′ cross–over region
URA5
3′ cross–over region
ALG3 terminator
PpATT1 gene
Endogenous chromosomal locus

FIG. 5

PpATT1 Deletion of Truncations Led
to Increased Thermal Tolerance

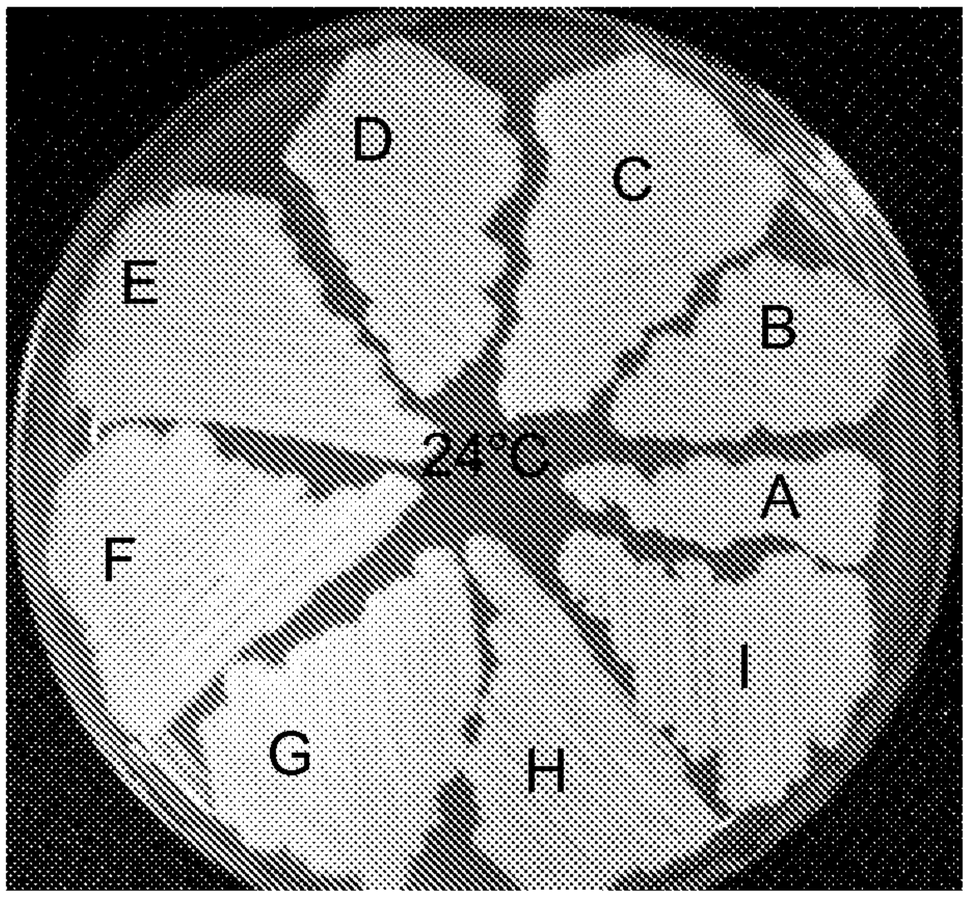

FIG.6A

PpATT1 Deletion of Truncations Led
to Increased Thermal Tolerance

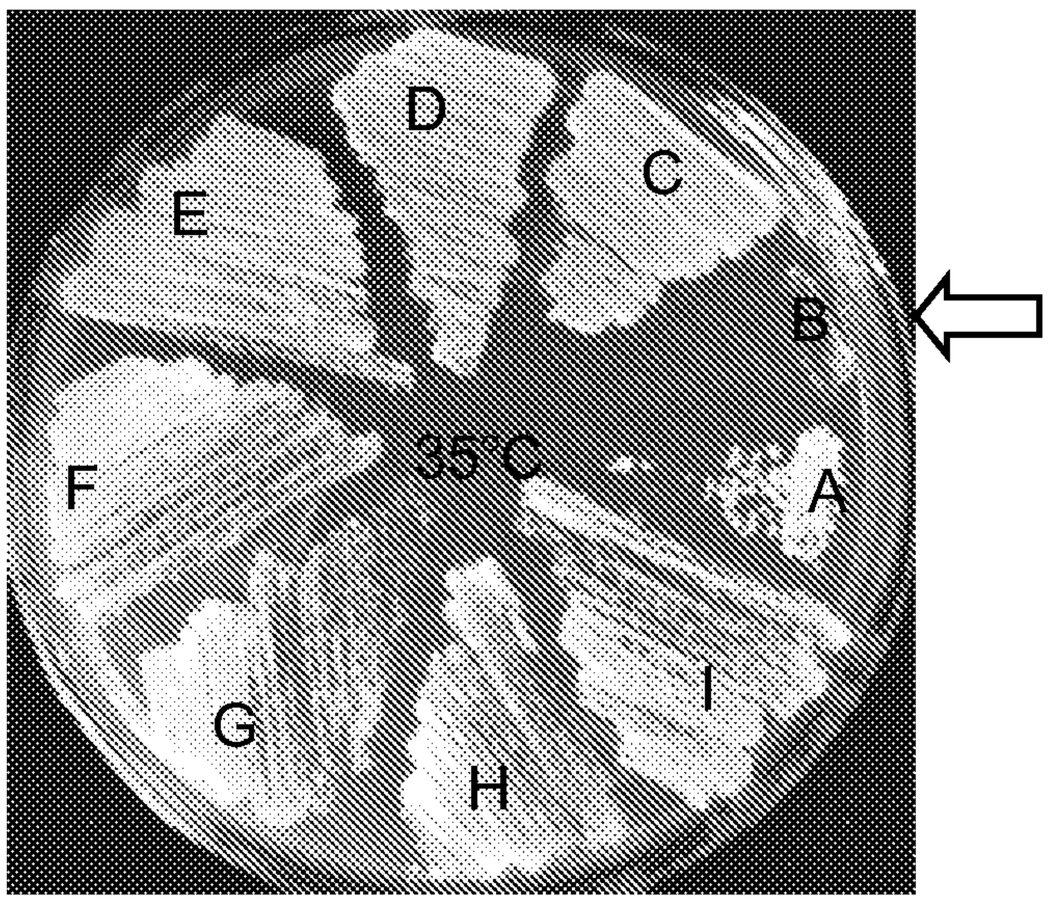

FIG.6B

PpATT1 Deletion of Truncations Led to Increased Thermal Tolerance

A	YGLY17172	PpATT1 UV derived mutant
B	YGLY1708	WT PpATT1 control (temperature-sensitive)
C	YGLY27611	ATT1 complete deletion
D	YGLY27601	ATT1 (1-107aa + 5bp insertion at 31aa)
E	YGLY27602	ATT1 (1-164aa + 5bp insertion at 31aa)
F	YGLY27603	ATT1 (1-655aa + 5bp insertion at 31aa)
G	YGLY27606	ATT1 (1-107aa) fragment
H	YGLY27608	ATT1 (1-164aa) fragment
I	YGLY27610	ATT1 (1-655aa) fragment

FIG.6C

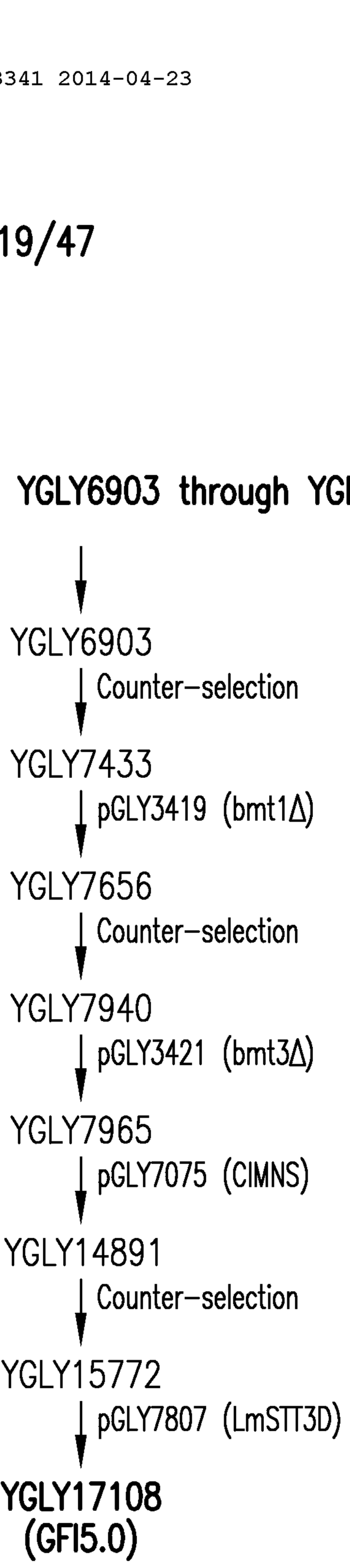
Strain lineages from YGLY6903 through YGLY17108
YGLY6903
Counter-selection
YGLY7433
pGLY3419 (bmt1Δ)
YGLY7656
Counter-selection
YGLY7940
pGLY3421 (bmt3Δ)
YGLY7965
pGLY7075 (CIMNS)
YGLY14891
Counter-selection
YGLY15772
pGLY7807 (LmSTT3D)
YGLY17108
(GFI5.0)

FIG.7

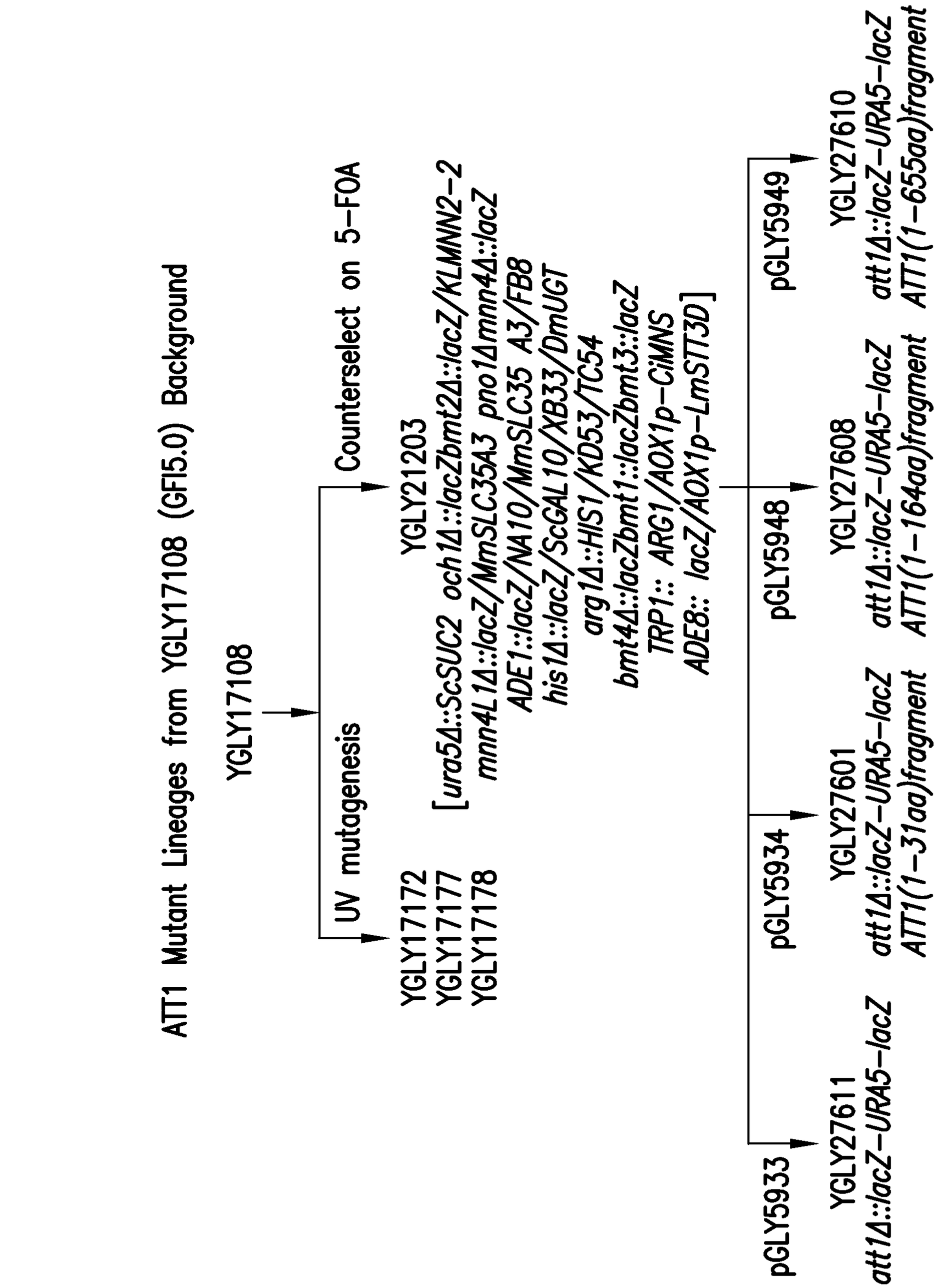
ATT1 Mutant Lineages from YGLY17108 (GFI5.0) Background
YGLY17108
UV mutagenesis
YGLY17172
YGLY17177
YGLY17178
[ura5Δ::ScSUC2 och1Δ::lacZbmt2Δ::lacZ/KLMN2-2
mnn4L1Δ::lacZ/MmSLC35A3 pno1Δmnn4Δ::lacZ
ADE1::lacZ/NA10/MmSLC35 A3/FB8
his1Δ::lacZ/ScGAL10/XB33/DmUGT
arg1Δ::HIS1/KD53/TC54
bmt4Δ::lacZbmt1::lacZbmt3::lacZ
TRP1:: ARG1/AOX1p-CiMNS
ADE8:: lacZ/AOX1p-LmSTT3D]
Counterselect on 5-FOA
YGLY21203
pGLY5948
YGLY27608
att1Δ::lacZ-URA5-lacZ
ATT1(1-164aa)fragment
pGLY5949
YGLY27610
att1Δ::lacZ-URA5-lacZ
ATT1(1-655aa)fragment
pGLY5934
YGLY27601
att1Δ::lacZ-URA5-lacZ
ATT1(1-31aa)fragment
pGLY5933
YGLY27611
att1Δ::lacZ-URA5-lacZ

FIG.8

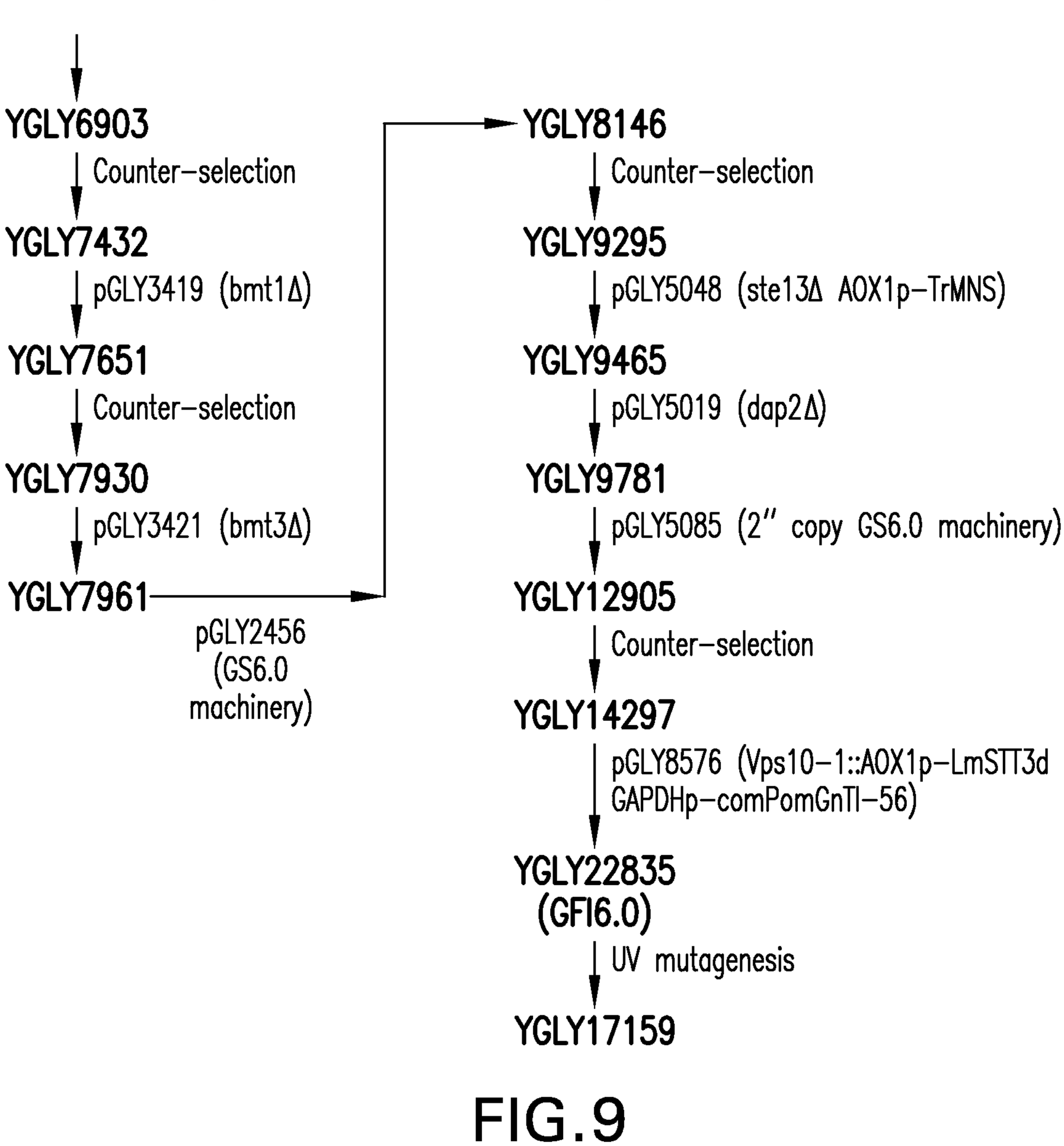
Strain lineages from YGLY6903 through YGLY22835 and YGLY17159
YGLY6903
Counter-selection
YGLY7432
pGLY3419 (bmt1Δ)
YGLY7651
Counter-selection
YGLY7930
pGLY3421 (bmt3Δ)
YGLY7961
pGLY2456 (GS6.0 machinery)
YGLY8146
Counter-selection
YGLY9295
pGLY5048 (ste13Δ AOX1p-TrMNS)
YGLY9465
pGLY5019 (dap2Δ)
YGLY9781
pGLY5085 (2″ copy GS6.0 machinery)
YGLY12905
Counter-selection
YGLY14297
pGLY8576 (Vps10-1::AOX1p-LmSTT3d GAPDHp-comPomGnTI-56)
YGLY22835 (GFI6.0)
UV mutagenesis
YGLY17159

FIG.9

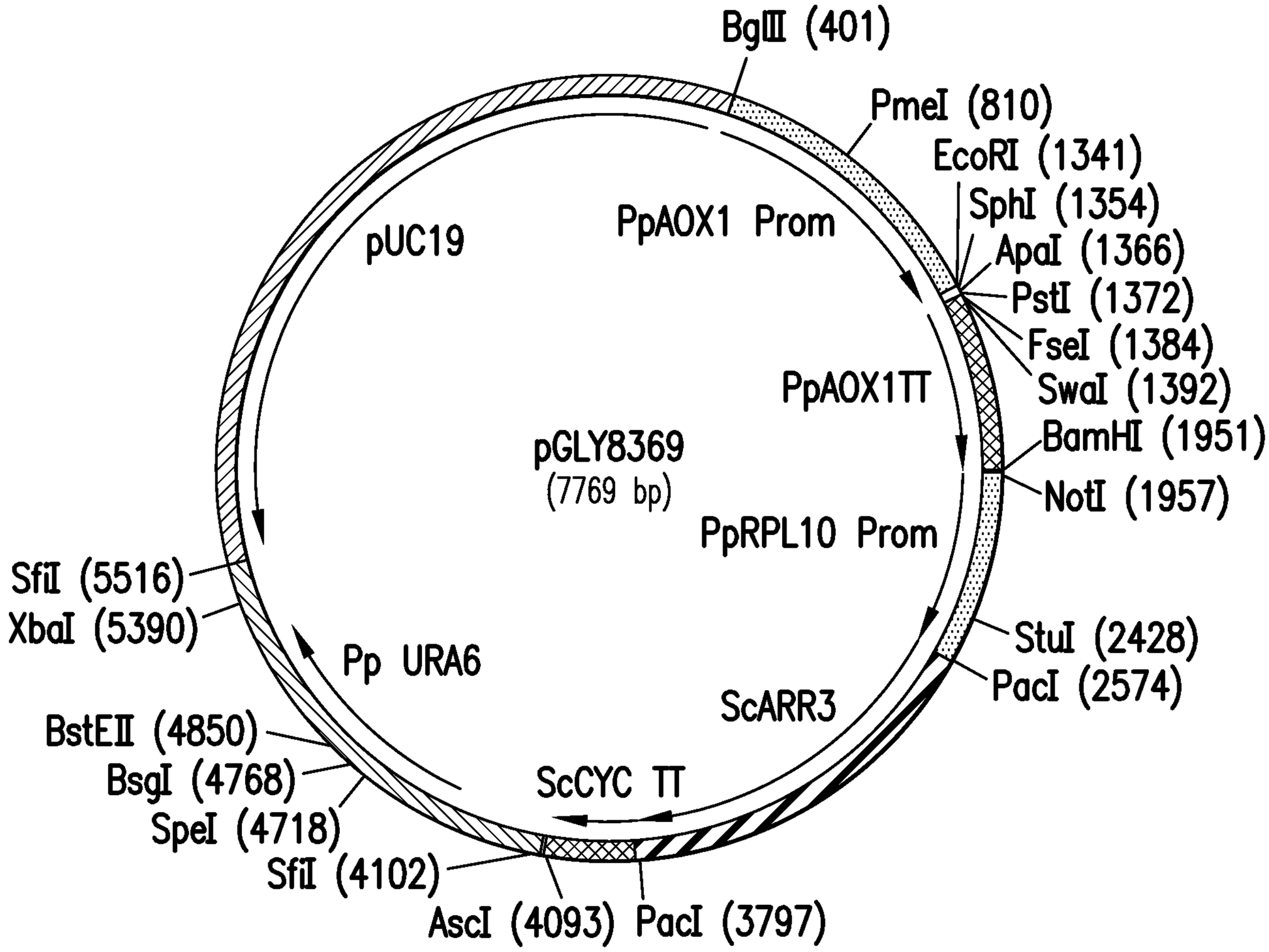
BglII (401)
PmeI (810)
EcoRI (1341)
SphI (1354)
ApaI (1366)
PstI (1372)
FseI (1384)
SwaI (1392)
BamHI (1951)
NotI (1957)
StuI (2428)
PacI (2574)
PacI (3797)
AscI (4093)
SfiI (4102)
SpeI (4718)
BsgI (4768)
BstEII (4850)
XbaI (5390)
SfiI (5516)
pUC19
PpAOX1 Prom
PpAOX1TT
pGLY8369
(7769 bp)
PpRPL10 Prom
ScARR3
ScCYC TT
Pp URA6

FIG. 10

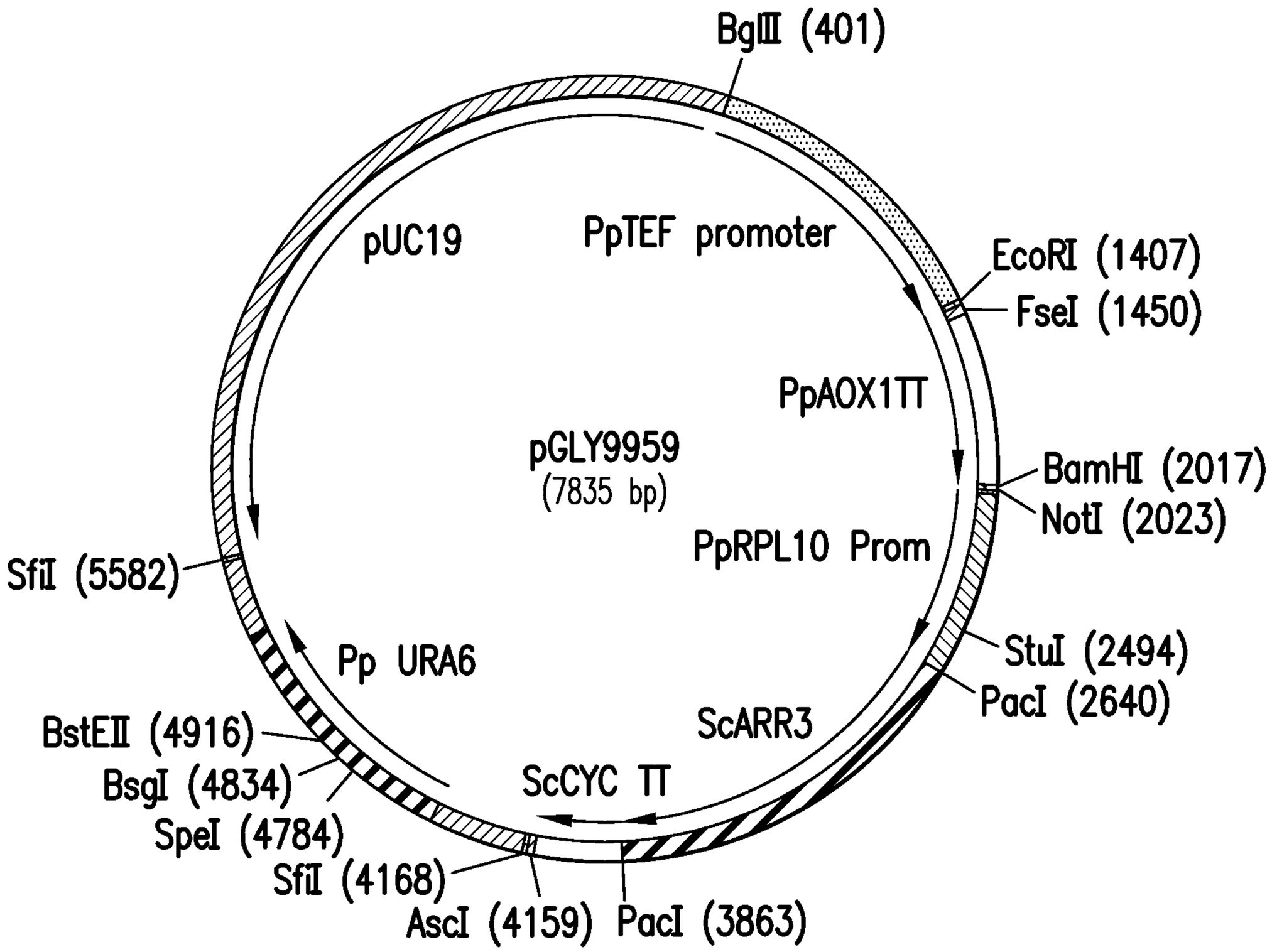
BglII (401)
pUC19
PpTEF promoter
EcoRI (1407)
FseI (1450)
PpAOX1TT
pGLY9959
(7835 bp)
BamHI (2017)
NotI (2023)
PpRPL10 Prom
SfiI (5582)
StuI (2494)
Pp URA6
PacI (2640)
BstEII (4916)
ScARR3
BsgI (4834)
ScCYC TT
SpeI (4784)
SfiI (4168)
AscI (4159)
PacI (3863)

FIG.11

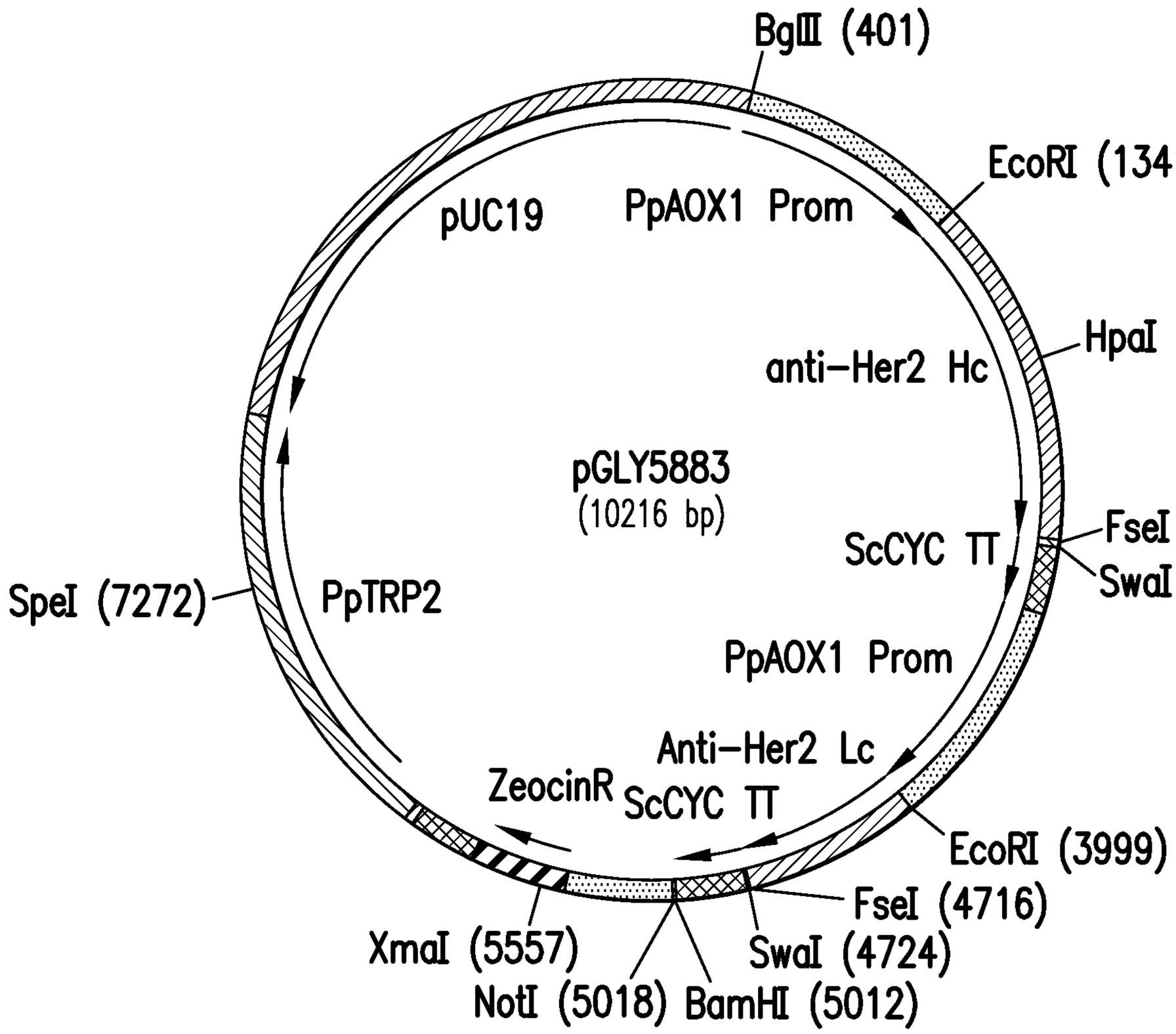
BglII (401)
EcoRI (1341)
pUC19
PpAOX1 Prom
HpaI (2022)
anti-Her2 Hc
pGLY5883
(10216 bp)
FseI (2763)
ScCYC TT
SwaI (2771)
SpeI (7272)
PpTRP2
PpAOX1 Prom
Anti-Her2 Lc
ZeocinR
ScCYC TT
EcoRI (3999)
FseI (4716)
XmaI (5557)
SwaI (4724)
NotI (5018)
BamHI (5012)

FIG.12

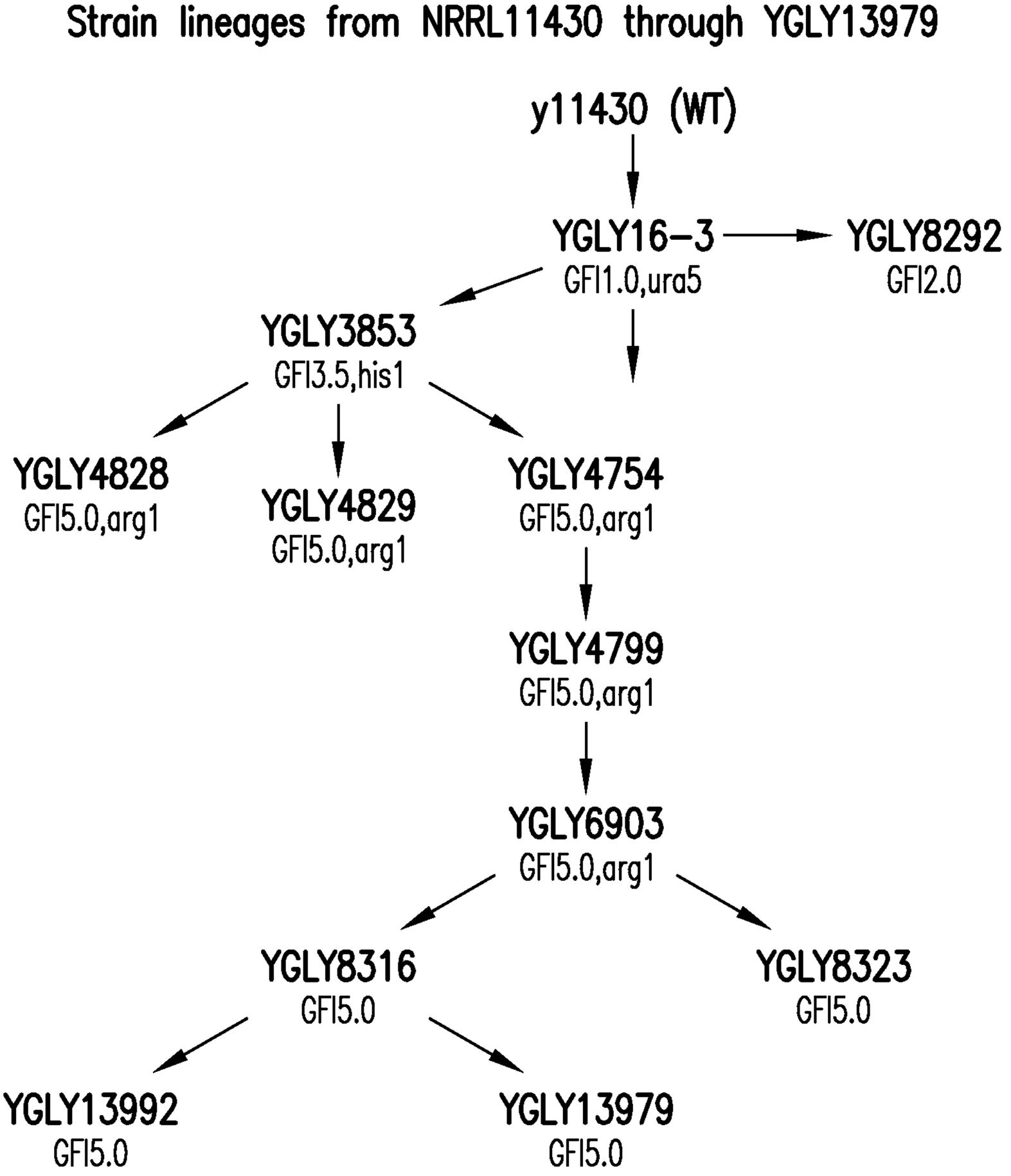
Strain lineages from NRRL11430 through YGLY13979
y11430 (WT)
YGLY16-3
GFI1.0,ura5
YGLY8292
GFI2.0
YGLY3853
GFI3.5,his1
YGLY4828
GFI5.0,arg1
YGLY4829
GFI5.0,arg1
YGLY4754
GFI5.0,arg1
YGLY4799
GFI5.0,arg1
YGLY6903
GFI5.0,arg1
YGLY8316
GFI5.0
YGLY8323
GFI5.0
YGLY13992
GFI5.0
YGLY13979
GFI5.0

FIG. 13

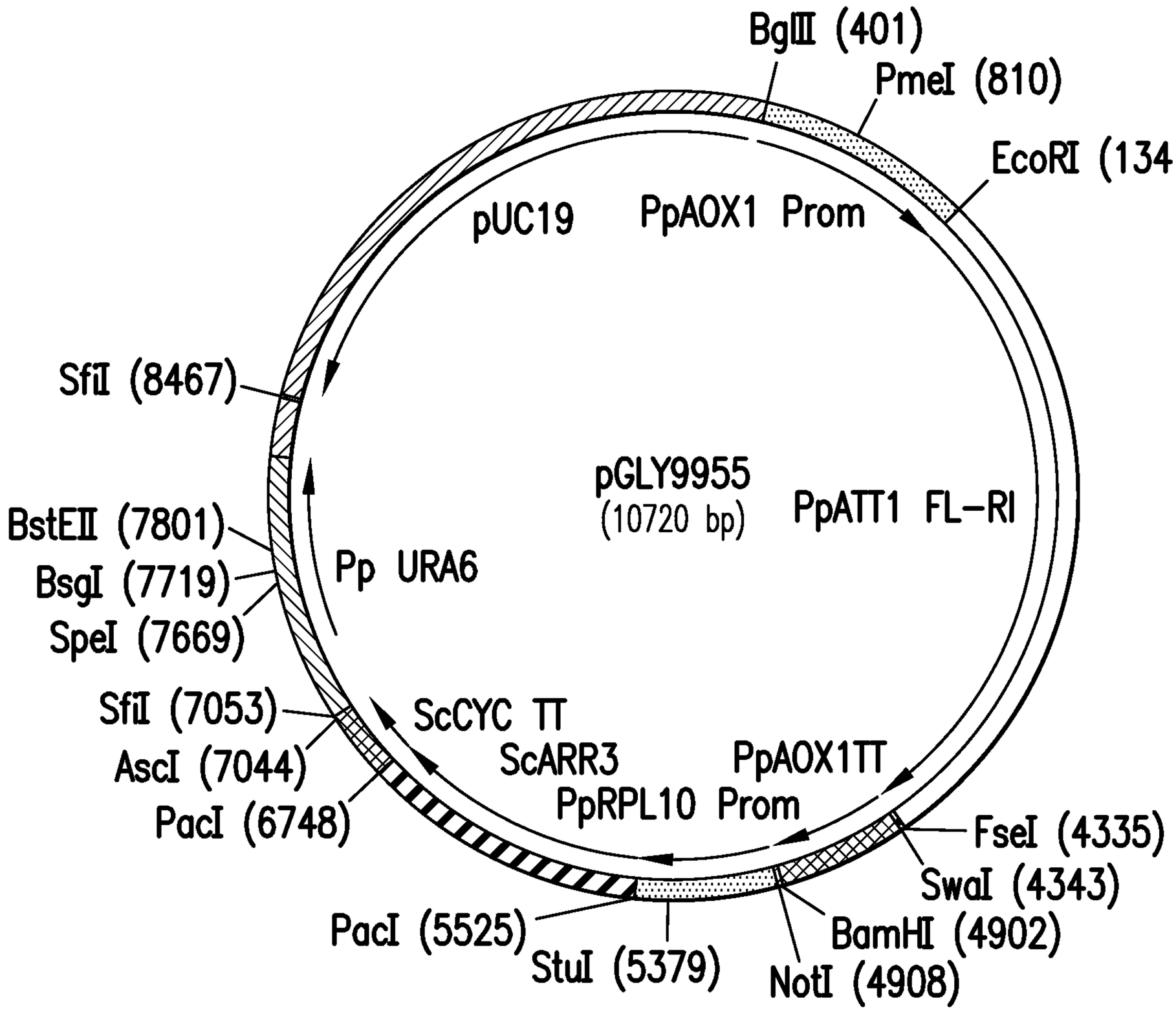
BglII (401)
PmeI (810)
EcoRI (1341)
pUC19
PpAOX1 Prom
SfiI (8467)
pGLY9955
(10720 bp)
PpATT1 FL-RI
BstEII (7801)
BsgI (7719)
SpeI (7669)
Pp URA6
SfiI (7053)
AscI (7044)
PacI (6748)
ScCYC TT
ScARR3
PpAOX1TT
PpRPL10 Prom
FseI (4335)
SwaI (4343)
PacI (5525)
StuI (5379)
BamHI (4902)
NotI (4908)

FIG.14A

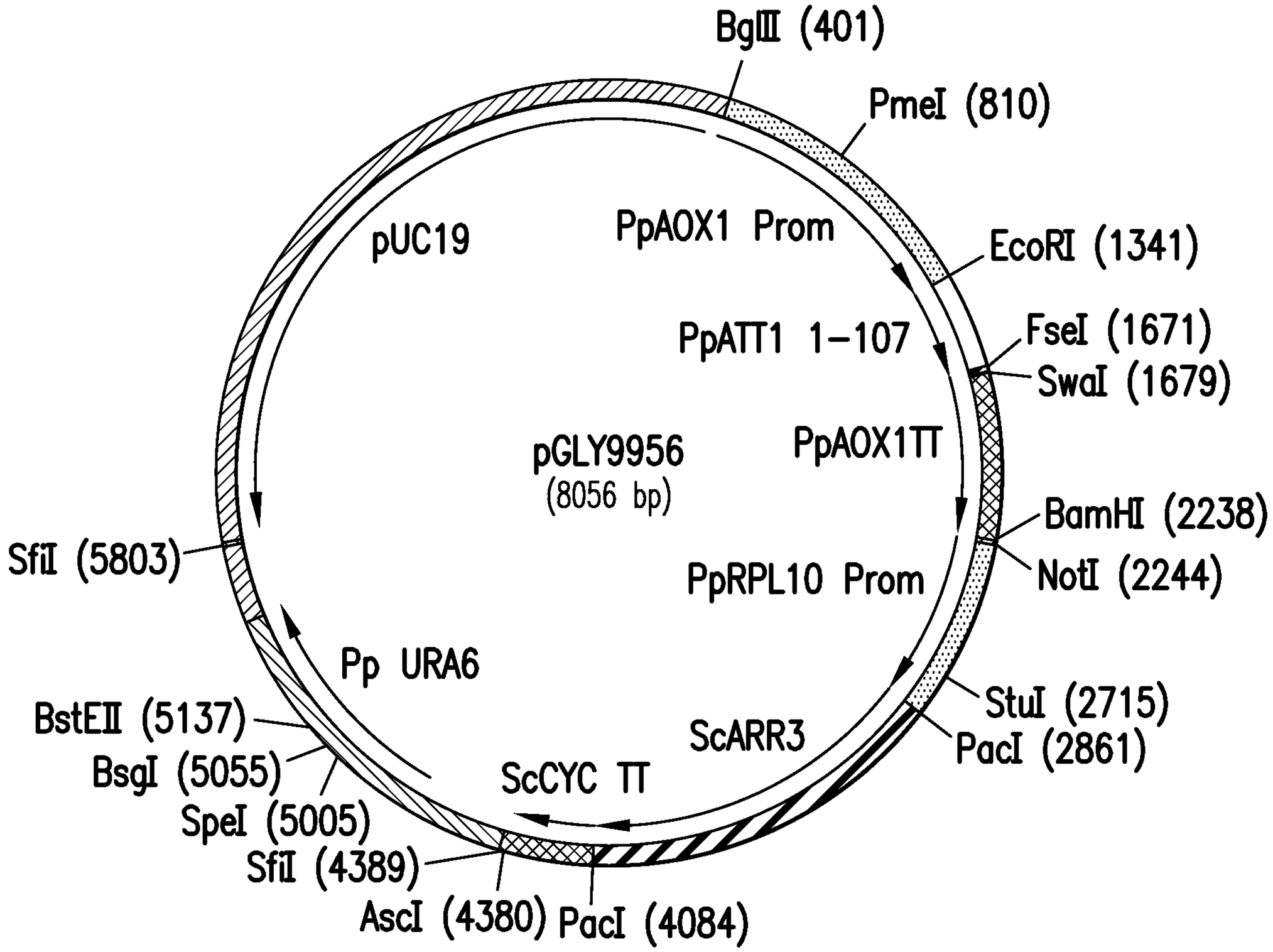
BglII (401)
PmeI (810)
pUC19
PpAOX1 Prom
EcoRI (1341)
PpATT1 1-107
FseI (1671)
SwaI (1679)
pGLY9956
(8056 bp)
PpAOX1TT
BamHI (2238)
NotI (2244)
SfiI (5803)
PpRPL10 Prom
Pp URA6
StuI (2715)
PacI (2861)
BstEII (5137)
ScARR3
BsgI (5055)
ScCYC TT
SpeI (5005)
SfiI (4389)
AscI (4380)
PacI (4084)

FIG.14B

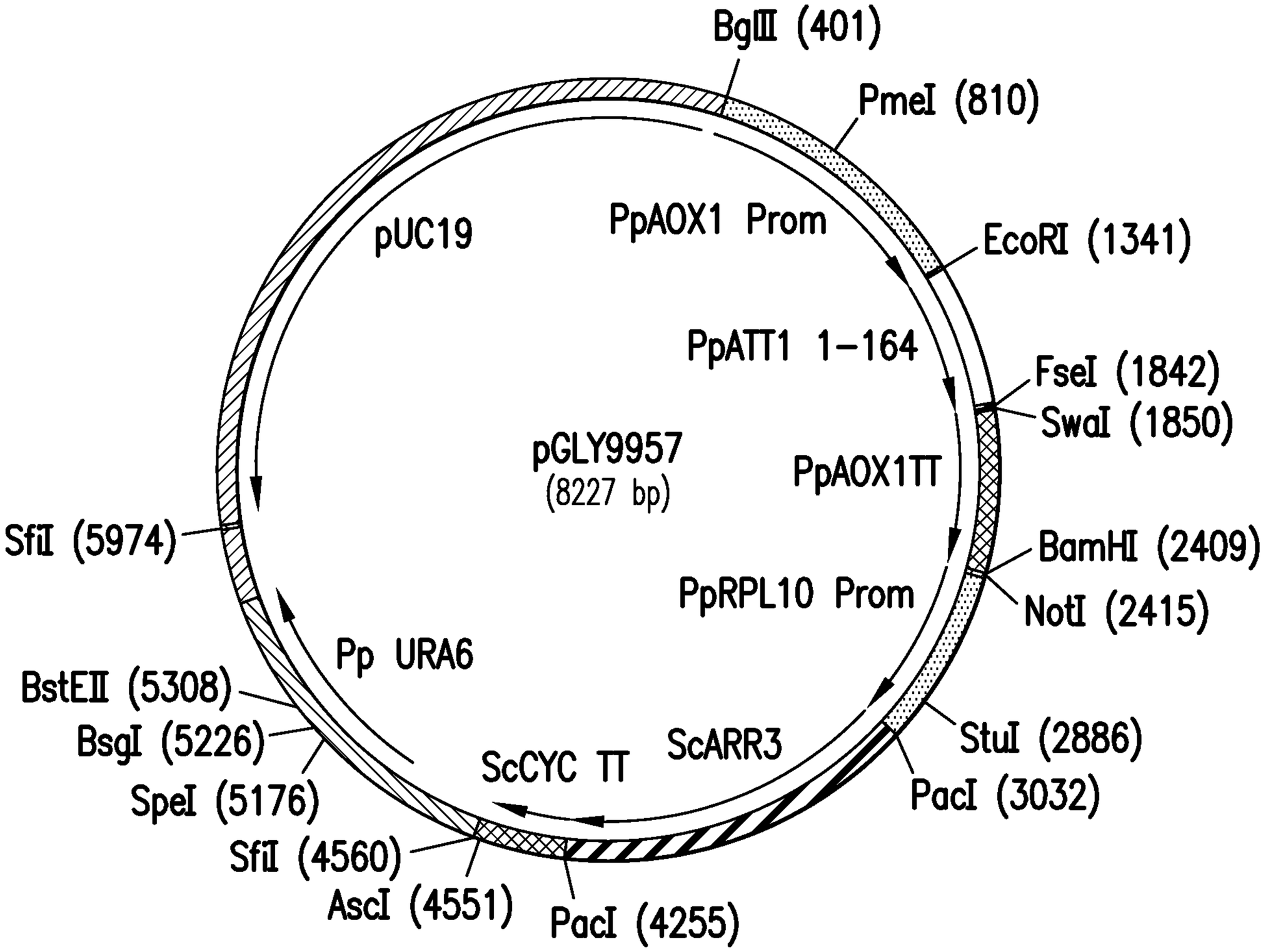
BglII (401)
PmeI (810)
EcoRI (1341)
FseI (1842)
SwaI (1850)
BamHI (2409)
NotI (2415)
StuI (2886)
PacI (3032)
PacI (4255)
AscI (4551)
SfiI (4560)
SpeI (5176)
BsgI (5226)
BstEII (5308)
Pp URA6
SfiI (5974)
pUC19
PpAOX1 Prom
PpATT1 1-164
pGLY9957
(8227 bp)
PpAOX1TT
PpRPL10 Prom
ScARR3
ScCYC TT

FIG.14C

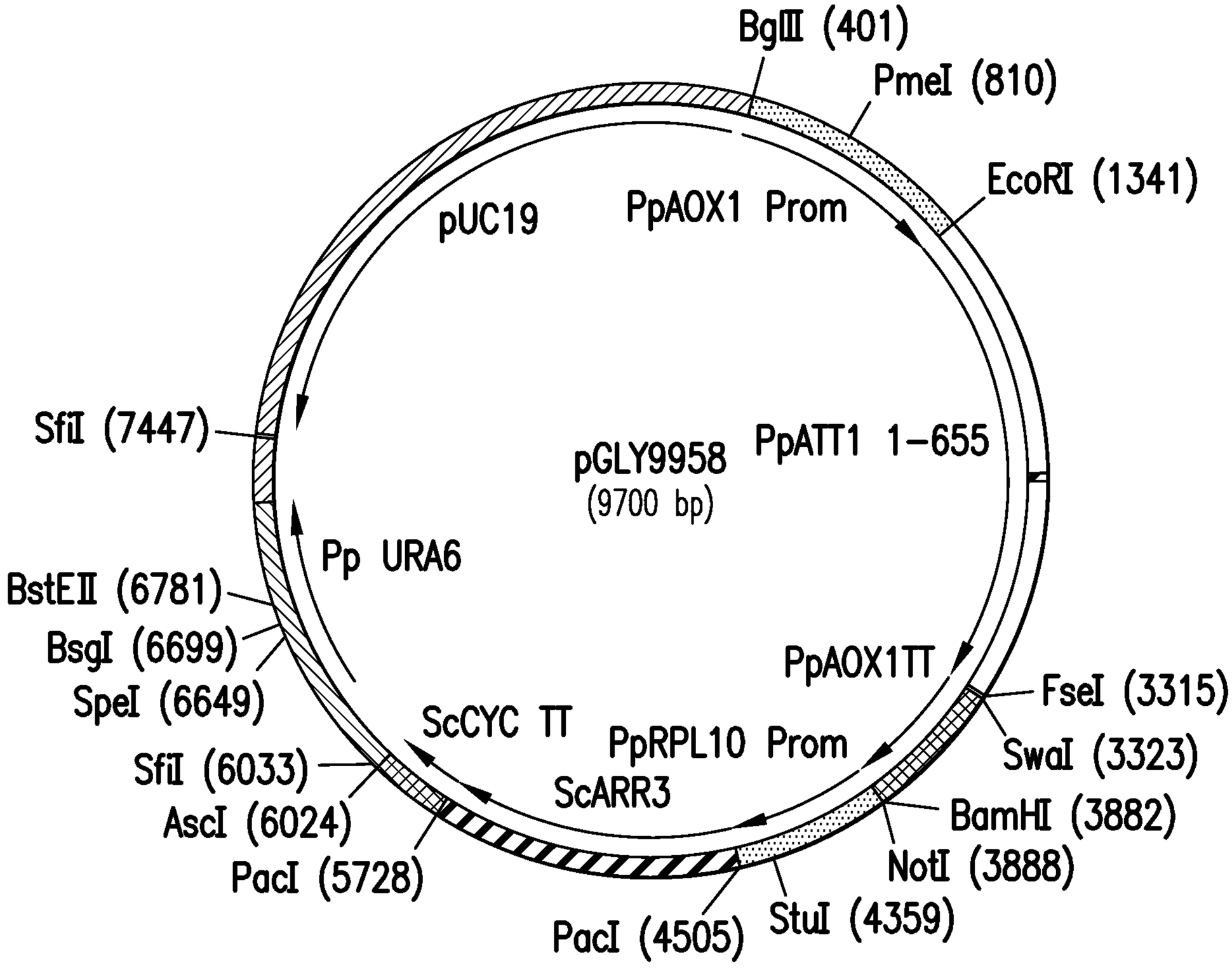
BglII (401)
PmeI (810)
EcoRI (1341)
pUC19
PpAOX1 Prom
SfiI (7447)
pGLY9958
(9700 bp)
PpATT1 1-655
Pp URA6
BstEII (6781)
BsgI (6699)
SpeI (6649)
PpAOX1TT
FseI (3315)
ScCYC TT
PpRPL10 Prom
SfiI (6033)
SwaI (3323)
ScARR3
AscI (6024)
BamHI (3882)
PacI (5728)
NotI (3888)
PacI (4505)
StuI (4359)

FIG.14D

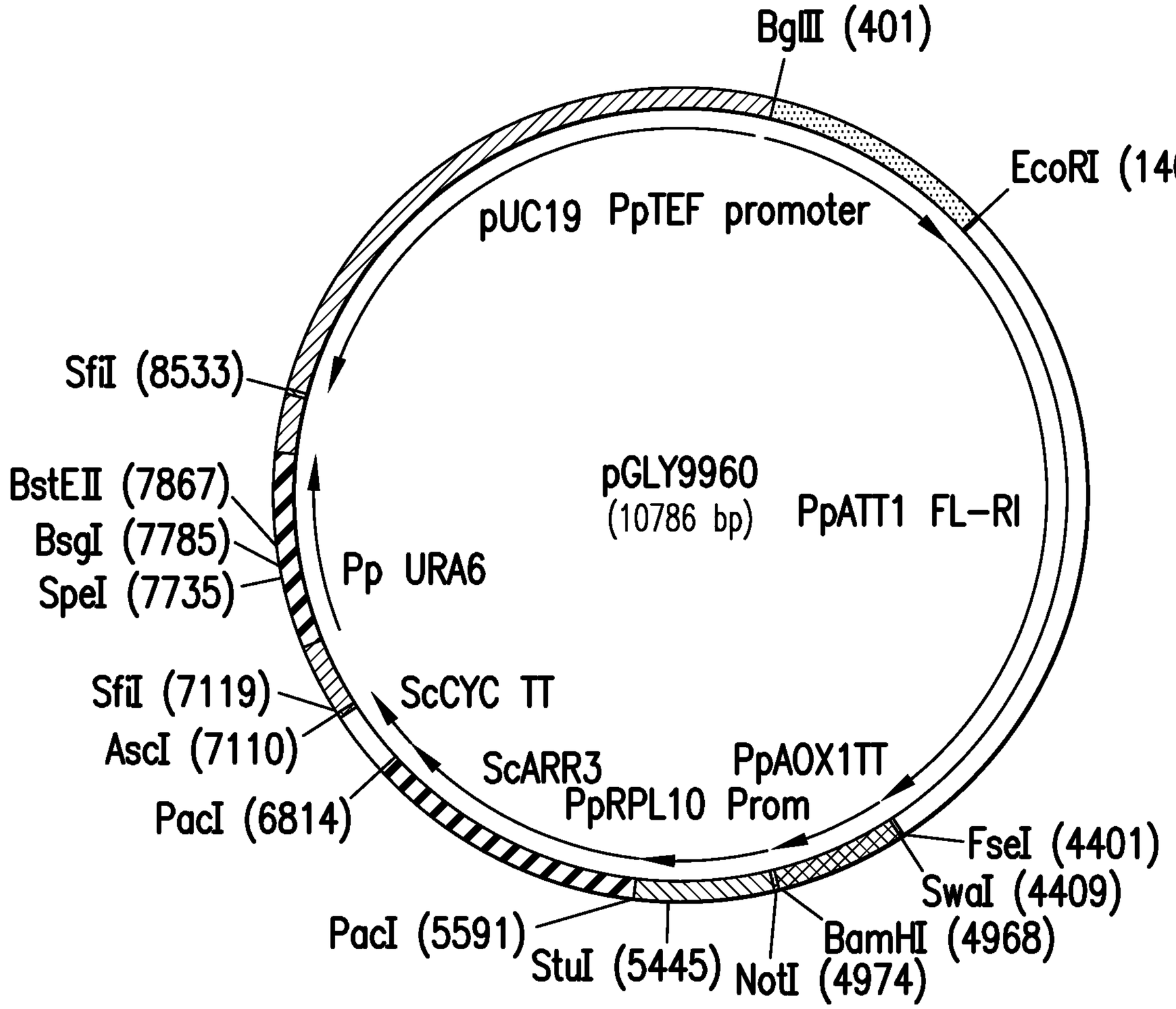
BglII (401)
EcoRI (1407)
pUC19 PpTEF promoter
SfiI (8533)
BstEII (7867)
BsgI (7785)
SpeI (7735)
pGLY9960
(10786 bp)
PpATT1 FL-RI
Pp URA6
SfiI (7119)
AscI (7110)
PacI (6814)
ScCYC TT
ScARR3
PpAOX1TT
PpRPL10 Prom
FseI (4401)
SwaI (4409)
BamHI (4968)
PacI (5591)
StuI (5445)
NotI (4974)

FIG.14E

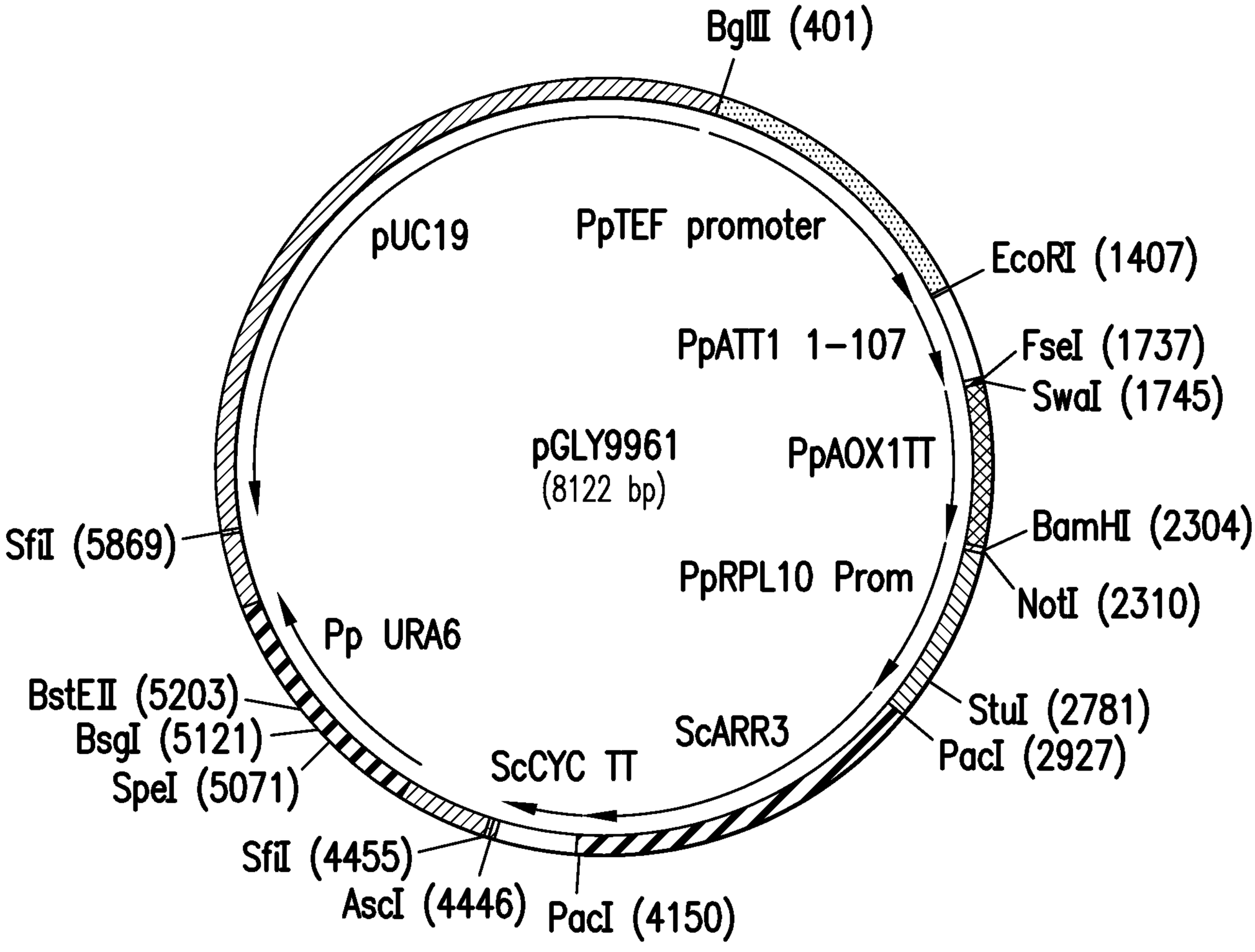
BglII (401)
pUC19
PpTEF promoter
EcoRI (1407)
PpATT1 1-107
FseI (1737)
SwaI (1745)
pGLY9961
(8122 bp)
PpAOX1TT
BamHI (2304)
SfiI (5869)
PpRPL10 Prom
NotI (2310)
Pp URA6
BstEII (5203)
StuI (2781)
BsgI (5121)
ScARR3
PacI (2927)
SpeI (5071)
ScCYC TT
SfiI (4455)
AscI (4446)
PacI (4150)

FIG.14F

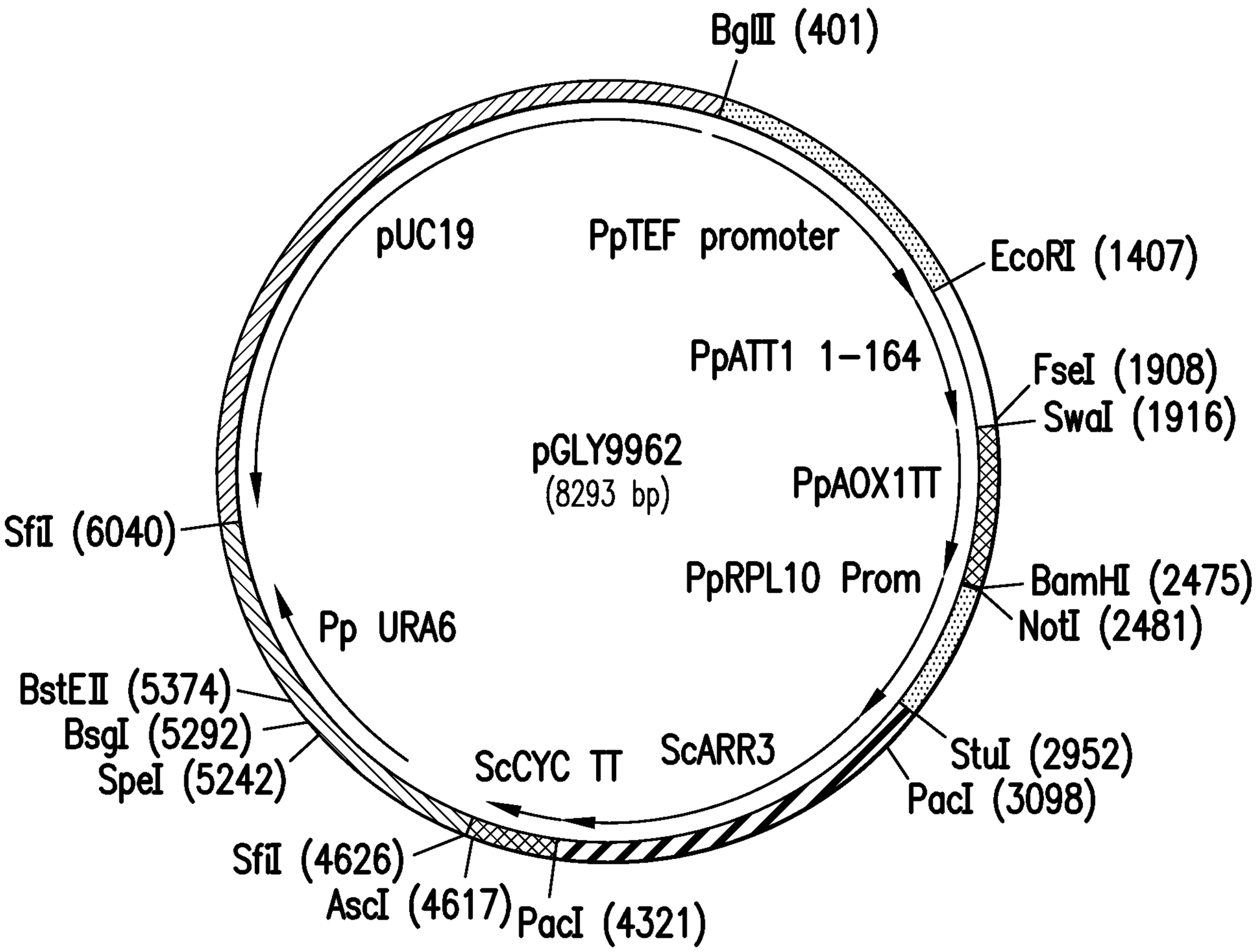
BglII (401)
pUC19
PpTEF promoter
EcoRI (1407)
PpATT1 1-164
FseI (1908)
SwaI (1916)
pGLY9962
(8293 bp)
PpAOX1TT
SfiI (6040)
PpRPL10 Prom
BamHI (2475)
NotI (2481)
Pp URA6
BstEII (5374)
BsgI (5292)
SpeI (5242)
ScCYC TT
ScARR3
StuI (2952)
PacI (3098)
SfiI (4626)
AscI (4617)
PacI (4321)

FIG.14G

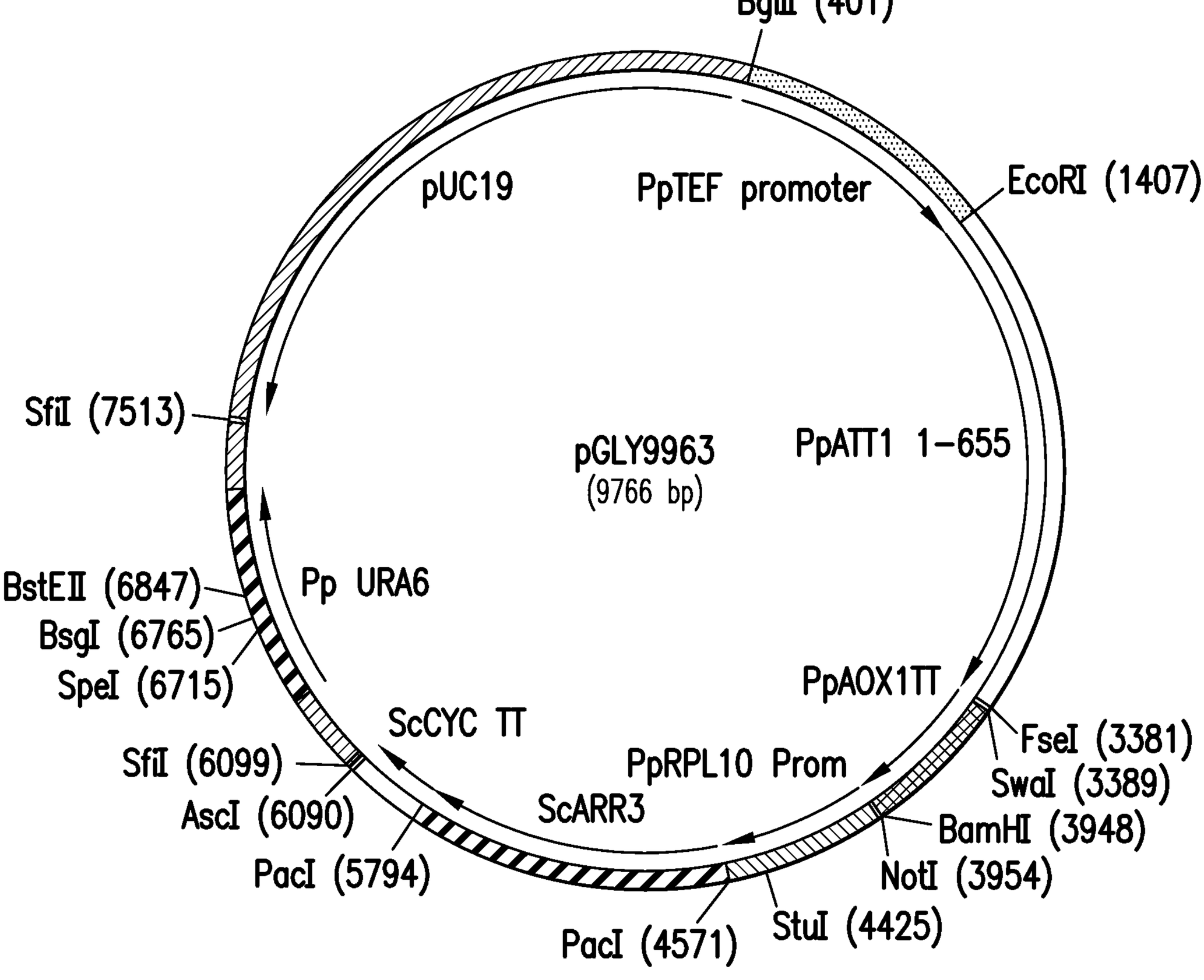
BglII (401)
pUC19
PpTEF promoter
EcoRI (1407)
SfiI (7513)
pGLY9963
(9766 bp)
PpATT1 1-655
Pp URA6
BstEII (6847)
BsgI (6765)
SpeI (6715)
PpAOX1TT
ScCYC TT
FseI (3381)
SfiI (6099)
PpRPL10 Prom
SwaI (3389)
AscI (6090)
ScARR3
BamHI (3948)
PacI (5794)
NotI (3954)
PacI (4571)
StuI (4425)

FIG.14H

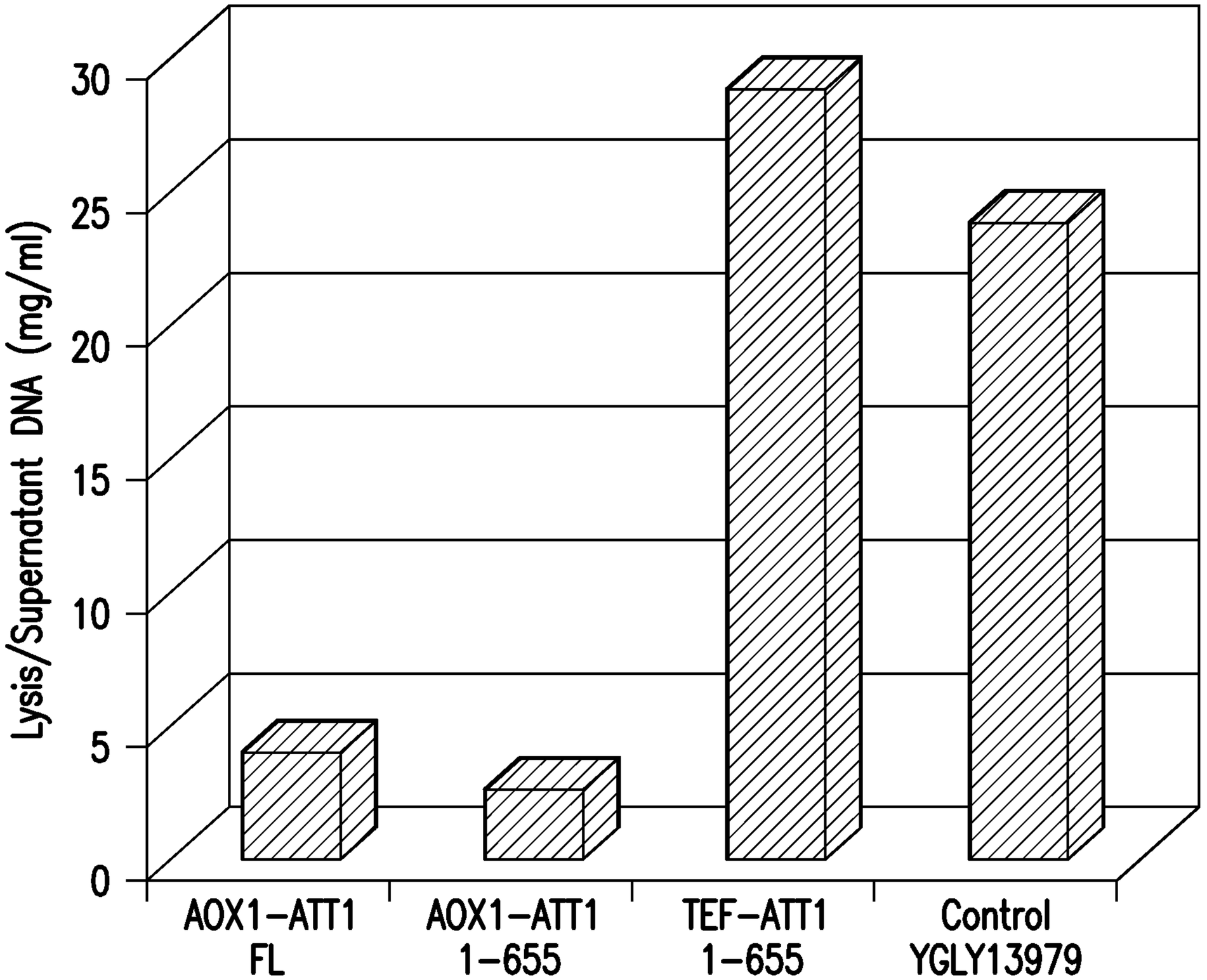
Lysis/Supernatant DNA (mg/ml)
30
25
20
15
10
5
0
AOX1-ATT1 FL
AOX1-ATT1 1-655
TEF-ATT1 1-655
Control YGLY13979

FIG.15

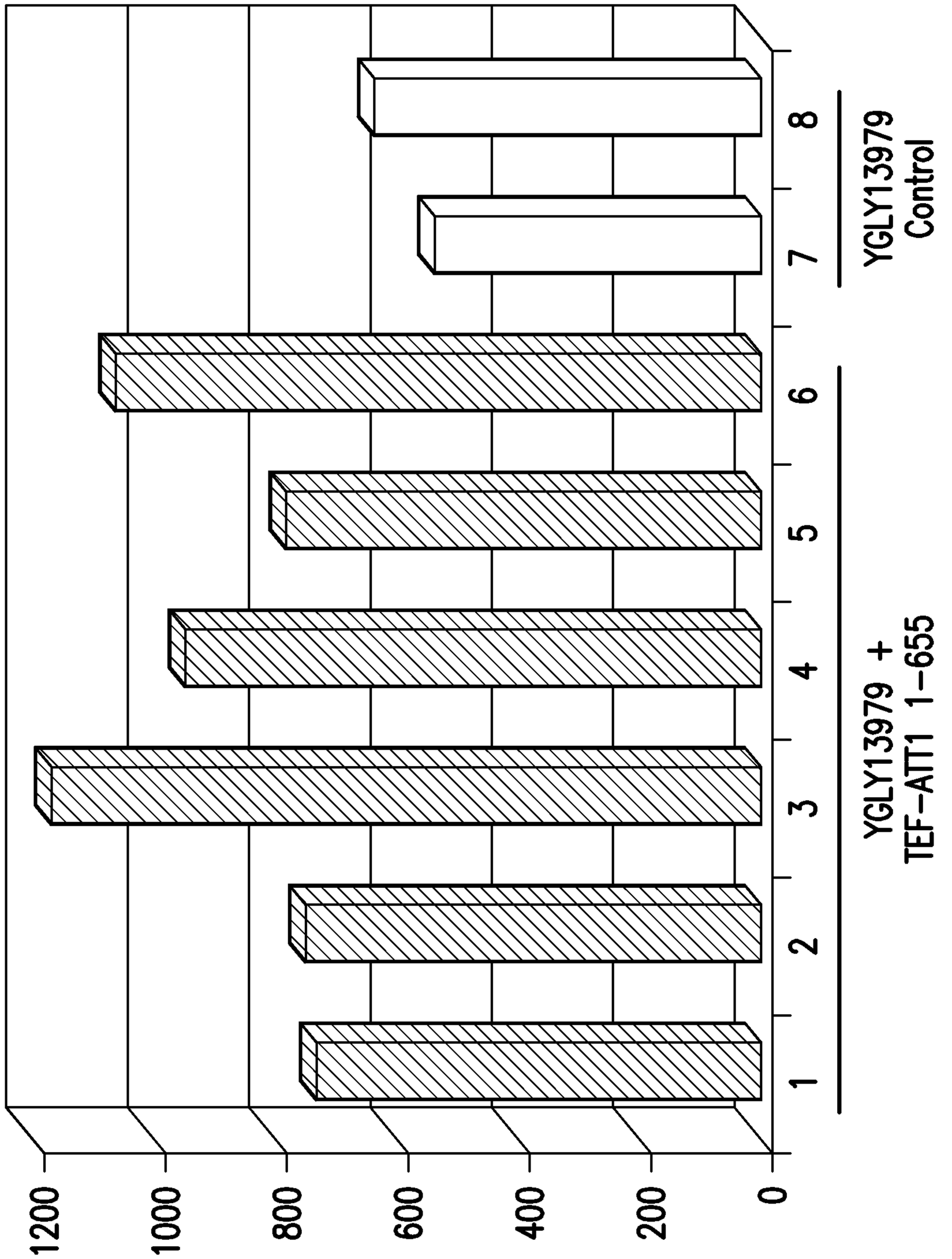
YGLY13979 + TEF-ATT1 1-655
YGLY13979 Control
1
2
3
4
5
6
7
8
0
200
400
600
800
1000
1200
HPLC Titer (mg/L)

FIG. 16

Run	Strain	Description	M24 clone	ind time (h)	Lysis	PicoGreen	HPLC Titer
D113325	YGLY27927	YGLY13979 + AOX1-ATT1	M1130-06 A1	111	2.5	23	707
D113326	YGLY27928	YGLY13979 + AOX1-ATT1	M1130-06 A2	64	4.25	28	605
D113327	YGLY27929	YGLY13979 + AOX1-ATT1	M1130-06 A3	112	1.25	4.5	1974
D113328	YGLY27930	YGLY13979 + AOX1-ATT1	M1130-06 A4	113	1	5.2	1960
D113329	YGLY27931	YGLY13979 + AOX1-ATT1	M1130-06 A5	67	3.5	7.4	648
D113330	YGLY27932	YGLY13979 + AOX1-ATT1	M1130-06 A6	66	4.25	38.9	837
D113331	YGLY13979	YGLY13979	N/A	66	4.25	26	671
D113332	YGLY27638	YGLY13979 att1::URA5	N/A	106	0.75	1.2	1256

FIG. 17

SUBSTITUTE SHEET (RULE 26)

WO 2013/062940 PCT/US2012/061432

CA 02853341 2014-04-23

PpATT1 Homologous to Saccharomyces Transcription Factor GAL4 and H. polymorpha ATT1–like

```
                    1                                                                                                  100
HpATT1 aa     (1)   MEGISKPVGFGENKESMVKRELSDEDLFVLESRLSESASSANLTNSPSPPQNSNLSNGHSTKPTISRTTSSGSSYVHRQHTPSHGHGSRHLAHHHHNNSV
PpATT1 aa     (1)   ----------------------------------------------------------------------------------------MHHKERL
ScGAL4 aa     (1)   ----------------------------------------------------------------------------------------------------
Consensus     (1)                                                                                            MHHKERL

                    101                                                                                                200
HpATT1 aa   (101)   FPQRTIKREPSDSLEDDAIEEAEGYENKSHSRNKPLIEQACDSCRRRKLRCTKELPKCSKCMDHGWECVYSPRAVRSPLTRAYLTKVENRVKQLETFLLK
PpATT1 aa     (8)   IDHISS--ESNFSLSTSSMPSFSHESNQSP--NPMLIEQACDSCRKRKLRCSKEYPKCSKCVTHKWSCVYSPRTVRSPLTRAHLTKVENRVRMLEDLLER
ScGAL4 aa     (1)   ----------------------------MKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVESRLERLEQLFLL
Consensus   (101)   IDHISS  ESNFSLSTSSMPSFSHESNQSP  NPMLIEQACDSCRKRKLRCSKEYPKCSKCVTHKWSCVYSPRTVRSPLTRAHLTKVENRVRMLEDLLER

                    201                                                                                                300
HpATT1 aa   (201)   AFPGEDLEQMLGGFSRTGSSIQSLCNSPNMSAYSLLSQNKQHCDNTSSTQTMQDFSLDASSTSIFKKETPQQILSRLPDEFMATDLSNNTNFDWSEDDEE
PpATT1 aa   (104)   VFPTQSVDQLL--EKRTSLSGNSTGHSPSYPNSNSVSPQN------SSPKVSDSSSTTAEPAPVLPSKPKSSFRPIVPDDYFLNDEIN--GFDWEEEDTP
ScGAL4 aa    (71)   IFPREDLDMILKMDSLQDIKALLTG-------------------------------------------------------LFVQDNVN-------KDAVT
Consensus   (201)   VFPTQSVDQLL  EKRTSLSGNSTGHSPSYPNSNSVSPQN      SSPKVSDSSSTTAEPAPVLPSKPKSSFRPIVPDDYFLNDEIN  GFDWEEEDTP

                    301                                                                                                400
HpATT1 aa   (301)   REKGLMMNGSPSSPSSITSLHEPKNSVISFNSLDHLHTSQAQSSTISTKTNNSSLCTSPYLRAVAPSFSTDGMGVNPTTKSGFLGVGSSSSFLRVMKIDK
PpATT1 aa   (194)   DQLLVMQQ--P--PTSVDSTNVSHSYWNHSRRSQKNSVTSLNSLAEHEQSGCSSLITSPSLQPLSQTTTNDSHPDGMAALSVNLKGGSG----------
ScGAL4 aa   (109)   DRLASVETDMP-----LTLRQHRISATSSSEESSNKGQRQLTVSIDSAAHHDNSTIPLDFMPRDALHGFDWSEEDDMSDGLPFLKTDPN----------
Consensus   (301)   DQLLVMQQ  P  PTSVDSTNVSHSYWNHSRRSQKNSVTSLNSLAEHEQSGCSSLITSPSLQPLSQTTTNDSHPDGMAALSVNLKGGSG
```

FIG.18-1

SUBSTITUTE SHEET (RULE 26)

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                401                                                
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SUBSTITUTE SHEET (RULE 26)

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                    801                                                
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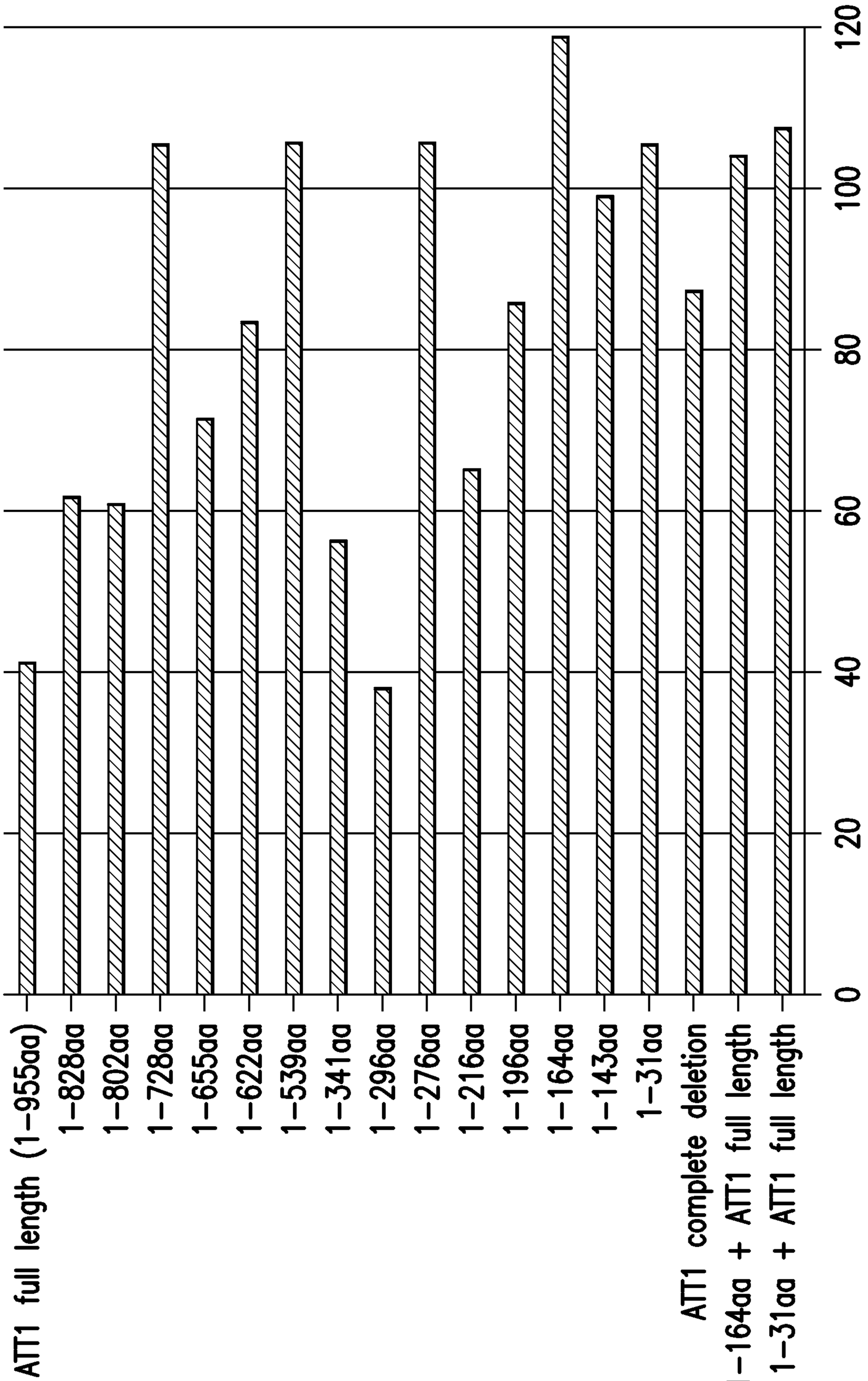

Effects of Different ATT1 Truncations on Cell Robustness
1L Bioreactor Induction Length at 32°C (hours)
0
20
40
60
80
100
120
ATT1 full length (1–955aa)
ATT1 full length 1–828aa
ATT1 full length 1–802aa
ATT1 full length 1–728aa
ATT1 complete deletion 1–655aa
1–622aa
1–539aa
1–341aa
1–296aa
1–276aa
1–216aa
1–196aa
1–164aa
1–143aa
1–31aa
ATT1 complete deletion
1–164aa + ATT1 full length
1–31aa + ATT1 full length

FIG.19A

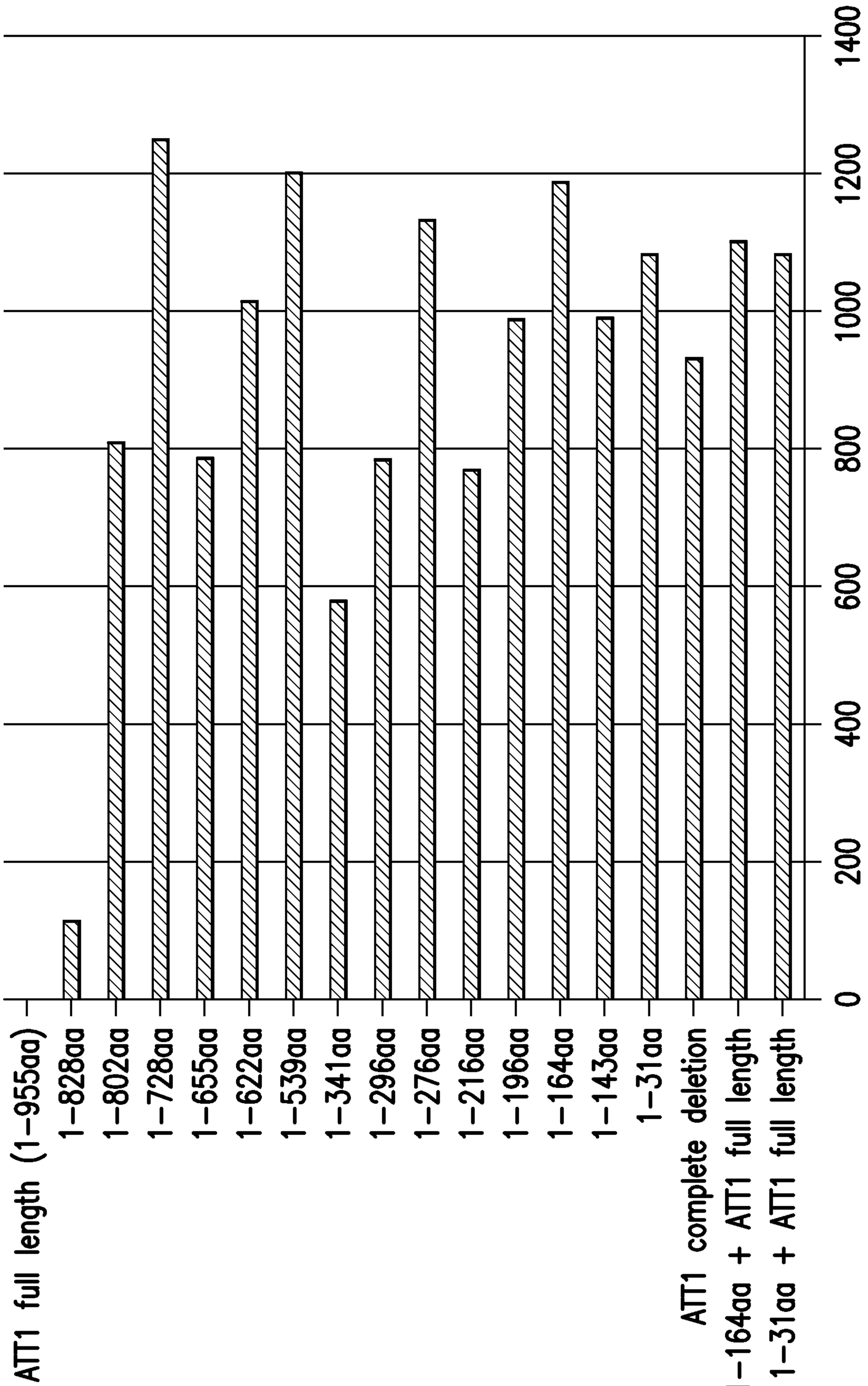

Effects of Different ATT1 Truncations on mAb Titer
anti-HER2 titer (mg/liter)
0
200
400
600
800
1000
1200
1400
ATT1 full length
(1–955aa)
1–828aa
1–802aa
1–728aa
1–655aa
1–622aa
1–539aa
1–341aa
1–296aa
1–276aa
1–216aa
1–196aa
1–164aa
1–143aa
1–31aa
complete deletion
full length
full length
ATT1 complete deletion
1–164aa + ATT1 full length
1–31aa + ATT1 full length

FIG.19B

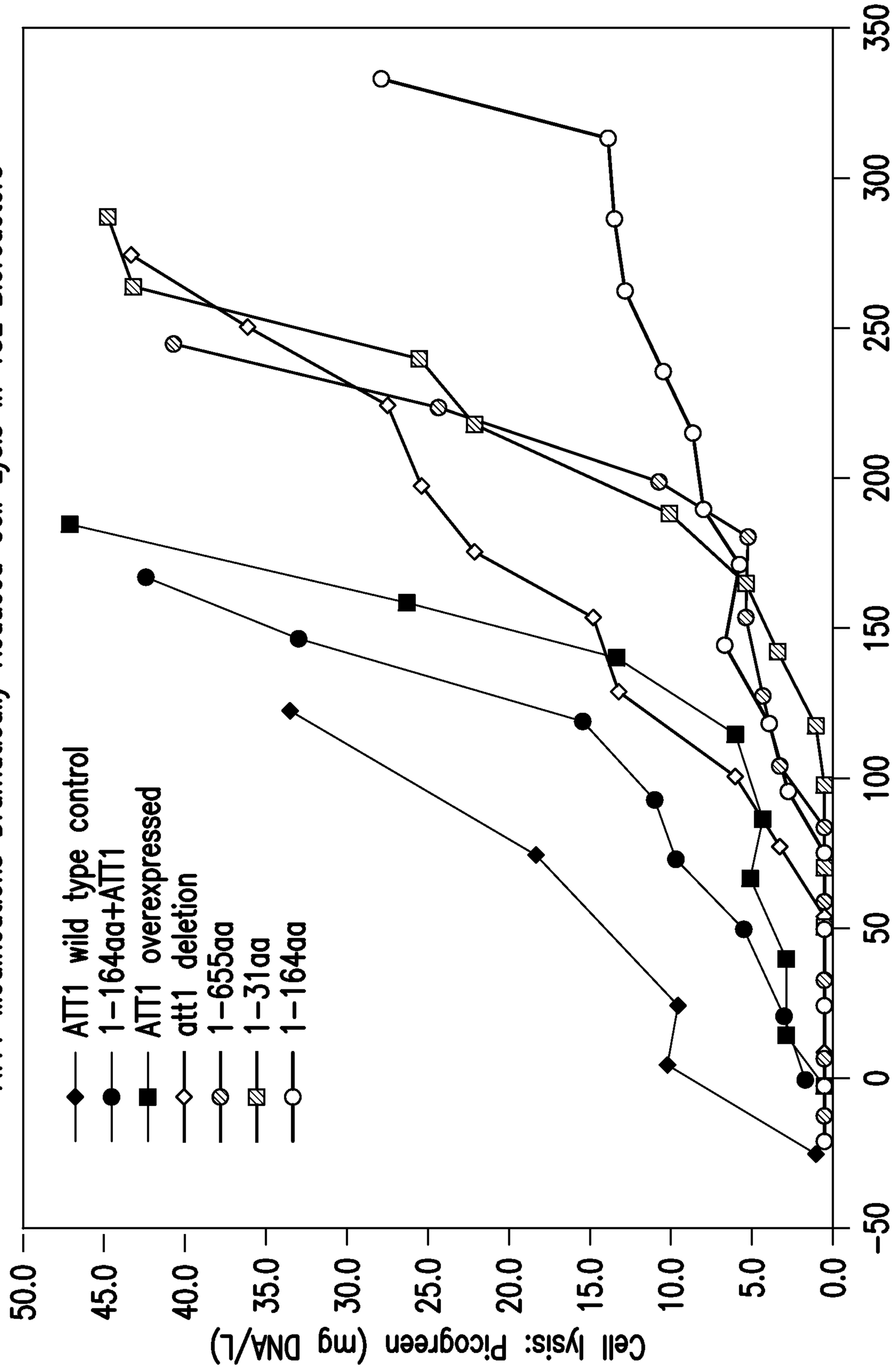

ATT1 Modifications Dramatically Reduced Cell Lysis in 15L Bioreactors
ATT1 wild type control
1-164aa+ATT1
ATT1 overexpressed
att1 deletion
1-655aa
1-31aa
1-164aa
Cell lysis: Picogreen (mg DNA/L)
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-50
0
50
100
150
200
250
300
Induction time (h)

FIG.20

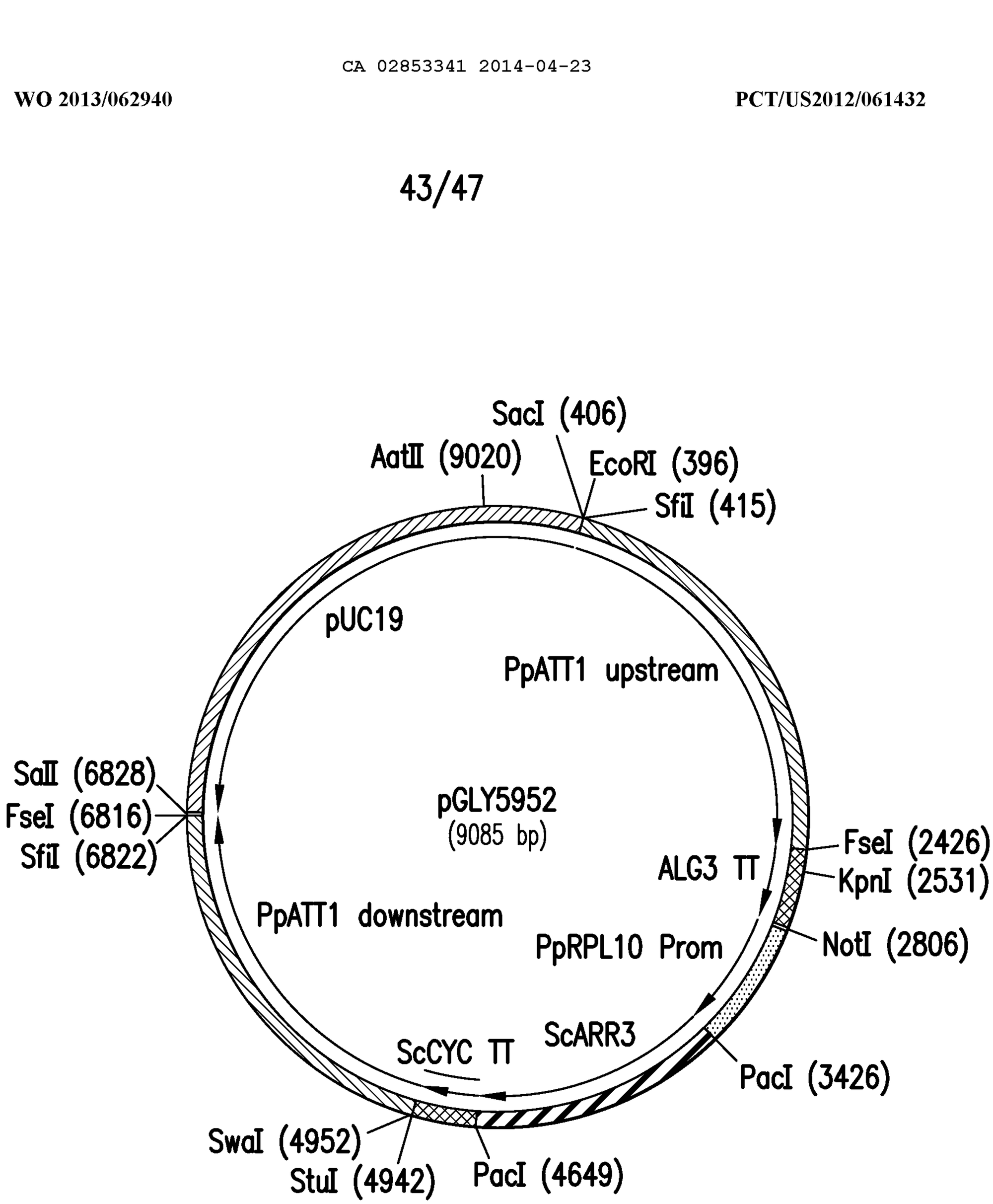
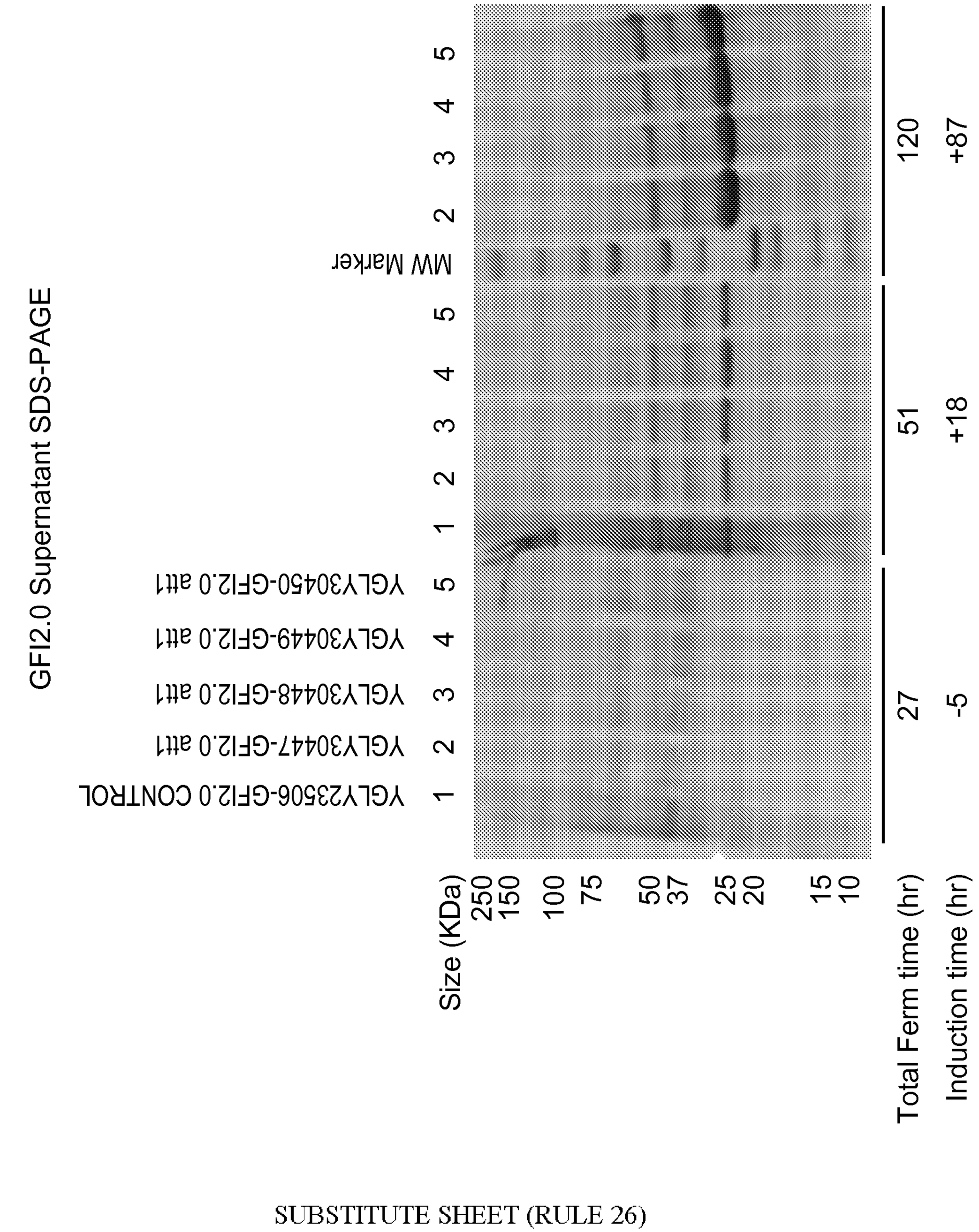

SacI (406)
AatII (9020)
EcoRI (396)
SfiI (415)
pUC19
PpATT1 upstream
SalI (6828)
FseI (6816)
SfiI (6822)
pGLY5952
(9085 bp)
FseI (2426)
KpnI (2531)
ALG3 TT
PpATT1 downstream
PpRPL10 Prom
NotI (2806)
ScARR3
ScCYC TT
PacI (3426)
SwaI (4952)
StuI (4942)
PacI (4649)

FIG.21

FIG.22


GFI2.0 Supernatant SDS-PAGE
MW Marker
3 4 5 2 3 4 5
1 2 1 2
YGLY23506-GFI2.0 CONTROL
YGLY30447-GFI2.0 att1
YGLY30448-GFI2.0 att1
YGLY30449-GFI2.0 att1
YGLY30450-GFI2.0 att1
Size (KDa)
250
150
100
75
50
37
25
20
15
10
51
120
+18
+87
27
-5
Total Ferm time (hr)
Induction time (hr)

Strain	Fermenter
YGLY23506 Control	Q121601
YGLY30447	Q121602
YGLY30448	Q121603
YGLY30449	Q121604
YGLY30450	Q121605

FIG.23

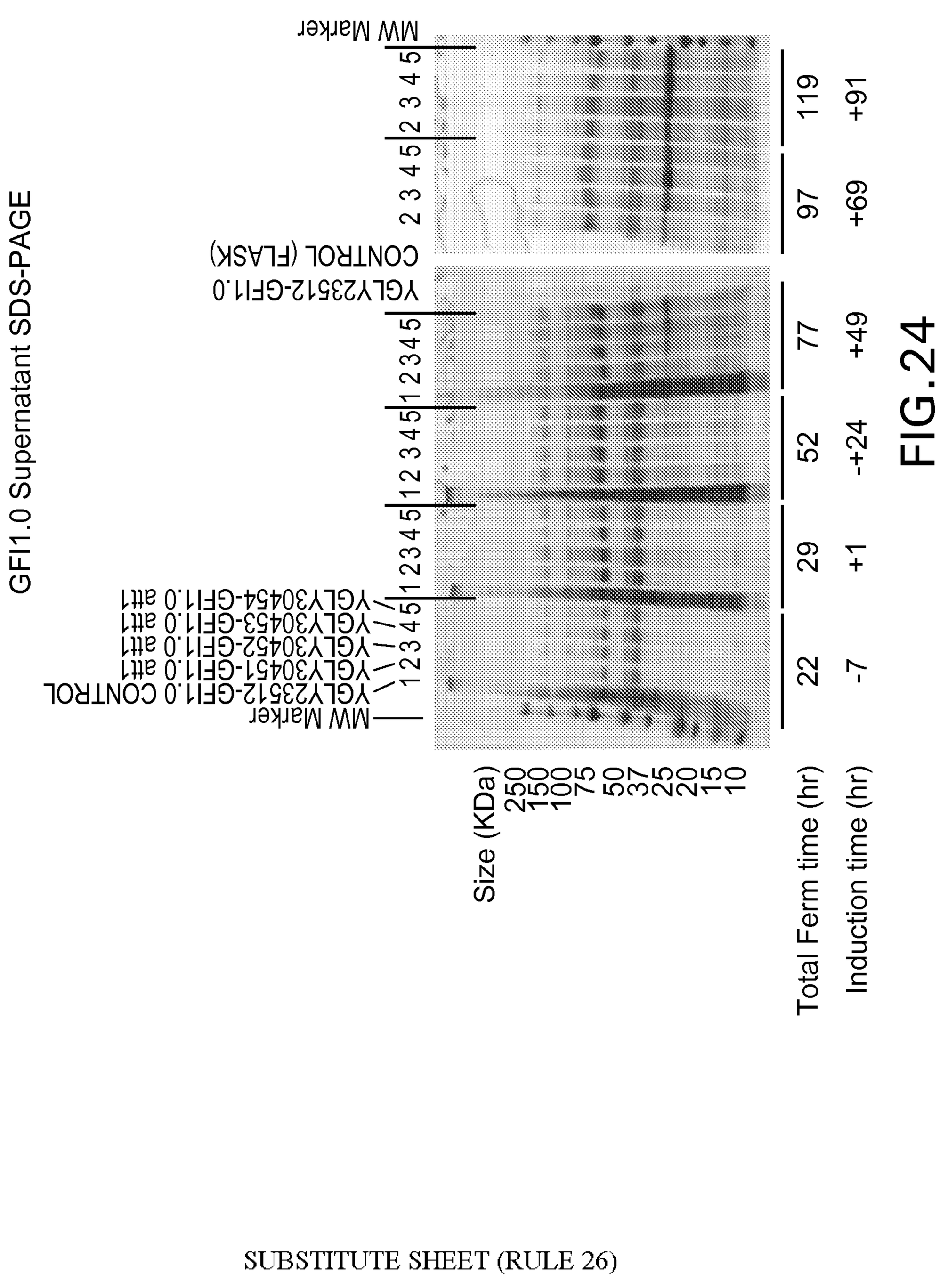

GFI1.0 Supernatant SDS-PAGE
MW Marker
YGLY23512-GFI1.0 CONTROL
YGLY30451-GFI1.0 att1
YGLY30452-GFI1.0 att1
YGLY30453-GFI1.0 att1
YGLY30454-GFI1.0 att1
1 2 3 4 5
MW Marker
YGLY23512-GFI1.0 CONTROL (FLASK)
1 2 3 4 5
2 3 4 5
MW Marker
1 2 3 4 5
Size (KDa)
250
150
100
75
50
37
25
20
15
10
Total Ferm time (hr)
22 29 52 77
-7 +1 +24 +49
Induction time (hr)
97 119
+69 +91

FIG.24

Strain	Fermenter
YGLY23512 Control	Q121401
YGLY30451	Q121402
YGLY30452	Q121403
YGLY30453	Q121404
YGLY30454	Q121405

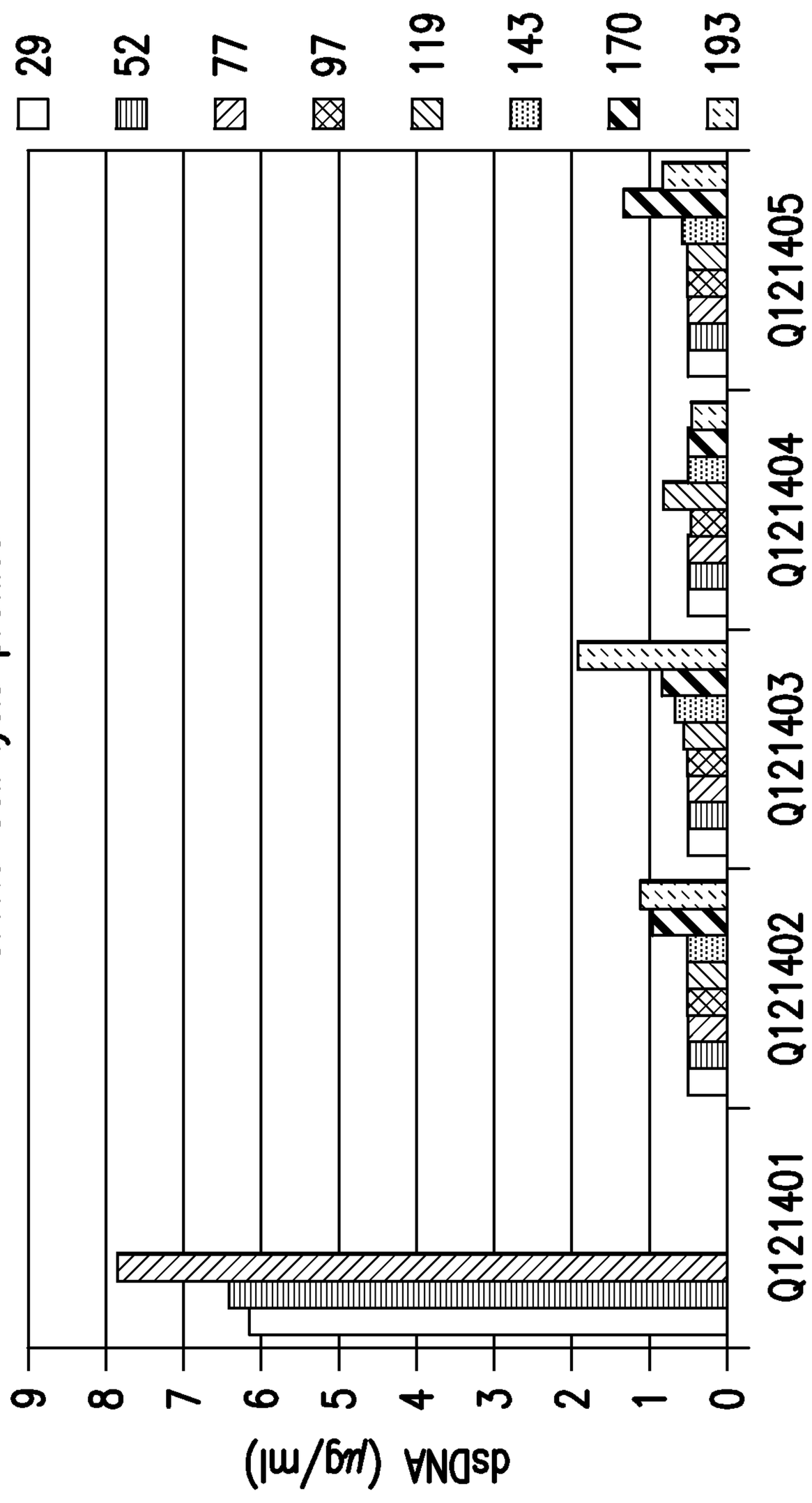


FIG.25

```
                1                                                    50
PpATT1    (1) MHHKERLIDHISSESNFSLSTSSMPSFSHESNQSPNPMLIEQACDSCRKR
ScGAL4    (1) ---------------------------------MKLLSSIEQACDICRLK
               51                                                   100
PpATT1   (51) KLRCSKEYPKCSKCVTHKWSCVYSPRTVRSPLTRAHLTKVENRVRMLEDL
ScGAL4   (18) KLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVESRLERLEQL

              101                                                  150
PpATT1  (101) LERVFPTQSVDQLLEKRTSLSGNSTGHSPSYPNSNSVSPQNSSPKVSDSS
ScGAL4   (68) FLLIFPREDLDMILKMDSLQDIKALLTGLFVQDN------------VNKD

              151                                                  200
PpATT1  (151) STTAEPAPVLPSKPKSSFRPIVPDDYFLNDEINGFDWEEEDTPDQLLVMQ
ScGAL4  (106) AVTDRLASVETDMP--------------------------------LTLR
              201                                                  250
PpATT1  (201) QPPTSVDSTNVSHSYWNHSRRSQKNSVTSLNSLAEHEQSGCSSLITSPSL
ScGAL4  (124) QHRISATSSSEES-----SNKGQRQLTVSIDSAAHHDNSTIPLDFMPRDA
              251                                                  300
PpATT1  (251) QPLSQTTTNDSHPDGMAALSVNLKGGSGYFGFSSSSGLLRALKLGQFDSA
ScGAL4  (169) LHGFDWSEEDDMSDGLPFLKTDPNN-NGFFGDGSLLCILRSIGFK-----
              301                                                  350
PpATT1  (301) SISPMSSVRNSVSKTNTEPTEPQSIRSLLGDPNDFLEPEKKAEFPGYDSH
ScGAL4  (213) ---PENYTNSNVNRLPTMITDR-------------------------YT
              351                                                  400
PpATT1  (351) LNDPNNQSQYLQAYFKYYHTSYPFIHKGSFLKHYAGELPIKNENHWQILL
ScGAL4  (234) LASRSTTSRLLQSYLNNFHPYCPIVHSPTLMMLYNNQIEIASKDQWQILF
              401                                                  450
PpATT1  (401) NVVLALGCWCLNGESSSIDLCYYNRAKMLLKQVGIFECGNIMLLESLILL
ScGAL4  (284) NCILAIGAWCIEGESTDIDVFYYQNAKSHLTSK-VFESGSIILVTALHLL
              451                                                  500
PpATT1  (451) SNYTQKRNKPNTGWSYLGIAIRMAMSLGLYKEFNLDHTEKDHYLNLEIRR
ScGAL4  (333) SRYTQWRQKTNTSYNFHSFSIRMAISLGLNRDLPSSFSDSS---ILEQRR
              501                                                  550
PpATT1  (501) RLWWGLYIFDAGASITFGRPITLPSRDSCDIQLCSNINDAELEELIEIKS
ScGAL4  (380) RIWWSVYSWEIQLSLLYGR------------------SIQLSQNTISFP
```

FIG.1A

```
              551                                                  600
PpATT1  (551) DSITTEDLNKPYPTAYSGLIQQTQFTELSMKIYNRLVSKPAP----TVEE
ScGAL4  (411) SSVDDVQRTTTGPTIYHGIIETARLLQVFTKIYELDKTVTAEKSPICAKK
              601                                                  650
PpATT1  (597) CLDMNMEIENFIKGLPAYFHESNEIAMSQFYKVTPSKYYDYDSNKQVDYT
ScGAL4  (461) CLMICNEIEEVSRQAPKFLQ-----------------MDISTTALTNLL

              651                                                  700
PpATT1  (647) RLPQWFDLSRSRLIWRYKNLQITLFRAFIWQRVIGVTNPKVLQQCKTS-R
ScGAL4  (493) KEHPWLSFTRFELKWKQLSLIIYVLRDFFTNFTQKKSQLEQDQNDHQSYE
              701                                                  750
PpATT1  (696) GKECRTICLRVAHETILSIQQFVNIDDDDDFSRLSVIGCWYATYFLFQAV
ScGAL4  (543) VKRCSIMLSDAAQRTVMSVSSYMDNHN------VTPYFAWNCSYYLFNAV
              751                                                  800
PpATT1  (746) LIPIACLCSEPDSKYAP-IWIEDIQISKKIFLKLNKLN----SLASKFAN
ScGAL4  (587) LVPIKTLLSNSKSNAENNETAQLLQQINTVLMLLKKLATFKIQTCEKYIQ
              801                                                  850
PpATT1  (791) VIDRSMSQVMPQFDTTSAKDSPLNINDLIDMHGLMGNS-------P-APG
ScGAL4  (637) VLEEVCAPFLLSQCAIPLPHISYNNSNGSAIKNIVGSATIAQYPTLPEEN
              851                                                  900
PpATT1  (833) SNNNSNTKSSPSTTNNTRTPNTINKNNSNMNNNSINNYFNNNS-----NN
ScGAL4  (687) VNNISVKYVSPGSVGPSPVPLKSGASFSDLVKLLSNRPPSRNSPVTIPRS
              901                                                  950
PpATT1  (878) NNSFSSSKAGPVKQEFEDYCLKLDP--------------EDEDMSALEF
ScGAL4  (737) TPSHRSVTPFLGQQQQLQSLVPLTPSALFGGANFNQSGNIADSSLSFTFT
              951                                                 1000
PpATT1  (913) TAVRFPNFSATTTAPPPTPVNCNSPENIKTSTVDDFLKATQDP-NNKEIL
ScGAL4  (787) NSSNGPNLITTQTNSQALSQPIASSNVHDNFMNNEITASKIDDGNNSKPL
              1001                                  1045
PpATT1  (962) NDIYSLIFDDSMDPMSFGSMEPRNDLEVPDTIMD-----------
ScGAL4  (837) SPGWTDQTAYNAFGITTGMFNTTTMDDVYNYLFDDEDTPPNPKKE
```

FIG.1B