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(72) Inventeurs/Inventors:
SLOMA, ALAN, US;
BEHR, REGINE, US;
WIDNER, WILLIAM, US;
TANG, MARIA, US;
STERNBERG, DAVID, US;
BROWN, STEPHEN, US

(73) Propriétaire/Owner:

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(54) Title: METHODS FOR PRODUCING HYALURONAN IN A RECOMBINANT HOST CELL

(57) **Abrégé/Abstract:**

The present invention relates to methods for producing a hyaluronic acid, comprising: (a) cultivating a *Bacillus* host cell under conditions suitable for production of the hyaluronic acid, wherein the *Bacillus* host cell comprises a nucleic acid construct comprising a hyaluronan synthase encoding sequence operably linked to a promoter sequence foreign to the hyaluronan synthase encoding sequence; and (b) recovering the hyaluronic acid from the cultivation medium. The present invention also relates to an isolated nucleic acid sequence encoding a hyaluronan synthase operon comprising a hyaluronan synthase gene and a UDP-glucose 6-dehydrogenase gene, and optionally one or more genes selected from the group consisting of a UDP-glucose pyrophosphorylase gene, UDP-N-acetylglucosamine pyrophosphorylase gene, and glucose-6-phosphate isomerase gene. The present invention also relates to isolated nucleic acid sequences encoding a UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, and UDP-N-acetylglucosamine pyrophosphorylase.



(73) **Propriétaires(suite)/Owners(continued):**NOVOZYMES BIOPHARMA DK A/S, DK

(74) **Agent:** NORTON ROSE CANADA S.E.N.C.R.L.,S.R.L./LLP

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- (74) Agent: **STARNES, Robert**; Novozymes Biotech, Inc.,
1445 Drew Avenue, Davis, CA 95616 (US).
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- (71) Applicant: **NOVOZYMES BIOTECH, INC.** [US/US];
1445 Drew Avenue, Davis, CA 95616 (US).
- (72) Inventors: **SLOMA, Alan**; 849 Donovan Court, Davis, CA 95616 (US). **BEHR, Regine**; 1017 Danielle Drive, Roseville, CA 95747 (US). **WIDNER, William**; 804 E. 9th Street, Apt. 107, Davis, CA 95616 (US). **TANG, Maria**; 2043 Cliffwood Drive, Fairfield, CA 94033 (US). **STERNBERG, David**; 3205 Breton Avenue, Davis, CA 95616 (US). **BROWN, Stephen**; 3708 Miwok Place, Davis, CA 95616 (US).
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(54) Title: METHODS FOR PRODUCING HYALURONAN IN A RECOMBINANT HOST CELL

(57) Abstract: The present invention relates to methods for producing a hyaluronic acid, comprising: (a) cultivating a *Bacillus* host cell under conditions suitable for production of the hyaluronic acid, wherein the *Bacillus* host cell comprises a nucleic acid construct comprising a hyaluronan synthase encoding sequence operably linked to a promoter sequence foreign to the hyaluronan synthase encoding sequence; and (b) recovering the hyaluronic acid from the cultivation medium. The present invention also relates to an isolated nucleic acid sequence encoding a hyaluronan synthase operon comprising a hyaluronan synthase gene and a UDP-glucose 6-dehydrogenase gene, and optionally one or more genes selected from the group consisting of a UDP-glucose pyrophosphorylase gene, UDP-N-acetylglucosamine pyrophosphorylase gene, and glucose-6-phosphate isomerase gene. The present invention also relates to isolated nucleic acid sequences encoding a UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, and UDP-N-acetylglucosamine pyrophosphorylase.

METHODS FOR PRODUCING HYALURONAN IN A RECOMBINANT HOST CELL

5

Background of the Invention

Field of the Invention

The present invention relates to methods for producing a hyaluronan in a
10 recombinant host cell.

Description of the Related Art

The most abundant heteropolysaccharides of the body are the
glycosaminoglycans. Glycosaminoglycans are unbranched carbohydrate polymers,
15 consisting of repeating disaccharide units (only keratan sulphate is branched in the core
region of the carbohydrate). The disaccharide units generally comprise, as a first
saccharide unit, one of two modified sugars - N-acetylgalactosamine (GalNAc) or N-
acetylglucosamine (GlcNAc). The second unit is usually an uronic acid, such as
glucuronic acid (GlcUA) or iduronate.

20 Glycosaminoglycans are negatively charged molecules, and have an extended
conformation that imparts high viscosity when in solution. Glycosaminoglycans are
located primarily on the surface of cells or in the extracellular matrix.
Glycosaminoglycans also have low compressibility in solution and, as a result, are ideal
as a physiological lubricating fluid, e.g., joints. The rigidity of glycosaminoglycans
25 provides structural integrity to cells and provides passageways between cells, allowing
for cell migration. The glycosaminoglycans of highest physiological importance are
hyaluronan, chondroitin sulfate, heparin, heparan sulfate, dermatan sulfate, and keratan
sulfate. Most glycosaminoglycans bind covalently to a proteoglycan core protein
through specific oligosaccharide structures. Hyaluronan forms large aggregates with
30 certain proteoglycans, but is an exception as free carbohydrate chains form non-
covalent complexes with proteoglycans.

Numerous roles of hyaluronan in the body have been identified (see, Laurent T.
C. and Fraser J. R. E., 1992, *FASEB J.* 6: 2397-2404; and Toole B.P., 1991,
"Proteoglycans and hyaluronan in morphogenesis and differentiation." In: *Cell Biology*
35 *of the Extracellular Matrix*, pp. 305-341, Hay E. D., ed., Plenum, New York).
Hyaluronan is present in hyaline cartilage, synovial joint fluid, and skin tissue, both
dermis and epidermis. Hyaluronan is also suspected of having a role in numerous

physiological functions, such as adhesion, development, cell motility, cancer, angiogenesis, and wound healing. Due to the unique physical and biological properties of hyaluronan, it is employed in eye and joint surgery and is being evaluated in other medical procedures. Products of hyaluronan have also been developed for use in
5 orthopaedics, rheumatology, and dermatology.

Rooster combs are a significant commercial source for hyaluronan. Microorganisms are an alternative source. U.S. Patent No. 4,801,539 discloses a fermentation method for preparing hyaluronic acid involving a strain of *Streptococcus zooepidemicus* with reported yields of about 3.6 g of hyaluronic acid per liter. European
10 Patent No. EP0694616 discloses fermentation processes using an improved strain of *Streptococcus zooepidemicus* with reported yields of about 3.5 g of hyaluronic acid per liter.

The microorganisms used for production of hyaluronic acid by fermentation are strains of pathogenic bacteria, foremost among them being several *Streptococcus* spp.
15 The group A and group C streptococci surround themselves with a nonantigenic capsule composed of hyaluronan, which is identical in composition to that found in connective tissue and joints. *Pasteurella multocida*, another pathogenic encapsulating bacteria, also surrounds its cells with hyaluronan.

Hyaluronan synthases have been described from vertebrates, bacterial
20 pathogens, and algal viruses (DeAngelis, P. L., 1999, *Cell. Mol. Life Sci.* 56: 670-682). WO 99/23227 discloses a Group I hyaluronate synthase from *Streptococcus equisimilis*. WO 99/51265 and WO 00/27437 describe a Group II hyaluronate synthase from *Pasturella multocida*. Ferretti *et al.* disclose the hyaluronan synthase operon of *Streptococcus pyogenes*, which is composed of three genes, *hasA*, *hasB*, and *hasC*,
25 that encode hyaluronate synthase, UDP glucose dehydrogenase, and UDP-glucose pyrophosphorylase, respectively (*Proc. Natl. Acad. Sci. USA.* 98, 4658-4663, 2001). WO 99/51265 describes a nucleic acid segment having a coding region for a *Streptococcus equisimilis* hyaluronan synthase.

Bacilli are well established as host cell systems for the production of native and
30 recombinant proteins. It is an object of the present invention to provide methods for producing a hyaluronan in a recombinant *Bacillus* host cell.

Brief Summary of the Invention

35 The present invention relates to methods for producing a hyaluronic acid, comprising: (a) cultivating a *Bacillus* host cell under conditions suitable for production of the hyaluronic acid, wherein the *Bacillus* host cell comprises a nucleic acid construct

comprising a hyaluronan synthase encoding sequence operably linked to a promoter sequence foreign to the hyaluronan synthase encoding sequence; and (b) recovering the hyaluronic acid from the cultivation medium.

In preferred embodiments, the nucleic acid construct further comprises one or more genes encoding enzymes in the biosynthesis of a precursor sugar of the hyaluronic acid or the *Bacillus* host cell further comprises one or more second nucleic acid constructs comprising one or more genes encoding enzymes in the biosynthesis of the precursor sugar.

In another preferred embodiment, the one or more genes encoding a precursor sugar are under the control of the same or a different promoter(s) as the hyaluronan synthase encoding sequence.

The present invention also relates to *Bacillus* host cells comprising a nucleic acid construct comprising a hyaluronan synthase encoding sequence operably linked to a promoter sequence foreign to the hyaluronan synthase encoding sequence, and to such nucleic acid constructs.

The present invention also relates to an isolated nucleic acid sequence encoding a hyaluronan synthase operon comprising a hyaluronan synthase gene or a portion thereof and a UDP-glucose 6-dehydrogenase gene, and optionally one or more genes selected from the group consisting of a UDP-glucose pyrophosphorylase gene, UDP-N-acetylglucosamine pyrophosphorylase gene, and glucose-6-phosphate isomerase gene.

The present invention also relates to isolated nucleic acid sequences encoding a UDP-glucose 6-dehydrogenase selected from the group consisting of: (a) a nucleic acid sequence encoding a polypeptide having an amino acid sequence which has at least about 75%, about 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 41; (b) a nucleic acid sequence having at least about 75%, about 80%, about 85%, about 90%, or about 95% homology to SEQ ID NO: 40; (c) a nucleic acid sequence which hybridizes under medium or high stringency conditions with (i) the nucleic acid sequence of SEQ ID NO: 40, (ii) the cDNA sequence contained in SEQ ID NO: 40, or (iii) a complementary strand of (i) or (ii); and (d) a subsequence of (a), (b), or (c), wherein the subsequence encodes a polypeptide fragment which has UDP-glucose 6-dehydrogenase activity.

The present invention also relates to isolated nucleic acid sequences encoding a UDP-glucose pyrophosphorylase selected from the group consisting of: (a) a nucleic acid sequence encoding a polypeptide having an amino acid sequence which has at least about 90%, about 95%, or about 97% identity to SEQ ID NO: 43; (b) a nucleic acid sequence having at least about 90%, about 95%, or about 97% homology to SEQ ID NO: 42; (c) a nucleic acid sequence which hybridizes under low, medium, or high

stringency conditions with (i) the nucleic acid sequence of SEQ ID NO: 42, (ii) the cDNA sequence contained in SEQ ID NO: 42, or (iii) a complementary strand of (i) or (ii); and (d) a subsequence of (a), (b), or (c), wherein the subsequence encodes a polypeptide fragment which has UDP-N-acetylglucosamine pyrophosphorylase activity.

5 The present invention also relates to isolated nucleic acid sequences encoding a UDP-N-acetylglucosamine pyrophosphorylase selected from the group consisting of: (a) a nucleic acid sequence encoding a polypeptide having an amino acid sequence which has at least about 75%, about 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 45; (b) a nucleic acid sequence having at least about 75%, about 80%,
10 about 85%, about 90%, or about 95% homology to SEQ ID NO: 44; (c) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with (i) the nucleic acid sequence of SEQ ID NO: 44, (ii) the cDNA sequence contained in SEQ ID NO: 44, or (iii) a complementary strand of (i) or (ii); and (d) a subsequence of (a), (b), or (c), wherein the subsequence encodes a polypeptide fragment which has UDP-N-
15 acetylglucosamine pyrophosphorylase activity.

Brief Description of the Figures

20 Figure 1 shows the chemical structure of hyaluronan.
 Figure 2 shows the biosynthetic pathway for hyaluronan synthesis.
 Figure 3 shows a restriction map of pCR2.1-*sehasA*.
 Figure 4 shows a restriction map of pCR2.1-*tuaD*.
 Figure 5 shows a restriction map of pCR2.1-*gtaB*.
25 Figure 6 shows a restriction map of pCR2.1-*gcaD*.
 Figure 7 shows a restriction map of pHA1.
 Figure 8 shows a restriction map of pHA2.
 Figure 9 shows a restriction map of pHA3.
 Figure 10 shows a restriction map of pHA4.
30 Figure 11 shows a restriction map of pHA5.
 Figure 12 shows a restriction map of pHA6.
 Figure 13 shows a restriction map of pHA7.
 Figure 14 shows a restriction map of pMRT106.
 Figure 15 shows a restriction map of pHA8.
35 Figure 16 shows a restriction map of pHA9.
 Figure 17 shows a restriction map of pHA10.
 Figure 18 shows a restriction map of pRB157.

Figure 19 shows a restriction map of pMRT084.

Figure 20 shows a restriction map of pMRT086.

Figure 21 shows a restriction map of pCJ791.

Figure 22 shows a restriction map of pMRT032.

5 Figure 23 shows a restriction map of pNNB194neo.

Figure 24 shows a restriction map of pNNB194neo-oriT.

Figure 25 shows a restriction map of pShV3.

Figure 26 shows a restriction map of pShV2.1-amyEΔB.

Figure 27 shows a restriction map of pShV3A.

10 Figure 28 shows a restriction map of pMRT036.

Figure 29 shows a restriction map of pMRT037.

Figure 30 shows a restriction map of pMRT041.

Figure 31 shows a restriction map of pMRT064.1.

Figure 32 shows a restriction map of pMRT068.

15 Figure 33 shows a restriction map of pMRT069.

Figure 34 shows a restriction map of pMRT071.

Figure 35 shows a restriction map of pMRT074.

Figure 36 shows a restriction map of pMRT120.

Figure 37 shows a restriction map of pMRT122.

20 Figure 38 shows a restriction map of pCR2.1-pel5'.

Figure 39 shows a restriction map of pCR2.1-pel3'.

Figure 40 shows a restriction map of pRB161.

Figure 41 shows a restriction map of pRB162.

Figure 42 shows a restriction map of pRB156.

25 Figure 43 shows a restriction map of pRB164.

Figure 44 shows a summary of fermentations of various hyaluronic acid producing *Bacillus subtilis* strains run under fed batch at approximately 2 g sucrose/L₀-hr, 37°C.

30 Figure 45 shows a summary of peak hyaluronic acid weight average molecular weights (MDa) obtained from fermentations of various hyaluronic acid producing *Bacillus subtilis* strains run under fed batch at approximately 2 g sucrose/L₀-hr, 37°C.

Detailed Description of the Invention

35 The present invention relates to methods for producing a hyaluronan, comprising: (a) cultivating a *Bacillus* host cell under conditions suitable for production of the hyaluronan, wherein the *Bacillus* host cell comprises a nucleic acid construct

comprising a hyaluronan synthase encoding sequence operably linked to a promoter sequence foreign to the hyaluronan synthase encoding sequence; and (b) recovering the hyaluronan from the cultivation medium.

The methods of the present invention represent an improvement over the production of hyaluronan from pathogenic, encapsulating bacteria. In encapsulating bacteria, a large quantity of the hyaluronan is produced in the capsule. In processing and purifying hyaluronan from such sources, it is first necessary to remove the hyaluronan from the capsule, such as by the use of a surfactant, or detergent, such as SDS. This creates a complicating step in commercial production of hyaluronan, as the surfactant must be added in order to liberate a large portion of the hyaluronan, and subsequently the surfactant must be removed prior to final purification.

The present invention allows the production of a large quantity of a hyaluronan, which is produced in a non-encapsulating host cell, as free hyaluronan. When viewed under the microscope, there is no visible capsule associated with the recombinant strains of *Bacillus*, whereas the pathogenic strains traditionally used in hyaluronan production comprise a capsule of hyaluronan that is at least twice the diameter of the cell itself.

Since the hyaluronan of the recombinant *Bacillus* cell is expressed directly to the culture medium, a simple process may be used to isolate the hyaluronan from the culture medium. First, the *Bacillus* cells and cellular debris are physically removed from the culture medium. The culture medium may be diluted first, if desired, to reduce the viscosity of the medium. Many methods are known to those skilled in the art for removing cells from culture medium, such as centrifugation or microfiltration. If desired, the remaining supernatant may then be filtered, such as by ultrafiltration, to concentrate and remove small molecule contaminants from the hyaluronan. Following removal of the cells and cellular debris, a simple precipitation of the hyaluronan from the medium is performed by known mechanisms. Salt, alcohol, or combinations of salt and alcohol may be used to precipitate the hyaluronan from the filtrate. Once reduced to a precipitate, the hyaluronan can be easily isolated from the solution by physical means. Alternatively, the hyaluronan may be dried or concentrated from the filtrate solution by using evaporative techniques known to the art, such as spray drying.

The methods of the present invention thus represent an improvement over existing techniques for commercially producing hyaluronan by fermentation, in not requiring the use of a surfactant in the purification of hyaluronan from cells in culture.

Hyaluronic Acid

"Hyaluronic acid" is defined herein as an unsulphated glycosaminoglycan

composed of repeating disaccharide units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA) linked together by alternating beta-1,4 and beta-1,3 glycosidic bonds (Figure 1). Hyaluronic acid is also known as hyaluronan, hyaluronate, or HA. The terms hyaluronan and hyaluronic acid are used interchangeably herein.

5 In a preferred embodiment, the hyaluronic acid obtained by the methods of the present invention has a molecular weight of about 10,000 to about 10,000,000 Da. In a more preferred embodiment, the hyaluronic acid obtained by the methods of the present invention has a molecular weight of about 25,000 to about 5,000,000 Da. In a most preferred embodiment, the hyaluronic acid obtained by the methods of the present
10 invention has a molecular weight of about 50,000 to about 3,000,000 Da.

The level of hyaluronic acid produced by a *Bacillus* host cell of the present invention may be determined according to the modified carbazole method (Bitter and Muir, 1962, *Anal Biochem.* 4: 330-334). Moreover, the average molecular weight of the hyaluronic acid may be determined using standard methods in the art, such as those
15 described by Ueno *et al.*, 1988, *Chem. Pharm. Bull.* 36, 4971-4975; Wyatt, 1993, *Anal. Chim. Acta* 272: 1-40; and Wyatt Technologies, 1999, "Light Scattering University DAWN Course Manual" and "DAWN EOS Manual" Wyatt Technology Corporation, Santa Barbara, California.

The hyaluronic acid obtained by the methods of the present invention may be
20 subjected to various techniques known in the art to modify the hyaluronic acid, such as crosslinking as described, for example, in U.S. Patent Nos. 5,616,568, 5,652,347, and 5,874,417. Moreover, the molecular weight of the hyaluronic acid may be altered using techniques known in the art.

25 Host Cells

In the methods of the present invention, the *Bacillus* host cell may be any *Bacillus* cell suitable for recombinant production of hyaluronic acid. The *Bacillus* host cell may be a wild-type *Bacillus* cell or a mutant thereof. *Bacillus* cells useful in the practice of the present invention include, but are not limited to, *Bacillus agaraderhens*,
30 *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis* cells. Mutant *Bacillus subtilis* cells particularly adapted for recombinant expression are described in WO
35 98/22598. Non-encapsulating *Bacillus* cells are particularly useful in the present invention.

In a preferred embodiment, the *Bacillus* host cell is a *Bacillus amyloliquefaciens*,

Bacillus clausii, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus stearothermophilus* or *Bacillus subtilis* cell. In a more preferred embodiment, the *Bacillus* cell is a *Bacillus amyloliquefaciens* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus clausii* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus lentus* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus licheniformis* cell. In another more preferred embodiment, the *Bacillus* cell is a *Bacillus subtilis* cell. In a most preferred embodiment, the *Bacillus* host cell is *Bacillus subtilis* A164Δ5 (see U.S. Patent No. 5,891,701) or *Bacillus subtilis* 168Δ4.

Transformation of the *Bacillus* host cell with a nucleic acid construct of the present invention may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), by using competent cells (see, e.g., Young and Spizizen, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5271-5278).

Nucleic Acid Constructs

"Nucleic acid construct" is defined herein as a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. The term nucleic acid construct may be synonymous with the term expression cassette when the nucleic acid construct contains all the control sequences required for expression of a coding sequence. The term "coding sequence" is defined herein as a sequence which is transcribed into mRNA and translated into an enzyme of interest when placed under the control of the below mentioned control sequences. The boundaries of the coding sequence are generally determined by a ribosome binding site located just upstream of the open reading frame at the 5' end of the mRNA and a transcription terminator sequence located just downstream of the open reading frame at the 3' end of the mRNA. A coding sequence can include, but is not limited to, DNA, cDNA, and recombinant nucleic acid sequences.

The techniques used to isolate or clone a nucleic acid sequence encoding a polypeptide are well known in the art and include, for example, isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleic acid sequences from such genomic DNA can be effected, e.g., by using antibody screening of expression libraries to detect cloned DNA fragments with shared structural features or

the well known polymerase chain reaction (PCR). See, for example, Innis *et al.*, 1990, *PCR Protocols: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction, ligated activated transcription, and nucleic acid sequence-based amplification may be used. The cloning
5 procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a *Bacillus* cell where clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semi-synthetic, synthetic origin, or any
10 combinations thereof.

An isolated nucleic acid sequence encoding an enzyme may be manipulated in a variety of ways to provide for expression of the enzyme. Manipulation of the nucleic acid sequence prior to its insertion into a construct or vector may be desirable or necessary depending on the expression vector or *Bacillus* host cell. The techniques for
15 modifying nucleic acid sequences utilizing cloning methods are well known in the art. It will be understood that the nucleic acid sequence may also be manipulated *in vivo* in the host cell using methods well known in the art.

A number of enzymes are involved in the biosynthesis of hyaluronic acid. These enzymes include hyaluronan synthase, UDP-glucose 6-dehydrogenase, UDP-glucose
20 pyrophosphorylase, UDP-N-acetylglucosamine pyrophosphorylase, glucose-6-phosphate isomerase, hexokinase, phosphoglucomutase, amidotransferase, mutase, and acetyl transferase. Hyaluronan synthase is the key enzyme in the production of hyaluronic acid.

"Hyaluronan synthase" is defined herein as a synthase that catalyzes the
25 elongation of a hyaluronan chain by the addition of GlcUA and GlcNAc sugar precursors. The amino acid sequences of streptococcal hyaluronan synthases, vertebrate hyaluronan synthases, and the viral hyaluronan synthase are distinct from the *Pasteurella* hyaluronan synthase, and have been proposed for classification as Group I and Group II hyaluronan synthases, the Group I hyaluronan synthases including
30 Streptococcal hyaluronan synthases (DeAngelis, 1999). For production of hyaluronan in *Bacillus* host cells, hyaluronan synthases of a eukaryotic origin, such as mammalian hyaluronan synthases, are less preferred.

The hyaluronan synthase encoding sequence may be any nucleic acid sequence capable of being expressed in a *Bacillus* host cell. The nucleic acid sequence may be of
35 any origin. Preferred hyaluronan synthase genes include any of either Group I or Group II, such as the Group I hyaluronan synthase genes from *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, and *Streptococcus equi* subsp.

zooepidemicus, or the Group II hyaluronan synthase genes of *Pasturella multocida*.

In a preferred embodiment, the hyaluronan synthase encoding sequence is selected from the group consisting of (a) a nucleic acid sequence encoding a polypeptide with an amino acid sequence having at least about 70%, about 75%, about 5 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 2, SEQ ID NO: 93, or SEQ ID NO: 103; (b) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with SEQ ID NO: 1, SEQ ID NO: 92, or SEQ ID NO: 102; and (c) a complementary strand of (a) or (b).

In a more preferred embodiment, the hyaluronan synthase encoding sequence 10 encodes a polypeptide having the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 93, or SEQ ID NO: 103; or a fragment thereof having hyaluronan synthase activity.

In another preferred embodiment, the hyaluronan synthase encoding sequence is selected from the group consisting of (a) a nucleic acid sequence encoding a polypeptide with an amino acid sequence having at least about 70%, about 75%, about 15 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 95; (b) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with SEQ ID NO: 94; and (c) a complementary strand of (a) or (b).

In another more preferred embodiment, the hyaluronan synthase encoding sequence encodes a polypeptide having the amino acid sequence of SEQ ID NO: 95, or 20 a fragment thereof having hyaluronan synthase activity.

The methods of the present invention also include constructs whereby precursor sugars of hyaluronan are supplied to the host cell, either to the culture medium, or by being encoded by endogenous genes, by non-endogenous genes, or by a combination of endogenous and non-endogenous genes in the *Bacillus* host cell. The precursor 25 sugar may be D-glucuronic acid or N-acetyl-glucosamine.

In the methods of the present invention, the nucleic acid construct may further comprise one or more genes encoding enzymes in the biosynthesis of a precursor sugar of a hyaluronan. Alternatively, the *Bacillus* host cell may further comprise one or more second nucleic acid constructs comprising one or more genes encoding enzymes in the 30 biosynthesis of the precursor sugar. Hyaluronan production is improved by the use of constructs with a nucleic acid sequence or sequences encoding a gene or genes directing a step in the synthesis pathway of the precursor sugar of hyaluronan. By, "directing a step in the synthesis pathway of a precursor sugar of hyaluronan" is meant that the expressed protein of the gene is active in the formation of N-acetyl-glucosamine 35 or D-glucuronic acid, or a sugar that is a precursor of either of N-acetyl-glucosamine and D-glucuronic acid (Figure 2).

In a preferred method for supplying precursor sugars, constructs are provided for

improving hyaluronan production in a host cell having a hyaluronan synthase, by culturing a host cell having a recombinant construct with a heterologous promoter region operably linked to a nucleic acid sequence encoding a gene directing a step in the synthesis pathway of a precursor sugar of hyaluronan. In a preferred method the host
5 cell also comprises a recombinant construct having a promoter region operably linked to a hyaluronan synthase, which may use the same or a different promoter region than the nucleic acid sequence to a synthase involved in the biosynthesis of N-acetylglucosamine. In a further preferred embodiment, the host cell may have a recombinant construct with a promoter region operably linked to different nucleic acid sequences
10 encoding a second gene involved in the synthesis of a precursor sugar of hyaluronan.

Thus, the present invention also relates to constructs for improving hyaluronan production by the use of constructs with a nucleic acid sequence encoding a gene directing a step in the synthesis pathway of a precursor sugar of hyaluronan. The nucleic acid sequence to the precursor sugar may be expressed from the same or a
15 different promoter as the nucleic acid sequence encoding the hyaluronan synthase.

The genes involved in the biosynthesis of precursor sugars for the production of hyaluronic acid include a UDP-glucose 6-dehydrogenase gene, UDP-glucose pyrophosphorylase gene, UDP-N-acetylglucosamine pyrophosphorylase gene, glucose-6-phosphate isomerase gene, hexokinase gene, phosphoglucomutase gene,
20 amidotransferase gene, mutase gene, and acetyl transferase gene.

In a cell containing a hyaluronan synthase, any one or combination of two or more of *hasB*, *hasC* and *hasD*, or the homologs thereof, such as the *Bacillus subtilis* *tuaD*, *gtaB*, and *gcaD*, respectively, as well as *hasE*, may be expressed to increase the pools of precursor sugars available to the hyaluronan synthase. The *Bacillus* genome is
25 described in Kunst, *et al.*, *Nature* 390, 249-256, "The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*" (20 November 1997). In some instances, such as where the host cell does not have a native hyaluronan synthase activity, the construct may include the *hasA* gene.

The nucleic acid sequence encoding the biosynthetic enzymes may be native to
30 the host cell, while in other cases heterologous sequence may be utilized. If two or more genes are expressed they may be genes that are associated with one another in a native operon, such as the genes of the HAS operon of *Streptococcus equisimilis*, which comprises *hasA*, *hasB*, *hasC* and *hasD*. In other instances, the use of some combination of the precursor gene sequences may be desired, without each element of
35 the operon included. The use of some genes native to the host cell, and others which are exogenous may also be preferred in other cases. The choice will depend on the available pools of sugars in a given host cell, the ability of the cell to accommodate

overproduction without interfering with other functions of the host cell, and whether the cell regulates expression from its native genes differently than exogenous genes.

As one example, depending on the metabolic requirements and growth conditions of the cell, and the available precursor sugar pools, it may be desirable to increase the production of N-acetyl-glucosamine by expression of a nucleic acid sequence encoding UDP-N-acetylglucosamine pyrophosphorylase, such as the *hasD* gene, the *Bacillus gcaD* gene, and homologs thereof. Alternatively, the precursor sugar may be D-glucuronic acid. In one such embodiment, the nucleic acid sequence encodes UDP-glucose 6-dehydrogenase. Such nucleic acid sequences include the *Bacillus tuaD* gene, the *hasB* gene of *Streptococcus*, and homologs thereof. The nucleic acid sequence may also encode UDP-glucose pyrophosphorylase, such as in the *Bacillus gtaB* gene, the *hasC* gene of *Streptococcus*, and homologs thereof.

In the methods of the present invention, the UDP-glucose 6-dehydrogenase gene may be a *hasB* gene or *tuaD* gene; or homologs thereof.

In a preferred embodiment, the *hasB* gene is selected from the group consisting of (a) a nucleic acid sequence encoding a polypeptide with an amino acid sequence having at least about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 41, SEQ ID NO: 97, or SEQ ID NO: 105; (b) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with SEQ ID NO: 40, SEQ ID NO: 96, or SEQ ID NO: 104; and (c) a complementary strand of (a) or (b).

In a more preferred embodiment, the *hasB* gene encodes a polypeptide having the amino acid sequence of SEQ ID NO: 41, SEQ ID NO: 97, or SEQ ID NO: 105; or a fragment thereof having UDP-glucose 6-dehydrogenase activity.

In another preferred embodiment, the *tuaD* gene is selected from the group consisting of (a) a nucleic acid sequence encoding a polypeptide with an amino acid sequence having at least about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 12; (b) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with SEQ ID NO: 11; and (c) a complementary strand of (a) or (b).

In another more preferred embodiment, the *tuaD* gene encodes a polypeptide having the amino acid sequence of SEQ ID NO: 12, or a fragment thereof having UDP-glucose 6-dehydrogenase activity.

In the methods of the present invention, the UDP-glucose pyrophosphorylase gene may be a *hasC* gene or *gtaB* gene; or homologs thereof.

In a preferred embodiment, the *hasC* gene is selected from the group consisting of (a) a nucleic acid sequence encoding a polypeptide with an amino acid sequence

having at least about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 43, SEQ ID NO: 99, or SEQ ID NO: 107; (b) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with SEQ ID NO: 42 or SEQ ID NO: 98, or SEQ ID NO: 106; and (c) a complementary strand of (a) or (b).

In another more preferred embodiment, the *hasC* gene encodes a polypeptide having the amino acid sequence of SEQ ID NO: 43 or SEQ ID NO: 99, or SEQ ID NO: 107; or a fragment thereof having UDP-glucose pyrophosphorylase activity.

In another preferred embodiment, the *gtaB* gene is selected from the group consisting of (a) a nucleic acid sequence encoding a polypeptide with an amino acid sequence having at least about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 22; (b) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with SEQ ID NO: 21; and (c) a complementary strand of (a) or (b).

In another more preferred embodiment, the *gtaB* gene encodes a polypeptide having the amino acid sequence of SEQ ID NO: 22, or a fragment thereof having UDP-glucose pyrophosphorylase activity.

In the methods of the present invention, the UDP-N-acetylglucosamine pyrophosphorylase gene may be a *hasD* or *gcaD* gene; or homologs thereof.

In a preferred embodiment, the *hasD* gene is selected from the group consisting of (a) a nucleic acid sequence encoding a polypeptide with an amino acid sequence having at least about 75%, about 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 45; (b) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with SEQ ID NO: 44; and (c) a complementary strand of (a) or (b).

In another more preferred embodiment, the *hasD* gene encodes a polypeptide having the amino acid sequence of SEQ ID NO: 45, or a fragment thereof having UDP-N-acetylglucosamine pyrophosphorylase activity.

In another preferred embodiment, the *gcaD* gene is selected from the group consisting of (a) a nucleic acid sequence encoding a polypeptide with an amino acid sequence having at least about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 30; (b) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with SEQ ID NO: 29; and (c) a complementary strand of (a) or (b).

In another more preferred embodiment, the *gcaD* gene encodes a polypeptide having the amino acid sequence of SEQ ID NO: 30, or a fragment thereof having UDP-N-acetylglucosamine pyrophosphorylase activity.

In the methods of the present invention, the glucose-6-phosphate isomerase gene may be a *hasE* or homolog thereof.

In a preferred embodiment, the *hasE* gene is selected from the group consisting of (a) a nucleic acid sequence encoding a polypeptide with an amino acid sequence having at least about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% identity to SEQ ID NO: 101; (b) a nucleic acid sequence which hybridizes under low, medium, or high stringency conditions with SEQ ID NO: 100; and (c) a complementary strand of (a) or (b).

In another more preferred embodiment, the *hasE* gene encodes a polypeptide having the amino acid sequence of SEQ ID NO: 101, or a fragment thereof having glucose-6-phosphate isomerase activity.

In the methods of the present invention, the hyaluronan synthase gene and the one or more genes encoding a precursor sugar are under the control of the same promoter. Alternatively, the one or more genes encoding a precursor sugar are under the control of the same promoter but a different promoter driving the hyaluronan synthase gene. A further alternative is that the hyaluronan synthase gene and each of the genes encoding a precursor sugar are under the control of different promoters. In a preferred embodiment, the hyaluronan synthase gene and the one or more genes encoding a precursor sugar are under the control of the same promoter.

The present invention also relates to a nucleic acid construct comprising an isolated nucleic acid sequence encoding a hyaluronan synthase operon comprising a hyaluronan synthase gene and a UDP-glucose 6-dehydrogenase gene, and optionally one or more genes selected from the group consisting of a UDP-glucose pyrophosphorylase gene, UDP-N-acetylglucosamine pyrophosphorylase gene, and glucose-6-phosphate isomerase gene. A nucleic acid sequence encoding most of the hyaluronan synthase operon of *Streptococcus equisimilis* is found in SEQ ID NO: 108. This sequence contains the *hasB* (SEQ ID NO: 40) and *hasC* (SEQ ID NO: 42) homologs of the *Bacillus subtilis tuaD* gene (SEQ ID NO: 11) and *gtaB* gene (SEQ ID NO: 21), respectively, as is the case for *Streptococcus pyogenes*, as well as a homolog of the *gcaD* gene (SEQ ID NO: 29), which has been designated *hasD* (SEQ ID NO: 44). The *Bacillus subtilis gcaD* encodes UDP-N-acetylglucosamine pyrophosphorylase, which is involved in the synthesis of N-acetyl-glucosamine, one of the two sugars of hyaluronan. The *Streptococcus equisimilis* homolog of *gcaD*, *hasD*, is arranged by *Streptococcus equisimilis* on the hyaluronan synthase operon. The nucleic acid sequence also contains a portion of the *hasA* gene (the last 1156 bp of SEQ ID NO: 1).

In some cases the host cell will have a recombinant construct with a heterologous promoter region operably linked to a nucleic acid sequence encoding a

gene directing a step in the synthesis pathway of a precursor sugar of hyaluronan, which may be in concert with the expression of hyaluronan synthase from a recombinant construct. The hyaluronan synthase may be expressed from the same or a different promoter region than the nucleic acid sequence encoding an enzyme involved in the biosynthesis of the precursor. In another preferred embodiment, the host cell may have a recombinant construct with a promoter region operably linked to a different nucleic acid sequence encoding a second gene involved in the synthesis of a precursor sugar of hyaluronan.

The nucleic acid sequence encoding the enzymes involved in the biosynthesis of the precursor sugar(s) may be expressed from the same or a different promoter as the nucleic acid sequence encoding the hyaluronan synthase. In the former sense, "artificial operons" are constructed, which may mimic the operon of *Streptococcus equisimilis* in having each *hasA*, *hasB*, *hasC* and *hasD*, or homologs thereof, or, alternatively, may utilize less than the full complement present in the *Streptococcus equisimilis* operon. The artificial operons" may also comprise a glucose-6-phosphate isomerase gene (*hasE*) as well as one or more genes selected from the group consisting of a hexokinase gene, phosphoglucomutase gene, amidotransferase gene, mutase gene, and acetyl transferase gene. In the artificial operon, at least one of the elements is heterologous to one other of the elements, such as the promoter region being heterologous to the encoding sequences.

In a preferred embodiment, the nucleic acid construct comprises *hasA*, *tuaD*, and *gtaB*. In another preferred embodiment, the nucleic acid construct comprises *hasA*, *tuaD*, *gtaB*, and *gcaD*. In another preferred embodiment, the nucleic acid construct comprises *hasA* and *tuaD*. In another preferred embodiment, the nucleic acid construct comprises *hasA*. In another preferred embodiment, the nucleic acid construct comprises *hasA*, *tuaD*, *gtaB*, *gcaD*, and *hasE*. In another preferred embodiment, the nucleic acid construct comprises *hasA*, *hasB*, *hasC*, and *hasD*. In another preferred embodiment, the nucleic acid construct comprises *hasA*, *hasB*, *hasC*, *hasD*, and *hasE*. Based on the above preferred embodiments, the genes noted can be replaced with homologs thereof.

In the methods of the present invention, the nucleic acid constructs comprise a hyaluronan synthase encoding sequence operably linked to a promoter sequence foreign to the hyaluronan synthase encoding sequence. The promoter sequence may be, for example, a single promoter or a tandem promoter.

"Promoter" is defined herein as a nucleic acid sequence involved in the binding of RNA polymerase to initiate transcription of a gene. "Tandem promoter" is defined herein as two or more promoter sequences each of which is operably linked to a coding

sequence and mediates the transcription of the coding sequence into mRNA. "Operably linked" is defined herein as a configuration in which a control sequence, e.g., a promoter sequence, is appropriately placed at a position relative to a coding sequence such that the control sequence directs the production of a polypeptide encoded by the coding sequence. As noted earlier, a "coding sequence" is defined herein as a nucleic acid sequence which is transcribed into mRNA and translated into a polypeptide when placed under the control of the appropriate control sequences. The boundaries of the coding sequence are generally determined by a ribosome binding site located just upstream of the open reading frame at the 5' end of the mRNA and a transcription terminator sequence located just downstream of the open reading frame at the 3' end of the mRNA. A coding sequence can include, but is not limited to, genomic DNA, cDNA, semisynthetic, synthetic, and recombinant nucleic acid sequences.

In a preferred embodiment, the promoter sequences may be obtained from a bacterial source. In a more preferred embodiment, the promoter sequences may be obtained from a gram positive bacterium such as a *Bacillus* strain, e.g., *Bacillus agaradherens*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis*; or a *Streptomyces* strain, e.g., *Streptomyces lividans* or *Streptomyces murinus*; or from a gram negative bacterium, e.g., *E. coli* or *Pseudomonas* sp.

Examples of suitable promoters for directing the transcription of a nucleic acid sequence in the methods of the present invention are the promoters obtained from the *E. coli lac* operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus lentus* or *Bacillus clausii* alkaline protease gene (*aprH*), *Bacillus licheniformis* alkaline protease gene (subtilisin Carlsberg gene), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* alpha-amylase gene (*amyE*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus subtilis* *xylA* and *xylB* genes, *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA gene (*cryIIIA*) or portions thereof, prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:3727-3731). Other examples are the promoter of the *spo1* bacterial phage promoter and the *tac* promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80:21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; and in Sambrook, Fritsch, and

Maniatus, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York.

The promoter may also be a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region. The consensus promoter may be obtained from any promoter which can function in a *Bacillus* host cell. The construction of a "consensus" promoter may be accomplished by site-directed mutagenesis to create a promoter which conforms more perfectly to the established consensus sequences for the "-10" and "-35" regions of the vegetative "sigma A-type" promoters for *Bacillus subtilis* (Voskuil *et al.*, 1995, *Molecular Microbiology* 17: 271-279).

In a preferred embodiment, the "consensus" promoter is obtained from a promoter obtained from the *E. coli lac* operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus clausii* or *Bacillus lentus* alkaline protease gene (*aprH*), *Bacillus licheniformis* alkaline protease gene (subtilisin Carlsberg gene), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis* alpha-amylase gene (*amyE*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus subtilis* *xylA* and *xylB* genes, *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA gene (*cryIIIA*) or portions thereof, or prokaryotic beta-lactamase gene *spo1* bacterial phage promoter. In a more preferred embodiment, the "consensus" promoter is obtained from *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*).

Widner, *et al.*, United States Patent Nos. 6,255,076 and 5,955,310, describe tandem promoters and constructs and methods for use in expression in *Bacillus* cells, including the short consensus *amyQ* promoter (also called scBAN). The use of the *cryIIIA* stabilizer sequence, and constructs using the sequence, for improved production in *Bacillus* are also described therein.

Each promoter sequence of the tandem promoter may be any nucleic acid sequence which shows transcriptional activity in the *Bacillus* cell of choice including a mutant, truncated, and hybrid promoter, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the *Bacillus* cell. Each promoter sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide and native or foreign to the *Bacillus* cell. The promoter sequences may be the same promoter sequence or different promoter sequences.

The two or more promoter sequences of the tandem promoter may simultaneously promote the transcription of the nucleic acid sequence. Alternatively, one or more of the promoter sequences of the tandem promoter may promote the

transcription of the nucleic acid sequence at different stages of growth of the *Bacillus* cell.

In a preferred embodiment, the tandem promoter contains at least the *amyQ* promoter of the *Bacillus amyloliquefaciens* alpha-amylase gene. In another preferred
5 embodiment, the tandem promoter contains at least a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region. In another preferred embodiment, the tandem promoter contains at least the *amyL* promoter of the *Bacillus licheniformis* alpha-amylase gene. In another preferred embodiment, the tandem promoter contains at least the *cryIIIA* promoter or portions thereof (Agaisse and
10 Lereclus, 1994, *Molecular Microbiology* 13: 97-107).

In a more preferred embodiment, the tandem promoter contains at least the *amyL* promoter and the *cryIIIA* promoter. In another more preferred embodiment, the tandem promoter contains at least the *amyQ* promoter and the *cryIIIA* promoter. In another more preferred embodiment, the tandem promoter contains at least a
15 "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region and the *cryIIIA* promoter. In another more preferred embodiment, the tandem promoter contains at least two copies of the *amyL* promoter. In another more preferred embodiment, the tandem promoter contains at least two copies of the *amyQ* promoter. In another more preferred embodiment, the tandem promoter contains
20 at least two copies of a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region. In another more preferred embodiment, the tandem promoter contains at least two copies of the *cryIIIA* promoter.

"An mRNA processing/stabilizing sequence" is defined herein as a sequence located downstream of one or more promoter sequences and upstream of a coding
25 sequence to which each of the one or more promoter sequences are operably linked such that all mRNAs synthesized from each promoter sequence may be processed to generate mRNA transcripts with a stabilizer sequence at the 5' end of the transcripts. The presence of such a stabilizer sequence at the 5' end of the mRNA transcripts increases their half-life (Agaisse and Lereclus, 1994, *supra*, Hue *et al.*, 1995, *Journal of*
30 *Bacteriology* 177: 3465-3471). The mRNA processing/stabilizing sequence is complementary to the 3' extremity of a bacterial 16S ribosomal RNA. In a preferred embodiment, the mRNA processing/stabilizing sequence generates essentially single-size transcripts with a stabilizing sequence at the 5' end of the transcripts. The mRNA processing/stabilizing sequence is preferably one, which is complementary to the 3'
35 extremity of a bacterial 16S ribosomal RNA. See, U.S. Patent Nos. 6,255,076 and 5,955,310.

In a more preferred embodiment, the mRNA processing/stabilizing sequence is the *Bacillus thuringiensis cryIIIA* mRNA processing/stabilizing sequence disclosed in WO 94/25612 and Agaisse and Lereclus, 1994, *supra*, or portions thereof which retain the mRNA processing/stabilizing function. In another more preferred embodiment, the
5 mRNA processing/stabilizing sequence is the *Bacillus subtilis* SP82 mRNA processing/stabilizing sequence disclosed in Hue *et al.*, 1995, *supra*, or portions thereof which retain the mRNA processing/stabilizing function.

When the *cryIIIA* promoter and its mRNA processing/stabilizing sequence are employed in the methods of the present invention, a DNA fragment containing the
10 sequence disclosed in WO 94/25612 and Agaisse and Lereclus, 1994, *supra*, or portions thereof which retain the promoter and mRNA processing/stabilizing functions, may be used. Furthermore, DNA fragments containing only the *cryIIIA* promoter or only the *cryIIIA* mRNA processing/stabilizing sequence may be prepared using methods well known in the art to construct various tandem promoter and mRNA processing/stabilizing
15 sequence combinations. In this embodiment, the *cryIIIA* promoter and its mRNA processing/stabilizing sequence are preferably placed downstream of the other promoter sequence(s) constituting the tandem promoter and upstream of the coding sequence of the gene of interest.

The isolated nucleic acid sequence encoding the desired enzyme(s) involved in
20 hyaluronic acid production may then be further manipulated to improve expression of the nucleic acid sequence. Expression will be understood to include any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion. The techniques for modifying nucleic acid sequences utilizing cloning methods are well
25 known in the art.

A nucleic acid construct comprising a nucleic acid sequence encoding an enzyme may be operably linked to one or more control sequences capable of directing the expression of the coding sequence in a *Bacillus* cell under conditions compatible with the control sequences.

30 The term "control sequences" is defined herein to include all components which are necessary or advantageous for expression of the coding sequence of a nucleic acid sequence. Each control sequence may be native or foreign to the nucleic acid sequence encoding the enzyme. In addition to promoter sequences described above, such control sequences include, but are not limited to, a leader, a signal sequence, and
35 a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of

the control sequences with the coding region of the nucleic acid sequence encoding an enzyme.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a *Bacillus* cell to terminate transcription. The terminator
5 sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the enzyme or the last enzyme of an operon. Any terminator which is functional in the *Bacillus* cell of choice may be used in the present invention.

The control sequence may also be a suitable leader sequence, a nontranslated region of a mRNA which is important for translation by the *Bacillus* cell. The leader
10 sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the enzyme. Any leader sequence which is functional in the *Bacillus* cell of choice may be used in the present invention.

The control sequence may also be a signal peptide coding region, which codes for an amino acid sequence linked to the amino terminus of a polypeptide which can
15 direct the expressed polypeptide into the cell's secretory pathway. The signal peptide coding region may be native to the polypeptide or may be obtained from foreign sources. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide.
20 Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which is foreign to that portion of the coding sequence which encodes the secreted polypeptide. The foreign signal peptide coding region may be required where the coding sequence does not normally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural
25 signal peptide coding region in order to obtain enhanced secretion of the polypeptide relative to the natural signal peptide coding region normally associated with the coding sequence. The signal peptide coding region may be obtained from an amylase or a protease gene from a *Bacillus* species. However, any signal peptide coding region capable of directing the expressed polypeptide into the secretory pathway of a *Bacillus*
30 cell of choice may be used in the present invention.

An effective signal peptide coding region for *Bacillus* cells is the signal peptide coding region obtained from the maltogenic amylase gene from *Bacillus* NCIB 11837, the *Bacillus stearothermophilus* alpha-amylase gene, the *Bacillus licheniformis* subtilisin gene, the *Bacillus licheniformis* beta-lactamase gene, the *Bacillus stearothermophilus*
35 neutral proteases genes (*nprT*, *nprS*, *nprM*), and the *Bacillus subtilis* *prsA* gene. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57:109-137.

The control sequence may also be a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*) and *Bacillus subtilis* neutral protease (*nprT*).

Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems.

Expression Vectors

In the methods of the present invention, a recombinant expression vector comprising a nucleic acid sequence, a promoter, and transcriptional and translational stop signals may be used for the recombinant production of an enzyme involved in hyaluronic acid production. The various nucleic acid and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the polypeptide or enzyme at such sites. Alternatively, the nucleic acid sequence may be expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression, and possibly secretion.

The recombinant expression vector may be any vector which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleic acid sequence. The choice of the vector will typically depend on the compatibility of the vector with the *Bacillus* cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal

entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the *Bacillus* cell, is
5 integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The vector system may be a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the *Bacillus* cell, or a transposon may be used.

The vectors of the present invention preferably contain an element(s) that
10 permits integration of the vector into the *Bacillus* host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the nucleic acid sequence encoding the polypeptide or any other element of the vector for integration of the vector into the genome by homologous or nonhomologous recombination.
15 Alternatively, the vector may contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the *Bacillus* cell. The additional nucleic acid sequences enable the vector to be integrated into the *Bacillus* cell genome at a precise location in the chromosome. To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a
20 sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the *Bacillus* cell. Furthermore,
25 the integrational elements may be non-encoding or encoding nucleic acid sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the *Bacillus* cell in question.
30 Examples of bacterial origins of replication are the origins of replication of plasmids pUB110, pE194, pTA1060, and pAM β 1 permitting replication in *Bacillus*. The origin of replication may be one having a mutation to make its function temperature-sensitive in the *Bacillus* cell (see, e.g., Ehrlich, 1978, *Proceedings of the National Academy of Sciences USA* 75:1433).

35 The vectors preferably contain one or more selectable markers which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide resistance, resistance to heavy metals, prototrophy to auxotrophs,

and the like. Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, selection may be accomplished by co-transformation, e.g., as described in WO 91/09129, where the selectable marker is on a separate vector.

More than one copy of a nucleic acid sequence may be inserted into the host cell to increase production of the gene product. An increase in the copy number of the nucleic acid sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the nucleic acid sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the nucleic acid sequence, can be selected for by cultivating the cells in the presence of the appropriate selectable agent. A convenient method for achieving amplification of genomic DNA sequences is described in WO 94/14968.

The procedures used to ligate the elements described above to construct the recombinant expression vectors are well known to one skilled in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

Production

In the methods of the present invention, the *Bacillus* host cells are cultivated in a nutrient medium suitable for production of the hyaluronic acid using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the enzymes involved in hyaluronic acid synthesis to be expressed and the hyaluronic acid to be isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). The secreted hyaluronic acid can be recovered directly from the medium.

The resulting hyaluronic acid may be isolated by methods known in the art. For example, the hyaluronic acid may be isolated from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The isolated hyaluronic acid may then be further purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion),

electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

In the methods of the present invention, the *Bacillus* host cells produce greater
5 than about 4 g, preferably greater than about 6 g, more preferably greater than about 8 g, even more preferably greater than about 10 g, and most preferably greater than about 12 g of hyaluronic acid per liter.

Deletions/Disruptions

10 Gene deletion or replacement techniques may be used for the complete removal of a selectable marker gene or other undesirable gene. In such methods, the deletion of the selectable marker gene may be accomplished by homologous recombination using a plasmid that has been constructed to contiguously contain the 5' and 3' regions flanking the selectable marker gene. The contiguous 5' and 3' regions may be introduced into a
15 *Bacillus* cell on a temperature-sensitive plasmid, e.g., pE194, in association with a second selectable marker at a permissive temperature to allow the plasmid to become established in the cell. The cell is then shifted to a non-permissive temperature to select for cells that have the plasmid integrated into the chromosome at one of the homologous flanking regions. Selection for integration of the plasmid is effected by selection for the
20 second selectable marker. After integration, a recombination event at the second homologous flanking region is stimulated by shifting the cells to the permissive temperature for several generations without selection. The cells are plated to obtain single colonies and the colonies are examined for loss of both selectable markers (see, for example, Perego, 1993, *In* A.L. Sonneshein, J.A. Hoch, and R. Losick, editors,
25 *Bacillus subtilis and Other Gram-Positive Bacteria*, Chapter 42, American Society of Microbiology, Washington, D.C., 1993).

A selectable marker gene may also be removed by homologous recombination by introducing into the mutant cell a nucleic acid fragment comprising 5' and 3' regions of the defective gene, but lacking the selectable marker gene, followed by selecting on
30 the counter-selection medium. By homologous recombination, the defective gene containing the selectable marker gene is replaced with the nucleic acid fragment lacking the selectable marker gene. Other methods known in the art may also be used.

U.S. Patent No. 5,891,701 discloses techniques for deleting several genes including *spolIAC*, *aprE*, *nprE*, and *amyE*.

35 Other undesirable biological compounds may also be removed by the above described methods such as the red pigment synthesized by *cypX* (accession no. BG12580) and/or *yvmC* (accession no. BG14121).

In a preferred embodiment, the *Bacillus* host cell is unmarked with any heterologous or exogenous selectable markers. In another preferred embodiment, the *Bacillus* host cell does not produce any red pigment synthesized by *cypX* and *yvmC*.

5 **Isolated Nucleic Acid Sequences Encoding Polypeptides Having UDP-Glucose 6-Dehydrogenase Activity, UDP-Glucose Pyrophosphorylase Activity, or UDP-N-Acetylglucosamine Pyrophosphorylase Activity**

The term "UDP-glucose 6-dehydrogenase activity" is defined herein as a UDP glucose:NAD⁺ 6-oxidoreductase activity which catalyzes the conversion of UDP-glucose
10 in the presence of 2NAD⁺ and water to UDP-glucuronate and 2NADH. For purposes of the present invention UDP-glucose 6-dehydrogenase activity is determined according to the procedure described by Jaenicke and Rudolph, 1986, *Biochemistry* 25: 7283-7287. One unit of UDP-glucose 6-dehydrogenase activity is defined as 1.0 μmole of UDP-glucuronate produced per minute at 25°C, pH 7.

15 The term "UDP-glucose pyrophosphorylase activity" is defined herein as a UTP:α-D-glucose-1-phosphate uridylyltransferase activity which catalyzes the conversion of glucose-1-phosphate in the presence of UTP to diphosphate and UDP-glucose. For purposes of the present invention UDP-glucose pyrophosphorylase activity is determined according to the procedure described by Kamogawa et al., 1965,
20 *J. Biochem. (Tokyo)* 57: 758-765 or Hansen et al., 1966, *Method Enzymol.* 8: 248-253. One unit of UDP-glucose pyrophosphorylase activity is defined as 1.0 μmole of UDP-glucose produced per minute at 25°C, pH 7.

The term "UDP-N-acetylglucosamine pyrophosphorylase activity" is defined herein as a UTP:N-acetyl-α-D-glucoamine-1-phosphate uridylyltransferase activity
25 which catalyzes the conversion of N-acetyl-α-D-glucosamine-1-phosphate in the presence of UTP to diphosphate and UDP-N-acetyl-α-D-glucoamine. For purposes of the present invention, UDP-N-acetylglucosamine pyrophosphorylase activity is determined according to the procedure described by Mangin-Lecreux et al., 1994, *J. Bacteriology* 176: 5788-5795. One unit of UDP-N-acetylglucosamine
30 pyrophosphorylase activity is defined as 1.0 μmole of UDP-N-acetyl-α-D-glucoamine produced per minute at 25°C, pH 7.

The term "isolated nucleic acid sequence" as used herein refers to a nucleic acid sequence which is essentially free of other nucleic acid sequences, e.g., at least about 20% pure, preferably at least about 40% pure, more preferably at least about 60% pure,
35 even more preferably at least about 80% pure, and most preferably at least about 90% pure as determined by agarose electrophoresis. For example, an isolated nucleic acid sequence can be obtained by standard cloning procedures used in genetic engineering

to relocate the nucleic acid sequence from its natural location to a different site where it will be reproduced. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a host cell where multiple copies or clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

In a first embodiment, the present invention relates to isolated nucleic acid sequences encoding polypeptides having an amino acid sequence which has a degree of identity to SEQ ID NO: 41 of at least about 75%, preferably at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have UDP-glucose 6-dehydrogenase activity (hereinafter "homologous polypeptides"). In a preferred embodiment, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from SEQ ID NO: 41.

In another first embodiment, the present invention relates to isolated nucleic acid sequences encoding polypeptides having an amino acid sequence which has a degree of identity to SEQ ID NO: 43 of at least about 90%, preferably at least about 95%, and more preferably at least about 97%, which have UDP-glucose pyrophosphorylase activity (hereinafter "homologous polypeptides"). In a preferred embodiment, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from SEQ ID NO: 43.

In another first embodiment, the present invention relates to isolated nucleic acid sequences encoding polypeptides having an amino acid sequence which has a degree of identity to SEQ ID NO: 45 of at least about 75%, preferably at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have UDP-N-acetylglucosamine pyrophosphorylase activity (hereinafter "homologous polypeptides"). In a preferred embodiment, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from SEQ ID NO: 45.

For purposes of the present invention, the degree of identity between two amino

acid sequences is determined by the Clustal method (Higgins, 1989, *CABIOS* 5: 151-153) using the Vector NTI AlignX software package (Informax Inc., Bethesda, MD) with the following defaults: pairwise alignment, gap opening penalty of 10, gap extension penalty of 0.1, and score matrix: blosum62mt2.

5 Preferably, the nucleic acid sequences of the present invention encode polypeptides that comprise the amino acid sequence of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45; or an allelic variant thereof; or a fragment thereof that has UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, or UDP-N-acetylglucosamine pyrophosphorylase activity, respectively. In a more preferred
10 embodiment, the nucleic acid sequence of the present invention encodes a polypeptide that comprises the amino acid sequence of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45. In another preferred embodiment, the nucleic acid sequence of the present invention encodes a polypeptide that consists of the amino acid sequence of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45; or an allelic variant thereof; or a fragment
15 thereof, wherein the polypeptide fragment has UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, or UDP-N-acetylglucosamine pyrophosphorylase activity, respectively. In another preferred embodiment, the nucleic acid sequence of the present invention encodes a polypeptide that consists of the amino acid sequence of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45.

20 The present invention also encompasses nucleic acid sequences which encode a polypeptide having the amino acid sequence of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45, which differ from SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44 by virtue of the degeneracy of the genetic code. The present invention also relates to subsequences of SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44 which encode
25 fragments of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45, respectively, which have UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, or UDP-N-acetylglucosamine pyrophosphorylase activity, respectively.

A subsequence of SEQ ID NO: 40 is a nucleic acid sequence encompassed by SEQ ID NO: 40 except that one or more nucleotides from the 5' and/or 3' end have been
30 deleted. Preferably, a subsequence contains at least 1020 nucleotides, more preferably at least 1080 nucleotides, and most preferably at least 1140 nucleotides. A fragment of SEQ ID NO: 41 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. Preferably, a fragment contains at least 340 amino acid residues, more preferably at least 360 amino acid residues, and
35 most preferably at least 380 amino acid residues.

A subsequence of SEQ ID NO: 42 is a nucleic acid sequence encompassed by SEQ ID NO: 42 except that one or more nucleotides from the 5' and/or 3' end have been

deleted. Preferably, a subsequence contains at least 765 nucleotides, more preferably at least 810 nucleotides, and most preferably at least 855 nucleotides. A fragment of SEQ ID NO: 43 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. Preferably, a fragment contains
5 at least 255 amino acid residues, more preferably at least 270 amino acid residues, and most preferably at least 285 amino acid residues.

A subsequence of SEQ ID NO: 44 is a nucleic acid sequence encompassed by SEQ ID NO: 44 except that one or more nucleotides from the 5' and/or 3' end have been deleted. Preferably, a subsequence contains at least 1110 nucleotides, more preferably
10 at least 1200 nucleotides, and most preferably at least 1290 nucleotides. A fragment of SEQ ID NO: 45 is a polypeptide having one or more amino acids deleted from the amino and/or carboxy terminus of this amino acid sequence. Preferably, a fragment contains at least 370 amino acid residues, more preferably at least 400 amino acid residues, and most preferably at least 430 amino acid residues.

15 An allelic variant denotes any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. The allelic variant of a polypeptide is a polypeptide
20 encoded by an allelic variant of a gene.

In a second embodiment, the present invention relates to isolated nucleic acid sequences which have a degree of homology to SEQ ID NO: 40 of at least about 75%, preferably at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at
25 least about 97%.

In another second embodiment, the present invention relates to isolated nucleic acid sequences which have a degree of homology to SEQ ID NO: 42 of at least about 90%, preferably at least about 95%, and more preferably at least about 97%.

In another second embodiment, the present invention relates to isolated nucleic
30 acid sequences which have a degree of homology to SEQ ID NO: 44 of at least about 75%, preferably at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%.

For purposes of the present invention, the degree of homology between two
35 nucleic acid sequences is determined by the Vector NTI AlignX software package (Informax Inc., Bethesda, MD) using the following defaults: pairwise alignment, gap opening penalty of 15, gap extension penalty of 6.6, and score matrix: swgapdnamt.

In a third embodiment, the present invention relates to isolated nucleic acid sequences encoding polypeptides having UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, or UDP-N-acetylglucosamine pyrophosphorylase activity, which hybridize under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with (i) the nucleic acid sequence of SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44, (ii) the cDNA sequence contained in SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44, or (iii) a complementary strand of (i) or (ii) (J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). The subsequence of SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44 may be at least 100 nucleotides or preferably at least 200 nucleotides. Moreover, the respective subsequence may encode a polypeptide fragment which has UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, or UDP-N-acetylglucosamine pyrophosphorylase activity.

The nucleic acid sequence of SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44, or subsequences thereof, as well as the amino acid sequence of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45, or a fragment thereof, may be used to design nucleic acid probes to identify and clone DNA encoding polypeptides having UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, or UDP-N-acetylglucosamine pyrophosphorylase activity, respectively, from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, preferably at least 25, and more preferably at least 35 nucleotides in length. Longer probes can also be used. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P , ^3H , ^{35}S , biotin, or avidin). Such probes are encompassed by the present invention.

Thus, a genomic DNA or cDNA library prepared from such other organisms may be screened for DNA which hybridizes with the probes described above and which encodes a polypeptide having UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, or UDP-N-acetylglucosamine pyrophosphorylase activity. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose

or other suitable carrier material. In order to identify a clone or DNA which is homologous with SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44, or a subsequence thereof, the carrier material is used in a Southern blot. For purposes of the present invention, hybridization indicates that the nucleic acid sequence hybridizes to a labeled
5 nucleic acid probe corresponding to the nucleic acid sequence shown in SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44, its complementary strand, or a subsequence thereof, under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions are detected using X-ray film.

In a preferred embodiment, the nucleic acid probe is a nucleic acid sequence
10 which encodes the polypeptide of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45; or a subsequence thereof. In another preferred embodiment, the nucleic acid probe is SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44. In another preferred embodiment, the nucleic acid probe is the nucleic acid sequence contained in plasmid pMRT106 which is contained in *Escherichia coli* NRRL B-30536, wherein the nucleic acid
15 sequence encodes polypeptides having UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, and UDP-N-acetylglucosamine pyrophosphorylase activity.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and either
20 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2 x SSC, 0.2% SDS preferably at least at
25 45°C (very low stringency), more preferably at least at 50°C (low stringency), more preferably at least at 55°C (medium stringency), more preferably at least at 60°C (medium-high stringency), even more preferably at least at 65°C (high stringency), and most preferably at least at 70°C (very high stringency).

For short probes which are about 15 nucleotides to about 70 nucleotides in
30 length, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at 5°C to 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic
35 phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures.

For short probes which are about 15 nucleotides to about 70 nucleotides in

length, the carrier material is washed once in 6X SCC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m .

In a fourth embodiment, the present invention relates to isolated nucleic acid sequences which encode variants of the polypeptide having an amino acid sequence of
5 SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45 comprising a substitution, deletion, and/or insertion of one or more amino acids.

The amino acid sequences of the variant polypeptides may differ from the amino acid sequence of SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45, by an insertion or deletion of one or more amino acid residues and/or the substitution of one or more
10 amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues;
15 or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine,
20 isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser,
25 Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly as well as these in reverse.

Modification of a nucleic acid sequence of the present invention may be necessary for the synthesis of polypeptides substantially similar to the polypeptide. The term "substantially similar" to the polypeptide refers to non-naturally occurring forms of
30 the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source, e.g., variants that differ in specific activity, thermostability, pH optimum, or the like. The variant sequence may be constructed on the basis of the nucleic acid sequence presented as the polypeptide encoding part of
SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44, e.g., a subsequence thereof, and/or
35 by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the polypeptide encoded by the nucleic acid sequence, but which corresponds to the codon usage of the host organism intended for production of the

enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, e.g., Ford *et al.*, 1991, *Protein Expression and Purification* 2: 95-107.

It will be apparent to those skilled in the art that such substitutions can be made
5 outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the polypeptide encoded by the isolated nucleic acid sequence of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, e.g., Cunningham and
10 Wells, 1989, *Science* 244: 1081-1085). In the latter technique, mutations are introduced at every positively charged residue in the molecule, and the resultant mutant molecules are tested for enzyme activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of the three-dimensional structure as determined by such techniques as
15 nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (see, e.g., de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *Journal of Molecular Biology* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Letters* 309: 59-64).

The polypeptides encoded by the isolated nucleic acid sequences of the present invention have at least 20%, preferably at least 40%, more preferably at least 60%, even
20 more preferably at least 80%, even more preferably at least 90%, and most preferably at least 100% of the UDP-glucose 6-dehydrogenase activity of the polypeptide of SEQ ID NO: 41, the UDP-glucose pyrophosphorylase activity of the polypeptide of SEQ ID NO: 43, or the UDP-N-acetylglucosamine pyrophosphorylase activity of the polypeptide of SEQ ID NO: 45.

25 The nucleic acid sequences of the present invention may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide encoded by the nucleic acid sequence is produced by the source or by a cell in which the nucleic acid sequence from the source has been inserted. In a preferred
30 embodiment, the polypeptide encoded by a nucleic acid sequence of the present invention is secreted extracellularly.

The nucleic acid sequences may be obtained from a bacterial source. For example, these polypeptides may be obtained from a gram positive bacterium such as a
35 *Bacillus* strain, e.g., *Bacillus agaradherens*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis*; or a

Streptomyces strain, e.g., *Streptomyces lividans* or *Streptomyces murinus*; or from a gram negative bacterium, e.g., *E. coli* or *Pseudomonas* sp.

In a preferred embodiment, the nucleic acid sequences are obtained from a *Streptococcus* or *Pastuerella* strain.

5 In a more preferred embodiment, the nucleic acid sequences are obtained from a *Streptococcus equisimilis*, *Streptococcus pyogenes*, *Streptococcus uberis*, or *Streptococcus equi* subs. *zooepidemicus* strain, or a *Pasteurella multocida* strain.

In a most preferred embodiment, the nucleic acid sequences are obtained from *Streptococcus equisimilis*, e.g., the nucleic acid sequence set forth in SEQ ID NO: 40,
10 SEQ ID NO: 42, or SEQ ID NO: 44. In another most preferred embodiment, the nucleic acid sequence is the sequence contained in plasmid pMRT106 which is contained in *Escherichia coli* NRRL B-30536. In further most preferred embodiment, the nucleic acid sequence is SEQ ID NO: 40, SEQ ID NO: 42, or SEQ ID NO: 44.

Strains of these species are readily accessible to the public in a number of
15 culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

Furthermore, such nucleic acid sequences may be identified and obtained from
20 other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art. The nucleic acid sequence may then be derived by similarly screening a genomic or cDNA library of another microorganism. Once a nucleic acid sequence encoding a polypeptide has
25 been detected with the probe(s), the sequence may be isolated or cloned by utilizing techniques which are known to those of ordinary skill in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

The present invention also relates to mutant nucleic acid sequences comprising at least one mutation in the polypeptide coding sequence of SEQ ID NO: 40, SEQ ID
30 NO: 42, and SEQ ID NO: 44, in which the mutant nucleic acid sequence encodes a polypeptide which consists of SEQ ID NO: 42, SEQ ID NO: 43, and SEQ ID NO: 45, respectively.

The techniques used to isolate or clone a nucleic acid sequence encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation
35 from cDNA, or a combination thereof. The cloning of the nucleic acid sequences of the present invention from such genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to

detect cloned DNA fragments with shared structural features. See, e.g., Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used. The nucleic acid sequence may be cloned from a strain of *Streptococcus*, or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleic acid sequence.

The present invention also relates to nucleic acid constructs comprising a nucleic acid sequence of the present invention operably linked to one or more control sequences which direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

The present invention also relates to recombinant expression vectors comprising a nucleic acid sequence of the present invention, a promoter, and transcriptional and translational stop signals.

The present invention also relates to recombinant host cells, comprising a nucleic acid sequence of the invention, which are advantageously used in the recombinant production of the polypeptides.

The present invention also relates to methods for producing a polypeptide having UDP-N-acetylglucosamine pyrophosphorylase activity comprising (a) cultivating a host cell under conditions suitable for production of the polypeptide; and (b) recovering the polypeptide.

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, and small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The polypeptides may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate.

For example, an enzyme assay may be used to determine the activity of the polypeptide as described herein.

The resulting polypeptide may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional
5 procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative
10 isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

The present invention further relates to the isolated polypeptides having UDP-glucose 6-dehydrogenase, UDP-glucose pyrophosphorylase, or UDP-N-
15 acetylglucosamine pyrophosphorylase activity encoded by the nucleic acid sequences described above.

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

20

Examples

Primers and Oligos

All primers and oligos were purchased (MWG Biotech Inc., High Point, NC)

25

Example 1: PCR amplification and cloning of the *Streptococcus equisimilis* *hasA* gene and the *Bacillus subtilis* *tuaD*, *gtaB*, and *gcaD* genes

The *Streptococcus equisimilis* hyaluronan synthase gene (*hasA*, accession
30 number AF023876, SEQ ID NOs: 1 [DNA sequence] and 2 [deduced amino acid sequence]) was PCR amplified from plasmid pKKseD (Weigel, 1997, *Journal of Biological Chemistry* 272: 32539-32546) using primers 1 and 2:

Primer 1:

5'-GAGCTCTATAAAAATGAGGAGGGAACCGAATGAGAACATTAAAAAACCT-3' (SEQ
35 ID NO: 3)

Primer 2:

5'-GTTAACGAATTCAGCTATGTAGGTACCTTATAATAATTTTTTACGTGT-3' (SEQ ID
NO: 4)

PCR amplifications were conducted in triplicate in 50 μ l reactions composed of 1 ng of pKKseD DNA, 0.4 μ M each of primers 1 and 2, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II (Applied Biosystems, Inc., Foster City, CA) with 2.5 mM $MgCl_2$, and 2.5 units of AmpliTaq Gold™ DNA polymerase (Applied Biosystems, Inc., Foster City, CA). The reactions were performed in a RoboCycler 40 thermocycler (Stratagene, Inc., La Jolla, CA) programmed for 1 cycle at 95°C for 9 minutes; 3 cycles each at 95°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute; 27 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 5 minutes. The PCR product was visualized using a 0.8% agarose gel with 44 mM Tris Base, 44 mM boric acid, 0.5 mM EDTA buffer (0.5X TBE). The expected fragment was approximately 1200 bp.

The 1200 bp PCR fragment was cloned into pCR2.1 using the TA-TOPO Cloning Kit (Stratagene, Inc., La Jolla, CA) and transformed into *E. coli* OneShot™ competent cells according to the manufacturers' instructions (Stratagene, Inc., La Jolla, CA). Transformants were selected at 37°C after 16 hours of growth on 2X yeast-tryptone (YT) agar plates supplemented with 100 μ g of ampicillin per ml. Plasmid DNA from these transformants was purified using a QIAGEN robot (QIAGEN, Valencia, CA) according to the manufacturer's instructions and the DNA sequence of the inserts confirmed by DNA sequencing using M13 (-20) forward and M13 reverse primers (Invitrogen, Inc, Carlsbad, CA) and the following internal primers. The plasmid harboring the 1200 bp PCR fragment was designated pCR2.1-sehasA (Figure 3).

Primer 3:

5'-GTTGACGATGGAAGTGCTGA-3' (SEQ ID NO: 5)

Primer 4:

5'-ATCCGTTACAGGTAATATCC-3' (SEQ ID NO: 6)

Primer 5:

5'-TCCTTTTGTAGCCCTATGGA-3' (SEQ ID NO: 7)

Primer 6:

5'-TCAGCACTTCCATCGTCAAC-3' (SEQ ID NO: 8)

Primer 7:

5'-GGATATTACCTGTAACGGAT-3' (SEQ ID NO: 9)

Primer 8:

5'-TCCATAGGGCTACAAAAGGA-3' (SEQ ID NO: 10)

The *Bacillus subtilis* UDP-glucose-6-dehydrogenase gene (*tuaD*, accession number BG12691, SEQ ID NOs: 11 [DNA sequence] and 12 [deduced amino acid sequence]) was PCR amplified from *Bacillus subtilis* 168 (BGSC 1A1, Bacillus Genetic Stock Center, Columbus, OH) using primers 9 and 10:

Primer 9:

5'-GGTACCGACACTGCGACCATTATAAA-3' (SEQ ID NO: 13)

Primer 10:

5'-GTTAACGAATTCCAGCTATGTATCTAGACAGCTTCAACCAAGTAACACT-3' (SEQ
5 ID NO: 14)

PCR amplifications were carried out in triplicate in 30 µl reactions composed of 50 ng of *Bacillus subtilis* 168 chromosomal DNA, 0.3 µM each of primers 9 and 10, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM MgCl₂, and 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a
10 RoboCycler 40 programmed for 1 cycle at 95°C for 9 minutes; 5 cycles each at 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes; 32 cycles each at 95°C for 1 minute, 54°C for 1 minute, and 72°C for 1.5 minute; and 1 cycle at 72°C for 7 minutes. The PCR product was visualized in a 0.8% agarose gel using 0.5X TBE buffer. The expected fragment was approximately 1400 bp.

15 The 1400 bp PCR fragment was cloned into pCR2.1 using the TA-TOPO Cloning Kit and transformed into *E. coli* OneShot™ competent cells according to the manufacturers' instructions. Plasmid DNA was purified using a QIAGEN robot according to the manufacturer's instructions and the DNA sequence of the inserts confirmed by DNA sequencing using M13 (-20) forward and M13 reverse primers and
20 the following internal primers. The plasmid harboring the 1400 bp PCR fragment was designated pCR2.1-*tuaD* (Figure 4).

Primer 11:

5'-AGCATCTTAACGGCTACAAA-3' (SEQ ID NO: 15)

Primer 12:

25 5'-TGTGAGCGAGTCGGCGCAGA-3' (SEQ ID NO: 16)

Primer 13:

5'-GGGCGCCCATGTAAAAGCAT-3' (SEQ ID NO: 17)

Primer 14:

5'-TTTGTAGCCGTTAAGATGCT-3' (SEQ ID NO: 18)

30 Primer 15:

5'-TCTGCGCCGACTCGCTCACA-3' (SEQ ID NO: 19)

Primer 16:

5'-ATGCTTTTACATGGGCGCCC-3' (SEQ ID NO: 20)

The *Bacillus subtilis* UTP-glucose-1-phosphate uridylyltransferase gene (*gtaB*,
35 accession number BG10402, SEQ ID NOs: 21 [DNA sequence] and 22 [deduced amino acid sequence]) was PCR amplified from *Bacillus subtilis* 168 using primers 17 and 18: Primer 17: 5'-TCTAGATTTTTCGATCATAAGGAAGGT-3' (SEQ ID NO: 23)

Primer 18: 5'-

GTTAACGAATTCCAGCTATGTAGGATCCAATGTCCAATAGCCTTTTTGT-3' (SEQ ID NO: 24)

PCR amplifications were carried out in triplicate in 30 μ l reactions composed of
 5 50 ng of *Bacillus subtilis* 168 chromosomal DNA, 0.3 μ M each of primers 17 and 18, 200
 μ M each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM MgCl₂, and
 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a
 RoboCycler 40 programmed for 1 cycle at 95°C for 9 minutes; 5 cycles each at 95°C for
 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes; 32 cycles each at 95°C for 1
 10 minute, 54°C for 1 minute, and 72°C for 1.5 minute; and 1 cycle at 72°C for 7 minutes.
 The PCR product was visualized in a 0.8% agarose-0.5X TBE gel. The expected
 fragment was approximately 900 bp.

The 900 bp PCR fragment was cloned into pCR2.1 using the TA-TOPO cloning
 kit and transformed into *E. coli* OneShot™ competent cells according to the
 15 manufacturer's instructions. Plasmid DNA was purified using a QIAGEN robot
 according to the manufacturer's instructions and the DNA sequence of the inserts
 confirmed by DNA sequencing using M13 (-20) forward and M13 reverse primers and
 the following internal primers. The plasmid harboring the 900 bp PCR fragment was
 designated pCR2.1-*gtaB* (Figure 5).

20 Primer 19:

5'-AAAAAGGCTTCTAACCTGGC-3' (SEQ ID NO: 25)

Primer 20:

5'-AAACCGCCTAAAGGCACAGC-3' (SEQ ID NO: 26)

Primer 21:

25 5'-GCCAGGTTAGAAGCCTTTTT-3' (SEQ ID NO: 27)

Primer 22:

5'-GCTGTGCCTTTAGGCGGTTT-3' (SEQ ID NO: 28)

The *Bacillus subtilis* UDP-N-acetylglucosamine pyrophosphorylase gene (*gcaD*,
 accession number BG10113, SEQ ID NOs: 29 [DNA sequence] and 30 [deduced amino
 30 acid sequence]) was PCR amplified from *Bacillus subtilis* 168 using primers 23 and 24:

Primer 23: 5'-GGATCCTTTCTATGGATAAAAGGGAT-3' (SEQ ID NO: 31)

Primer 24: 5'-GTTAACAGGATTATTTTTTATGAATATTTTT-3' (SEQ ID NO: 32)

PCR amplifications were carried out in triplicate in 30 μ l reactions composed of
 50 ng of *Bacillus subtilis* 168 chromosomal DNA, 0.3 μ M each of primers 23 and 24, 200
 35 μ M each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM MgCl₂, and
 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a
 RoboCycler 40 programmed for 1 cycle at 95°C for 9 minutes; 5 cycles each at 95°C for

1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes; 32 cycles each at 95°C for 1 minute, 54°C for 1 minute, and 72°C for 1.5 minute; and 1 cycle at 72°C for 7 minutes. The PCR product was visualized in a 0.8% agarose-0.5X TBE gel. The expected fragment was approximately 1500 bp.

5 The 1500 bp PCR fragment was cloned into pCR2.1 using the TA-TOPO cloning kit and transformed into *E. coli* OneShot™ competent cells according to the manufacturer's instructions. Plasmid DNA was purified using a QIAGEN robot according to the manufacturer's instructions and the DNA sequence of the inserts confirmed by DNA sequencing using M13 (-20) forward and M13 reverse primers and
10 the following internal primers. The plasmid harboring the 900 bp PCR fragment was designated pCR2.1-*gcaD* (Figure 6).

Primer 25:

5'-CAGAGACGATGGAACAGATG-3' (SEQ ID NO: 33)

Primer 26:

15 5'-GGAGTTAATGATAGAGTTGC-3' (SEQ ID NO: 34)

Primer 27:

5'-GAAGATCGGGAATTTTGTAG-3' (SEQ ID NO: 35)

Primer 28:

5'-CATCTGTTCCATCGTCTCTG-3' (SEQ ID NO: 36)

20 Primer 29:

5'-GCAACTCTATCATTA ACTCC-3' (SEQ ID NO: 37)

Primer 30:

5'-CTACAAAATTCCCGATCTTC-3' (SEQ ID NO: 38)

25 **Example 2: Construction of the *hasA/tuaD/gtaB* operon**

Plasmids pDG268Δneo-cryIIIAstab/Sav (U.S. Patent No. 5,955,310) and pCR2.1-*tuaD* (Example 1, Figure 4) were digested with *KpnI* and *HpaI*. The digestions were resolved on a 0.8% agarose gel using 0.5X TBE buffer and the larger vector fragment (approximately 7700 bp) from pDG268Δneo-cryIIIAstab/Sav and the smaller
30 *tuaD* fragment (approximately 1500 bp) from pCR2.1-*tuaD* were gel-purified using the QIAquick DNA Extraction kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). The two purified fragments were ligated together with T4 DNA ligase according to the manufacturer's instructions (Roche Applied Science; Indianapolis, IN) and the ligation mix was transformed into *E. coli* SURE competent cells (Stratagene,
35 Inc., La Jolla, CA). Transformants were selected on 2X YT agar plates supplemented with 100 μg of ampicillin per ml.

Plasmid DNA was purified from several transformants using a QIAGEN robot

according to the manufacturer's instructions and analyzed by *KpnI* plus *HpaI* digestion on a 0.8% agarose gel using 0.5X TBE buffer. The correct plasmid was identified by the presence of an approximately 1500 bp *KpnI/HpaI* *tuaD* fragment and was designated pHA1 (Figure 7).

5 Plasmids pHA1 and pCR2.1-*gtaB* (Example 1, Figure 5) were digested with *XbaI* and *HpaI*. The digestions were resolved on a 0.8% agarose gel using 0.5X TBE buffer and the larger vector fragment from pHA1 (approximately 9200 bp) and the smaller *gtaB* fragment (approximately 900 bp) from pCR2.1-*gtaB* were gel-purified from a 0.8% agarose-0.5X TBE buffer gel using the QIAquick DNA Extraction Kit according to the
10 manufacturer's instructions. These two purified fragments were ligated together with T4 DNA ligase and the ligation mix was used to transform *E. coli* SURE competent cells. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml at 37°C

Plasmids were purified from several transformants using a QIAGEN robot
15 according to the manufacturer's instructions and analyzed by *XbaI* plus *HpaI* digestion. The digestions were resolved on a 0.8% agarose-0.5X TBE buffer gel. The correct plasmid was identified by the presence of an approximately 900 bp *XbaI/HpaI* *gtaB* fragment and was designated pHA2 (Figure 8).

Plasmids pHA2 and pCR2.1-*sehasA* (Example 1, Figure 3) were digested with
20 *SacI* plus *KpnI*. The digestions were resolved on a 0.8% agarose-0.5X TBE buffer gel. The larger vector fragment (approximately 10000 bp) from pHA2 and the smaller *hasA* fragment (approximately 1300 bp) from pCR2.1-*sehasA* were gel-purified from a 0.8% agarose-0.5X TBE buffer gel using the QIAquick DNA Extraction kit according to the manufacturer's instructions. The two purified fragments were ligated together with T4
25 DNA ligase and the ligation mix was used to transform *E. coli* SURE competent cells. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml at 37°C. Plasmids were purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *SacI* plus *KpnI* digestion. The digestions were resolved on a 0.8% agarose-0.5X TBE buffer gel.
30 The correct plasmid was identified by the presence of an approximately 1300 bp *SacI/KpnI* *hasA* fragment and was designated pHA3 (Figure 9).

Example 3: Construction of the *hasA/tuaD/gtaB/gcaD* operon

Plasmids pHA2 (Example 2, Figure 8) and pCR2.1-*gcaD* (Example 1, Figure 6)
35 were digested with *BamHI* and *HpaI*. The digestions were resolved on a 0.8% agarose gel using 0.5X TBE buffer and the larger vector fragment (approximately 10,000 bp) from pHA2 and the smaller *gcaD* fragment (approximately 1,400 bp) from pCR2.1-*gcaD*

were gel-purified from a 0.8% agarose-0.5X TBE buffer gel using the QIAquick DNA Extraction Kit according to the manufacturer's instructions. These two purified fragments were ligated together with T4 DNA ligase and the ligation mix was used to transform *E. coli* SURE competent cells. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml at 37°C.

Plasmids were purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *Xba*I plus *Hpa*I digestion. The digestions were resolved on a 0.8% agarose-0.5X TBE buffer gel. The correct plasmid was identified by the presence of an approximately 1400 bp *Bam*HI/*Hpa*I *gcaD* fragment and was designated pHA4 (Figure 10).

Plasmids pHA4 and pCR2.1-*sehasA* (Example 1, Figure 3) were digested with *Sac*I and *Kpn*I. The digestions were resolved on a 0.8% agarose-0.5X TBE buffer gel. The larger vector fragment (approximately 11,000 bp) from pHA4 and the smaller *hasA* fragment (approximately 1,300 bp) from pCR2.1-*sehasA* were gel-purified from a 0.8% agarose-0.5X TBE buffer gel using the QIAquick DNA Extraction kit according to the manufacturer's instructions. The two purified fragments were ligated together with T4 DNA ligase and the ligation mix was used to transform *E. coli* SURE competent cells. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml at 37°C. Plasmids were purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *Sac*I plus *Kpn*I digestion. The digestions were resolved on a 0.8% agarose-0.5X TBE buffer gel. The correct plasmid was identified by the presence of an approximately 1,300 bp *Sac*I/*Kpn*I *hasA* fragment and was designated pHA5 (Figure 11).

Example 4: Construction of the *hasA/tuaD/gcaD* operon

Plasmids pHA1 (Example 2, Figure 7) and pCR2.1-*gcaD* (Example 1, Figure 6) were digested with *Bam*HI and *Hpa*I. The digestions were resolved on a 0.8% agarose gel using 0.5X TBE buffer and the larger vector fragment from pHA1 (approximately 9,200 bp) and the smaller *gcaD* fragment (approximately 1400 bp) from pCR2.1-*gcaD* were gel-purified from a 0.8% agarose-0.5X TBE buffer gel using the QIAquick DNA Extraction Kit according to the manufacturer's instructions. These two purified fragments were ligated together with T4 DNA ligase and the ligation mix was used to transform *E. coli* SURE competent cells. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml at 37°C.

Plasmids were purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *Bam*HI plus *Hpa*I digestion. The digestions were resolved on a 0.8% agarose-0.5X TBE buffer gel. The

correct plasmid was identified by the presence of an approximately 1400 bp *Bam*HI/*Hpa*I *gtaB* fragment and was designated pHA6 (Figure 12).

Plasmids pHA6 and pCR2.1-*sehasA* (Example 1, Figure 3) were digested with *Sac*I plus *Kpn*I. The digestions were resolved on a 0.8% agarose-0.5X TBE buffer gel.

5 The larger vector fragment (approximately 10,200 bp) from pHA6 and the smaller *hasA* fragment (approximately 1,300 bp) from pCR2.1-*sehasA* were gel-purified from a 0.8% agarose-0.5X TBE buffer gel using the QIAquick DNA Extraction kit according to the manufacturer's instructions. The two purified fragments were ligated together with T4 DNA ligase and the ligation mix was used to transform *E. coli* SURE competent cells.

10 Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml. Plasmids were purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *Sac*I plus *Kpn*I digestion. The digestions were resolved on a 0.8% agarose-0.5X TBE buffer gel. The correct plasmid was identified by the presence of an approximately 1300 bp *Sac*I/*Kpn*I

15 *hasA* fragment and was designated pHA7 (Figure 13).

Example 5: Construction of *Bacillus subtilis* RB161

Plasmid pDG268MCSΔneo/scBAN/Sav (U.S. Patent No. 5,955,310) was digested with *Sac*I. The digested plasmid was then purified using a QIAquick DNA

20 Purification Kit according to the manufacturer's instructions, and finally digested with *Not*I. The largest plasmid fragment of approximately 6800 bp was gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions (QIAGEN, Valencia, CA). The recovered vector DNA was then ligated with the DNA insert described below.

25 Plasmid pHA3 (Example 2, Figure 9) was digested with *Sac*I. The digested plasmid was then purified as described above, and finally digested with *Not*I. The smallest plasmid fragment of approximately 3800 bp was gel-purified as described above. The recovered vector and DNA insert were ligated using the Rapid DNA Cloning Kit (Roche Applied Science; Indianapolis, IN) according to the manufacturer's

30 instructions. Prior to transformation in *Bacillus subtilis*, the ligation described above was linearized using *Sca*I to ensure double cross-over integration in the chromosome rather than single cross-over integration in the chromosome. Competent cells of *Bacillus subtilis* 168Δ4 were transformed with the ligation products digested with *Sca*I. *Bacillus subtilis* 168Δ4 is derived from the *Bacillus subtilis* type strain 168 (BGSC 1A1, Bacillus

35 Genetic Stock Center, Columbus, OH) and has deletions in the *spo*IIAC, *aprE*, *nprE*, and *amyE* genes. The deletion of these four genes was performed essentially as described for *Bacillus subtilis* A164Δ5, which is described in detail in U.S. Patent No.

5,891,701.

Bacillus subtilis chloramphenicol-resistant transformants were selected at 34°C after 16 hours of growth on Tryptose blood agar base (TBAB) plates supplemented with 5 µg of chloramphenicol per ml. To screen for integration of the plasmid by double cross-over at the *amyE* locus, *Bacillus subtilis* primary transformants were patched on TBAB plates supplemented with 6 µg of neomycin per ml and on TBAB plates supplemented with 5 µg of chloramphenicol per ml. Integration of the plasmid by double cross-over at the *amyE* locus does not incorporate the neomycin resistance gene and therefore renders the strain neomycin sensitive. Isolates were also patched onto minimal plates to visualize whether or not these were producing hyaluronic acid. Hyaluronic acid producing isolates have a “wet” phenotype on minimal plates. Using this plate screen, chloramphenicol resistant and neomycin sensitive “wet” transformants (due to hyaluronic acid production) were isolated at 37°C.

Genomic DNA was isolated from the “wet”, chloramphenicol resistant, and neomycin sensitive *Bacillus subtilis* 168Δ4 transformants using a QIAGEN tip-20 column (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. PCR amplifications were performed on these transformants using the synthetic oligonucleotides below, which are based on the *hasA*, *tuaD*, and *gtaB* gene sequences, to confirm the presence and integrity of these genes in the operon of the *Bacillus subtilis* transformants.

The amplification reactions (25 µl) were composed of approximately 50 ng of genomic DNA of the *Bacillus subtilis* 168Δ4 transformants, 0.5 µM of each primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II, 3 mM MgCl₂, and 0.625 units of AmpliTaq Gold™ DNA polymerase. The reactions were incubated in a RoboCycler 40 Temperature Cycler programmed for one cycle at 95°C for 9 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes; and a final cycle at 72°C for 7 minutes.

Primers 3 and 8 were used to confirm the presence of the *hasA* gene, primers 3 and 16 to confirm the presence of the *tuaD* gene, and primers 3 and 22 to confirm the presence of the *gtaB* gene. The *Bacillus subtilis* 168Δ4 *hasA/tuaD/gtaB* integrant was designated *Bacillus subtilis* RB158.

Genomic DNA was isolated from *Bacillus subtilis* RB158 using a QIAGEN tip-20 column according to the manufacturer’s instructions, and was used to transform competent *Bacillus subtilis* A164Δ5 (deleted at the *spoIIAC*, *aprE*, *nprE*, *amyE*, and *srfC* genes; see U.S. Patent No. 5,891,701). Transformants were selected on TBAB plates supplemented with 5 µg of chloramphenicol per ml at 37°C. A *Bacillus subtilis* A164Δ5 *hasA/tuaD/gtaB* integrant was identified by its “wet” phenotype and designated *Bacillus*

subtilis RB161.

Example 6: Construction of *Bacillus subtilis* RB163

Plasmid pDG268MCS Δ neo/scBAN/Sav (U.S. Patent No. 5,955,310) was
5 digested with *SacI*. The digested plasmid was then purified using a QIAquick DNA
Purification Kit according to the manufacturer's instructions, and finally digested with
NotI. The largest plasmid fragment of approximately 6,800 bp was gel-purified using a
QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the
manufacturer's instructions. The recovered vector DNA was then ligated with the DNA
10 insert described below.

Plasmid pHA7 (Example 4, Figure 13) was digested with *SacI*. The digested
plasmid was then purified as described above, and finally digested with *NotI*. The
smallest plasmid fragment of approximately 4,300 bp was gel-purified as described
above. The recovered vector and DNA insert were ligated using the Rapid DNA Cloning
15 Kit according to the manufacturer's instructions. Prior to transformation in *Bacillus*
subtilis, the ligation described above was linearized using *ScaI* to ensure double cross-
over integration in the chromosome rather than single cross-over integration in the
chromosome. *Bacillus subtilis* 168 Δ 4 competent cells were transformed with the ligation
digested with the restriction enzyme *ScaI*.

20 *Bacillus subtilis* chloramphenicol-resistant transformants were selected on TBAB
plates supplemented with 5 μ g of chloramphenicol per ml at 37°C. To screen for
integration of the plasmid by double cross-over at the *amyE* locus, *Bacillus subtilis*
primary transformants were patched on TBAB plates supplemented with 6 μ g of
neomycin per ml and on TBAB plates supplemented with 5 μ g of chloramphenicol per ml
25 to isolate chloramphenicol resistant and neomycin sensitive "wet" transformants (due to
hyaluronic acid production).

Genomic DNA was isolated from the "wet", chloramphenicol resistant, and
neomycin sensitive *Bacillus subtilis* 168 Δ 4 transformants using a QIAGEN tip-20 column
according to the manufacturer's instructions. PCR amplifications were performed on
30 these transformants using primers 3, 8, 16, 22 and primer 30 (Example 1) to confirm the
presence and integrity of these genes in the operon of the *Bacillus subtilis*
transformants. The amplification reactions (25 μ l) were composed of approximately 50
ng of genomic DNA of the *Bacillus subtilis* 168 Δ 4 transformants, 0.5 μ M of each primer,
200 μ M each of dATP, dCTP, dGTP, and dTTP, 1X PCR buffer, 3 mM MgCl₂, and 0.625
35 units of AmpliTaq Gold™ DNA polymerase. The reactions were incubated in a
RoboCycler 40 Temperature Cycler programmed for one cycle at 95°C for 9 minutes; 30
cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes; and a final

cycle at 72°C for 7 minutes.

Primers 3 and 8 were used to confirm the presence of the *hasA* gene, primers 3 and 16 to confirm the presence of the *tuaD* gene, primers 3 and 22 to confirm the presence of the *gtaB* gene, and primers 3 and 30 to confirm the presence of the *gcaD* gene. The *Bacillus subtilis* 168Δ4 *hasA/tuaD/gcaD* integrant was designated *Bacillus subtilis* RB160.

Genomic DNA was isolated from *Bacillus subtilis* RB160 using a QIAGEN tip-20 column according to the manufacturer's instructions, and was used to transform competent *Bacillus subtilis* A164Δ5. Transformants were selected on TBAB plates containing 5 μg of chloramphenicol per ml, and grown at 37°C for 16 hours. The *Bacillus subtilis* A164Δ5 *hasA/tuaD/gcaD* integrant was identified by its "wet" phenotype and designated *Bacillus subtilis* RB163.

Example 7: Construction of *Bacillus subtilis* TH-1

The hyaluronan synthase (*has*) operon was obtained from *Streptococcus equisimilis* using the following procedure. The *has* operon is composed of the *hasA*, *hasB*, *hasC*, and *hasD* genes. Approximately 20 μg of *Streptococcus equisimilis* D181 (Kumari and Weigel, 1997, *Journal of Biological Chemistry* 272: 32539-32546) chromosomal DNA was digested with *HindIII* and resolved on a 0.8% agarose-0.5X TBE gel. DNA in the 3-6 kb range was excised from the gel and purified using the QIAquick DNA Gel Extraction Kit according to the manufacturer's instructions. The recovered DNA insert was then ligated with the vector DNA described below.

Plasmid pUC18 (2 μg) was digested with *HindIII* and the 5' protruding ends were dephosphorylated with shrimp alkaline phosphatase according to the manufacturer's instructions (Roche Applied Science; Indianapolis, IN). The dephosphorylated vector and DNA insert were ligated using the Rapid DNA Cloning Kit according to the manufacturer's instructions. The ligation was used to transform *E. coli* XL10 Gold Kan competent cells (Stratagene, Inc., La Jolla, CA). Cells were plated onto Luria broth plates (100 μg/ml ampicillin) and incubated overnight at 37°C. Five plates containing approximately 500 colonies/plate were probed with oligo 952-55-1, shown below, which is a 54 bp sequence identical to the coding strand near the 3' end of the *Streptococcus equisimilis* D181 *hasA* gene (nucleotides 1098-1151 with respect to the A residue of the ATG translation start codon).

Primer 31:

5'-GTGTCGGAACATTCATTACATGCTTAAGCACCCGCTGTCCTTCTTGTTATCTCC-3'
(SEQ ID NO: 39)

The oligonucleotide probe was DIG-labeled using the DIG Oligonucleotide 3'-end

Labeling Kit according to the manufacturer's instructions (Roche Applied Science; Indianapolis, IN). Colony hybridization and chemiluminescent detection were performed as described in "THE DIG SYSTEM USER'S GUIDE FOR FILTER HYBRIDIZATION", Boehringer Mannheim GmbH.

5 Seven colonies were identified that hybridized to the probe. Plasmid DNA from one of these transformants was purified using a QIAGEN robot (QIAGEN, Valencia, CA) according to the manufacturer's instructions, digested with *Hind*III, and resolved on a 0.8% agarose gel using 0.5X TBE buffer. The DNA insert was shown to be approximately 5 kb in size. This plasmid was designated pMRT106 (Figure 14).

10 The DNA sequence of the cloned fragment was determined using the EZ::TN™ <TET-1> Insertion Kit according to the manufacturer's instructions (Epicenter Technologies, Madison, WI). The sequencing revealed that the cloned DNA insert contained the last 1156 bp of the *Streptococcus equisimilis* *hasA* gene followed by three other genes designated *hasB*, *hasC*, and *hasD*; presumably all four genes are contained
15 within a single operon and are therefore co-transcribed. The *Streptococcus equisimilis* *hasB* gene is contained in nucleotides 1411-2613 (SEQ ID NOs: 40 [DNA sequence] and 41 [deduced amino acid sequence]) of the fragment, and *Streptococcus equisimilis* *hasC* gene in nucleotides 2666-3565 (SEQ ID NOs: 42 [DNA sequence] and 43 [deduced amino acid sequence]) of the fragment, and *Streptococcus equisimilis* *hasD*
20 gene in nucleotides 3735-5114 (SEQ ID NOs: 44 [DNA sequence] and 45 [deduced amino acid sequence]) of the fragment.

 The polypeptides encoded by the *Streptococcus equisimilis* *hasB* and *hasC* genes show some homology to those encoded by the *hasB* and *hasC* genes, respectively, from the *Streptococcus pyogenes* *has* operon sequence (Ferretti *et al.*,
25 2001, *Proc. Natl. Acad. Sci. U.S.A.* 98 (8), 4658-4663). The degree of identity was determined by the Clustal method (Higgins, 1989, *CABIOS* 5: 151-153) using using the Vector NTI AlignX software (Informax Inc., Bethesda, MD) with the following defaults: pairwise alignment, gap opening penalty of 10, gap extension penalty of 0.1, and score matrix: blosum62mt2.

30 Amino acid sequence comparisons showed that the *Streptococcus equisimilis* HasB protein has 70% identity to the HasB protein from *Streptococcus uberis* (SEQ ID NO: 105); the *Streptococcus equisimilis* HasC protein has 91% identity to the HasC protein from *Streptococcus pyogenes* (SEQ ID NO: 99); and the *Streptococcus equisimilis* HasD protein has 73% identity to the GlmU protein (a putative UDP-N-acetylglucosamine pyrophosphorylase) of *Streptococcus pyogenes* (accession #
35 Q8P286). The *Streptococcus equisimilis* *hasD* gene encodes a polypeptide that shows 50.7% identity to the UDP-N-acetyl-glucosamine pyrophosphorylase enzyme encoded

by the *gcaD* gene of *Bacillus subtilis*.

Plasmid pHA5 (Example 3, Figure 11) was digested with *HpaI* and *BamHI*. The digestion was resolved on a 0.8% agarose gel using 0.5X TBE buffer and the larger vector fragment (approximately 11,000 bp) was gel-purified using the QIAquick DNA
5 Extraction Kit according to the manufacturer's instructions. Plasmid pMRT106 was digested with *HindIII*, the sticky ends were filled in with Klenow fragment, and the DNA was digested with *BamHI*. The digestion was resolved on a 0.8% agarose gel using 0.5X TBE buffer and the smaller insert fragment (approximately 1000 bp, the last 2/3 of the *Streptococcus equisimilis hasD* gene) was gel-purified using the QIAquick DNA
10 Extraction kit according to the manufacturer's instructions.

The two purified fragments were ligated together with T4 DNA ligase and the ligation mix was transformed into *E. coli* SURE competent cells. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml at 37°C.

Plasmid DNA was purified from several transformants using a QIAGEN robot
15 according to the manufacturer's instructions and analyzed by *BamHI* plus *NotI* digestion on a 0.8% agarose gel using 0.5X TBE buffer. The correct plasmid was identified by the presence of an approximately 1,100 bp *BamHI/NotI hasD* fragment and was designated pHA8 (Figure 15). This plasmid was digested with *HindIII* and ligated together with T4 DNA ligase and the ligation mix was transformed into *E. coli* SURE competent cells.
20 Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml. Plasmid DNA was purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *HindIII* digestion on a 0.8% agarose gel using 0.5X TBE buffer. The correct plasmid was identified by the presence of a single band of approximately 9,700 bp and was
25 designated pHA9 (Figure 16).

Plasmid pHA9 was digested with *SacI* and *NotI*. The digestion was resolved on a 0.8% agarose gel using 0.5X TBE buffer and the smaller fragment of approximately 2,500 bp was gel-purified using the QIAquick DNA Extraction kit according to the manufacturer's instructions. Plasmid pDG268MCSΔneo/scBAN/Sav (U.S. Patent No.
30 5,955,310) was digested with *SacI* and *NotI*. The digestion was resolved on a 0.8% agarose gel using 0.5X TBE buffer and the larger vector fragment of approximately 6,800 bp was gel-purified using the QIAquick DNA Extraction kit according to the manufacturer's instructions. The two purified fragments were ligated together with T4 DNA ligase and the ligation mix was transformed into *E. coli* SURE competent cells
35 (Stratagene, Inc., La Jolla, CA). Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml.

Plasmid DNA was purified from several transformants using a QIAGEN robot

according to the manufacturer's instructions and analyzed by *Sall* plus *HindIII* digestion on a 0.8% agarose gel using 0.5X TBE buffer. The correct plasmid was identified by the presence of an approximately 1600 bp *Sall/HindIII* fragment and was designated pHA10 (Figure 17).

5 Plasmid pHA10 was digested with *HindIII* and *BamHI*. The digestion was resolved on a 0.8% agarose gel using 0.5X TBE buffer and the larger vector fragment (approximately 8100 bp) was gel-purified using the QIAquick DNA Extraction kit according to the manufacturer's instructions. Plasmid pMRT106 was digested with *HindIII* and *BamHI*. The digestion was resolved on a 0.8% agarose gel using 0.5X TBE
10 buffer and the larger insert fragment of approximately 4,100 bp was gel-purified using the QIAquick DNA Extraction kit according to the manufacturer's instructions. The two purified fragments were ligated together with T4 DNA ligase and the ligation mix was used to transform *Bacillus subtilis* 168Δ4. Transformants were selected on TBAB agar plates supplemented with 5 μg of chloramphenicol per ml at 37°C. Approximately 100
15 transformants were patched onto TBAB supplemented with chloramphenicol (5 μg/ml) and TBAB supplemented with neomycin (10 μg/ml) to score chloramphenicol resistant, neomycin sensitive colonies; this phenotype is indicative of a double crossover into the *amyE* locus. A few such colonies were identified, all of which exhibited a "wet" phenotype indicating that hyaluronic acid was being produced. One colony was chosen
20 and designated *Bacillus subtilis* 168Δ4::scBAN/se hasA/hasB/hasC/hasD.

Genomic DNA was isolated from *Bacillus subtilis* 168Δ4::scBAN/se hasA/hasB/hasC/hasD using a QIAGEN tip-20 column according to the manufacturer's instructions, and used to transform competent *Bacillus subtilis* A164Δ5. Transformants were selected on TBAB plates containing 5 μg of chloramphenicol per ml, and grown at
25 37°C for 16 hours. The *Bacillus subtilis* A164Δ5 hasA/hasB/hasC/hasD integrant was identified by its "wet" phenotype and designated *Bacillus subtilis* TH-1.

Example 8: Construction of *Bacillus subtilis* RB184

The *hasA* gene from *Streptococcus equisimilis* (Example 1) and *tuaD* gene (a
30 *Bacillus subtilis* *hasB* homologue) (Example 1) were cloned to be under the control of a short "consensus" *amyQ* (scBAN) promoter (U.S. Patent No. 5,955,310).

Plasmid pDG268MCSΔneo/scBAN/Sav (U.S. Patent No. 5,955,310) was digested with *SacI*. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's instructions, and finally digested with
35 *NotI*. The largest plasmid fragment of approximately 6,800 bp was gel-purified from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Gel Extraction Kit according to the manufacturer's instructions. The recovered vector DNA was then ligated with the DNA

insert described below.

Plasmid pHA5 (Example 3, Figure 11) was digested with *HpaI*. The digested plasmid was then purified as described above, and finally digested with *XbaI*. The double-digested plasmid was then blunted by first inactivating *XbaI* at 85°C for 30 minutes. Blunting was performed by adding 0.5 µl of 10 mM each dNTPs, 1 µl of 1 U/µl T4 DNA polymerase (Roche Applied Science; Indianapolis, IN) and incubating at 11°C for 10 minutes. Finally the polymerase was inactivated by incubating the reaction at 75°C for 10 minutes. The largest plasmid fragment of approximately 11,000 bp was then gel-purified as described above and religated using the Rapid DNA Cloning Kit according to the manufacturer's instructions. The ligation mix was transformed into *E. coli* SURE competent cells. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml at 37°C. Plasmid DNA was purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *ScaI* digestion on a 0.8% agarose gel using 0.5X TBE buffer. The correct plasmid was identified by the presence of an approximately 11 kb fragment and was designated pRB157 (Figure 18).

pRB157 was digested with *SacI*. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's instructions, and finally digested with *NotI*. The smallest plasmid fragment of approximately 2,632 bp was gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered DNA insert was then ligated with the vector DNA described above.

Prior to transformation in *Bacillus subtilis*, the ligation described above was linearized using *ScaI* to ensure double cross-over integration in the chromosome rather than single cross-over integration in the chromosome. *Bacillus subtilis* 168Δ4 competent cells were transformed with the ligation digested with the restriction enzyme *ScaI*.

Bacillus subtilis chloramphenicol-resistant transformants were selected on TBAB plates supplemented with 5 µg of chloramphenicol per ml. To screen for integration of the plasmid by double cross-over at the *amyE* locus, *Bacillus subtilis* primary transformants were patched on TBAB plates supplemented with 6 µg of neomycin per ml and on TBAB plates supplemented with 5 µg of chloramphenicol per ml to isolate chloramphenicol resistant and neomycin sensitive "wet" transformants (due to hyaluronic acid production).

Genomic DNA was isolated from the "wet", chloramphenicol resistant, and neomycin sensitive *Bacillus subtilis* 168Δ4 transformants using a QIAGEN tip-20 column according to the manufacturer's instructions. PCR amplifications were performed on

these transformants using primers 3, 8, and 16 (Example 1) to confirm the presence and integrity of *hasA* and *tuaD* in the operon of the *Bacillus subtilis* transformants. The amplification reactions (25 μ l) were composed of approximately 50 ng of genomic DNA of the *Bacillus subtilis* 168 Δ 4 transformants, 0.5 μ M of each primer, 200 μ M each of
5 dATP, dCTP, dGTP, and dTTP, 1X PCR buffer, 3 mM MgCl₂, and 0.625 units of AmpliTaq Gold™ DNA polymerase. The reactions were incubated in a RoboCycler 40 Temperature Cycler programmed for one cycle at 95°C for 9 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes; and a final cycle at 72°C for 7 minutes.

10 Primers 3 and 8 were used to confirm the presence of the *hasA* gene and primers 3 and 16 to confirm the presence of the *tuaD* gene. A *Bacillus subtilis* 168 Δ 4 *hasA/tuaD* integrant was designated *Bacillus subtilis* RB183.

Bacillus subtilis RB183 genomic DNA was also used to transform competent *Bacillus subtilis* A164 Δ 5. Transformants were selected on TBAB plates containing 5 μ g
15 of chloramphenicol per ml, and grown at 37°C for 16 hours. The *Bacillus subtilis* A164 Δ 5 *hasA/tuaD* integrant was identified by its "wet" phenotype and designated *Bacillus subtilis* RB184.

Example 9: Construction of *Bacillus subtilis* RB187

20 *Bacillus subtilis* RB161 was made competent and transformed with the *cat* deletion plasmid pRB115 (Widner *et al.*, 2000, *Journal of Industrial Microbiology & Biotechnology* 25: 204-212). Selection for direct integration into the chromosome was performed at the non-permissive temperature of 45°C using erythromycin (5 μ g/ml) selection. At this temperature, the pE194 origin of replication is inactive. Cells are able
25 to maintain erythromycin resistance only by integration of the plasmid into the *cat* gene on the bacterial chromosome. These so-called "integrants" were maintained at 45°C to ensure growth at this temperature with selection. To allow for loss or "looping out" of the plasmid, which will result in the deletion of most of the *cat* gene from the chromosome, the integrants were grown in Luria-Bertani (LB) medium without selection at the
30 permissive temperature of 34°C for many generations. At this temperature the pE194 origin of replication is active and promotes excision of the plasmid from the genome (*Molecular Biological Methods for Bacillus*, edited by C.R. Harwood and S.M. Cutting, 1990, John Wiley and Sons Ltd.).

The cells were then plated on non-selective LB agar plates and colonies which
35 contained deletions in the *cat* gene and loss of the pE194-based replicon were identified by the following criteria: (1) chloramphenicol sensitivity indicated the presence of the *cat* deletion; (2) erythromycin sensitivity indicated the absence of the erythromycin

resistance gene encoded by the vector pRB115; and (3) PCR confirmed the presence of the *cat* deletion in the strain of interest. PCR was performed to confirm deletion of the *cat* gene at the *amyE* locus by using primers 32 and 33:

Primer 32: 5'-GCGGCCGCGGTACCTGTGTTACACCTGTT-3' (SEQ ID NO: 46)

5 Primer 33: 5'-GTCAAGCTTAATTCTCATGTTTGACAGCTTATCATCGG-3' (SEQ ID NO: 47)

Chromosomal DNA from potential deletants was isolated using the REDextract-N-Amp™ Plant PCR kits (Sigma Chemical Company, St. Louis, MO) as follows: Single *Bacillus* colonies were inoculated into 100 µl of Extraction Solution (Sigma Chemical
10 Company, St. Louis, MO), incubated at 95°C for 10 minutes, and then diluted with an equal volume of Dilution Solution (Sigma Chemical Company, St. Louis, MO). PCR was performed using 4 µl of extracted DNA in conjunction with the REDextract-N-Amp PCR Reaction Mix and the desired primers according to the manufacturer's instructions, with PCR cycling conditions described in Example 5. PCR reaction products were visualized
15 in a 0.8% agarose-0.5X TBE gel. The verified strain was named *Bacillus subtilis* RB187.

Example 10: Construction of *Bacillus subtilis* RB192

Bacillus subtilis RB184 was made unmarked by deleting the chloramphenicol resistance gene (*cat* gene). This was accomplished using the method described
20 previously in Example 9. The resultant strain was designated *Bacillus subtilis* RB192.

Example 11: Construction of *Bacillus subtilis* RB194

Bacillus subtilis RB194 was constructed by deleting the *cypX* region of the chromosome of *Bacillus subtilis* RB187 (Example 9). The *cypX* region includes the
25 *cypX* gene which encodes a cytochrome P450-like enzyme that is involved in the synthesis of a red pigment during fermentation. In order to delete this region of the chromosome plasmid pMRT086 was constructed.

The region of the chromosome which harbors the *cypX-yvmC* and *yvmB-yvmA* operons was PCR amplified from *Bacillus subtilis* BRG-1 as a single fragment using
30 primers 34 and 35. *Bacillus subtilis* BRG1 is essentially a chemically mutagenized isolate of an amylase-producing strain of *Bacillus subtilis* which is based on the *Bacillus subtilis* A164Δ5 genetic background that was described in Example 5. The sequence of this region is identical to the published sequence for the *Bacillus subtilis* 168 type strain.

Primer 34: 5'-CATGGGAGAGACCTTTGG-3' (SEQ ID NO: 48)

35 Primer 35: 5'-GTCGGTCTTCCATTTGC-3' (SEQ ID NO: 49)

The amplification reactions (50 µl) were composed of 200 ng of *Bacillus subtilis* BRG-1 chromosomal DNA, 0.4 µM each of primers 34 and 35, 200 µM each of dATP,

dCTP, dGTP, and dTTP, 1X Expand™ High Fidelity buffer (Roche Applied Science; Indianapolis, IN) with 1.5 mM MgCl₂, and 2.6 units of Expand™ High Fidelity PCR System enzyme mix (Roche Applied Science; Indianapolis, IN). *Bacillus subtilis* BRG-1 chromosomal DNA was obtained using a QIAGEN tip-20 column according to the manufacturer's instructions. Amplification reactions were performed in a RoboCycler 40 thermacycler (Stratagene, Inc, La Jolla, CA) programmed for 1 cycle at 95°C for 3 minutes; 10 cycles each at 95°C for 1 minute, 58°C for 1 minute, and 68°C for 4 minutes; 20 cycles each at 95°C for 1 minute, 58°C for 1 minute, 68°C for 4 minutes plus 20 seconds per cycle, followed by 1 cycle at 72°C for 7 minutes. Reaction products were analyzed by agarose gel electrophoresis using a 0.8% agarose gel using 0.5X TBE buffer.

The resulting fragment comprising the *cypX-yvmC* and *yvmB-yvmA* operons was cloned into pCR2.1 using the TA-TOPO Cloning Kit and transformed into *E. coli* OneShot™ cells according to the manufacturer's instructions (Invitrogen, Inc., Carlsbad, CA). Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml. Plasmid DNA from several transformants was isolated using QIAGEN tip-20 columns according to the manufacturer's instructions and verified by DNA sequencing with M13 (-20) forward, M13 reverse and primers 36 to 51 shown below. The resulting plasmid was designated pMRT084 (Figure 19).

- 20 Primer 36: 5'-CGACCACTGTATCTTGG-3' (SEQ ID NO: 50)
- Primer 37: 5'-GAGATGCCAAACAGTGC-3' (SEQ ID NO: 51)
- Primer 38: 5'-CATGTCCATCGTGACG-3' (SEQ ID NO: 52)
- Primer 39: 5'-CAGGAGCATTGATACG-3' (SEQ ID NO: 53)
- Primer 40: 5'-CCTTCAGATGTGATCC-3' (SEQ ID NO: 54)
- 25 Primer 41: 5'-GTGTTGACGTCAACTGC-3' (SEQ ID NO: 55)
- Primer 42: 5'-GTTCAGCCTTTCCTCTCG-3' (SEQ ID NO: 56)
- Primer 43: 5'-GCTACCTTCTTTCTTAGG-3' (SEQ ID NO: 57)
- Primer 44: 5'-CGTCAATATGATCTGTGC-3' (SEQ ID NO: 58)
- Primer 45: 5'-GGAAAGAAGGTCTGTGC-3' (SEQ ID NO: 59)
- 30 Primer 46: 5'-CAGCTATCAGCTGACAG-3' (SEQ ID NO: 60)
- Primer 47: 5'-GCTCAGCTATGACATATTCC-3' (SEQ ID NO: 61)
- Primer 48: 5'-GATCGTCTTGATTACCG-3' (SEQ ID NO: 62)
- Primer 49: 5'-AGCTTTATCGGTGACG-3' (SEQ ID NO: 63)
- Primer 50: 5'-TGAGCACGATTGCAGG-3' (SEQ ID NO: 64)
- 35 Primer 51: 5'-CATTGCGGAGACATTGC-3' (SEQ ID NO: 65)

Plasmid pMRT084 was digested with *BsgI* to delete most of the *cypX-yvmC* and *yvmB-yvmA* operons, leaving about 500 bases at each end. The digested *BsgI* DNA

was treated with T4 DNA polymerase. Plasmid pECC1 (Youngman *et al.*, 1984, *Plasmid* 12: 1-9) was digested with *Sma*I. A fragment of approximately 5,100 bp from pMRT084 and a fragment of approximately 1,600 bp fragment from pECC1 were isolated from a 0.8% agarose-0.5X TBE gel using the QIAquick DNA Extraction Kit according to the manufacturer's instructions, ligated together, and transformed into *E. coli* XL1 Blue cells according to the manufacturer's instructions (Stratagene, Inc., La Jolla, CA). Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml. Transformants carrying the correct plasmid with most of the *cypX-yvmC* and *yvmB-yvmA* operons deleted were identified by PCR amplification using primers 52 and 53. PCR amplification was conducted in 50 µl reactions composed of 1 ng of plasmid DNA, 0.4 µM of each primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM MgCl₂, and 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 10 minutes; 25 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 7 minutes. The PCR product was visualized using a 0.8% agarose-0.5X TBE gel. This construct was designated pMRT086 (Figure 20).

Primer 52: 5'-TAGACAATTGGAAGAGAAAAGAGATA-3' (SEQ ID NO: 66)

Primer 53: 5'-CCGTCGCTATTGTAACCAGT-3' (SEQ ID NO: 67)

Plasmid pMRT086 was linearized with *Sca*I and transformed into *Bacillus subtilis* RB128 competent cells in the presence of 0.2 µg of chloramphenicol per ml. Transformants were selected on TBAB plates containing 5 µg of chloramphenicol per ml after incubation at 37°C for 16 hours. Chromosomal DNA was prepared from several transformants using a QIAGEN tip-20 column according to the manufacturer's instructions. Chloramphenicol resistant colonies were screened by PCR for deletion of the *cypX-yvmC* and *yvmB-yvmA* operons via PCR using primers 36 and 52, 36 and 53, 37 and 52, and 37 and 53. PCR amplification was conducted in 50 µl reactions composed of 50 ng of chromosomal DNA, 0.4 µM of each primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM MgCl₂, and 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 10 minutes; 25 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 7 minutes. The PCR products were visualized using a 0.8% agarose-0.5X TBE gel. The resulting *Bacillus subtilis* RB128 *cypX-yvmC* and *yvmB-yvmA* deleted strain was designated *Bacillus subtilis* MaTa17.

Competent cells of *Bacillus subtilis* RB187 (Example 9) were transformed with genomic DNA from *Bacillus subtilis* MaTa17. Genomic DNA was obtained from this

strain using a QIAGEN tip-20 column according to the manufacturer's instructions. *Bacillus subtilis* chloramphenicol resistant transformants were selected on TBAB plates supplemented with 5 µg of chloramphenicol per ml at 37°C. Primary transformants were streaked for single colony isolations on TBAB plates containing 5 µg of chloramphenicol per ml at 37°C. The resulting *cypX-yvmC* and *yvmB-yvmA* deleted strain was designated *Bacillus subtilis* RB194.

Example 12: Construction of *Bacillus subtilis* RB197

Bacillus subtilis RB197 is very similar to *Bacillus subtilis* RB194, the only difference being that RB197 contains a smaller deletion in the *cypX* region: only a portion of the *cypX* gene is deleted in this strain to generate a *cypX* minus phenotype. In order to accomplish this task a plasmid, pMRT122, was constructed as described below.

Plasmid pCJ791 (Figure 21) was constructed by digestion of plasmid pSJ2739 (WO 96/23073) with *EcoRI/HindIII* and ligation to a fragment containing a deleted form of the *wprA* gene (cell wall serine protease) from *Bacillus subtilis*. The 5' region of *wprA* was amplified using primers 54 and 55 see below, and the 3' region was amplified using primers 56 and 57 shown below from chromosomal DNA obtained from *Bacillus subtilis* DN1885 (Diderichsen *et al.*, 1990, *Journal of Bacteriology* 172: 4315-4321). PCR amplification was conducted in 50 µl reactions composed of 1 ng of *Bacillus subtilis* DN1885 chromosomal DNA, 0.4 µM each of primers 39 and 40, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM MgCl₂, and 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 10 minutes; 25 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 7 minutes.

The 5' and 3' *wprA* PCR fragments were linked by digestion with *BglII* followed by ligation, and PCR amplification was performed on the ligation mixture fragments using primers 54 and 57. PCR amplification was conducted in 50 µl reactions composed of 1 ng of the ligated fragment, 0.4 µM of each primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM MgCl₂, and 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 10 minutes; 25 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 7 minutes. The PCR product was visualized using a 0.8% agarose-0.5X TBE gel. The resulting PCR fragment was cloned into pSJ2739 as an *EcoRI/HindIII* fragment, resulting in plasmid pCJ791 (Figure 21). Transformants were selected on TBAB-agar plates supplemented with 1 µg of erythromycin and 25 µg of kanamycin per ml after incubation

at 28°C for 24-48 hours. Plasmid DNA from several transformants was isolated using QIAGEN tip-20 columns according to the manufacturer's instructions and verified by PCR amplification with primers 54 and 57 using the conditions above.

Primer 54: 5'-GGAATTCCAAAGCTGCAGCGGCCGGCGCG-3' (SEQ ID NO: 68)

5 Primer 55: 5'-GAAGATCTCGTATACTTGGCTTCTGCAGCTGC-3' (SEQ ID NO: 69)

Primer 56: 5'-GAAGATCTGGTCAACAAGCTGGAAAGCACTC-3' (SEQ ID NO: 70)

Primer 57: 5'-CCCAAGCTTCGTGACGTACAGCACCGTTCCGGC-3' (SEQ ID NO: 71)

The *amyL* upstream sequence and 5' coding region from plasmid pDN1981 (U.S. Patent No. 5,698,415) were fused together by SOE using the primer pairs 58/59 and
 10 60/61 shown below. The resulting fragment was cloned into vector pCR2.1 to generate plasmid pMRT032 as follows. PCR amplifications were conducted in triplicate in 50 µl reactions composed of 1 ng of pDN1981 DNA, 0.4 µM each of appropriate primers, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM MgCl₂, and 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a
 15 RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 9 minutes; 3 cycles each at 95°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute; 27 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 5 minutes. The PCR product was visualized in a 0.8% agarose-0.5X TBE gel. The expected fragments were approximately 530 and 466 bp, respectively. The final SOE
 20 fragment was generated using primer pair 59/60 and cloned into pCR2.1 vector using the TA-TOPO Cloning Kit. Transformants were selected on 2X YT agar plates supplemented with 100 µg/ml ampicillin after incubation at 37°C for 16 hours. Plasmid DNA from several transformants was isolated using QIAGEN tip-20 columns according to the manufacturer's instructions and verified by DNA sequencing with M13 (-20) forward and M13 reverse primers. The plasmid harboring the *amyL* upstream
 25 sequence/5'coding sequence fusion fragment was designated pMRT032 (Figure 22).

Primer 58:

5'-CCTTAAGGGCCGAATATTTATACGGAGCTCCCTGAAACAACAAAAACGGC-3'
 (SEQ ID NO: 72)

30 Primer 59: 5'-GGTGTCTCTAGAGCGGCCGCGGTTGCGGTCAGC-3' (SEQ ID NO: 73)

Primer 60: 5'-GTCCTTCTTGGTACCTGGAAGCAGAGC-3' (SEQ ID NO: 74)

Primer 61: 5'-GTATAAATATTCGGCCCTTAAGGCCAGTACCATTTC-3' (SEQ ID NO: 75)

35 Plasmid pNNB194 (pSK⁺/pE194; U.S. Patent No. 5,958,728) was digested with *NsiI* and *NotI*, and plasmid pBEST501 (Itaya *et al.* 1989 *Nucleic Acids Research* 17: 4410) was digested with *PstI* and *NotI*. The 5,193 bp vector fragment from pNNB194

and the 1,306 bp fragment bearing the *neo* gene from pBEST501 were isolated from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Purification Kit according to the manufacturer's instructions. The isolated fragments were ligated together and used to transform *E. coli* SURE competent cells according to the manufacturer's instructions. Ampicillin-resistant transformants were selected on 2X YT plates supplemented with 100 µg of ampicillin per ml. Plasmid DNA was isolated from one such transformant using the QIAGEN Plasmid Kit (QIAGEN Inc., Valencia, CA), and the plasmid was verified by digestion with *Nsi*I and *Not*I. This plasmid was designated pNNB194neo (Figure 23).

Plasmid pNNB194neo was digested with *Sac*I/*Not*I and treated with T4 DNA polymerase and dNTPs to generate blunt ends using standard protocols. Plasmid pPL2419 (U.S. Patent No. 5,958,728) was digested with *Ecl*136II. The 6,478 bp vector fragment from pNNB194neo and the 562 bp fragment bearing *oriT* from pPL2419 were isolated from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Purification Kit according to the manufacturer's instructions. The gel-purified fragments were ligated together and used to transform *E. coli* SURE cells according to the manufacturer's instructions. Ampicillin-resistant transformants were selected on 2X YT plates supplemented with 100 µg of ampicillin per ml at 37°C. Plasmid DNA was isolated from one such transformant using the QIAGEN Plasmid Kit, and the plasmid was verified by digestion with *Nsi*I, *Sac*I, and *Bsc*I. This plasmid was designated pNNB194neo-oriT (Figure 24).

Plasmid pNNB194neo-oriT was digested with *Bam*HI and treated with T4 DNA polymerase and dNTPs to generate blunt ends using standard protocols. The digested plasmid was gel-purified from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Purification Kit according to the manufacturer's instructions. The purified plasmid was treated with T4 DNA ligase and used to transform *E. coli* SURE cells according to the manufacturer's instructions. Ampicillin-resistant transformants were selected on 2X YT plates supplemented with 100 µg of ampicillin per ml at 37°C. Plasmid DNA was isolated from one such transformant using the QIAGEN Plasmid Kit, and disruption of the *Bam*HI site was confirmed by digestion with *Bam*HI and *Sca*I. The resulting plasmid was designated pShV3 (Figure 25).

Plasmid pShV2.1-amyEΔ (U.S. Patent No. 5,958,728) was digested with *Sfi*I and *Not*I, and the 8696 bp vector fragment was gel-purified from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Purification Kit according to the manufacturer's instructions. In order to insert a *Bam*HI site between the *Sfi*I and *Not*I sites of pShV2.1-amyEΔ, a synthetic linker was constructed as follows: primers 62 and 63 were annealed by mixing 50 µM of each, boiling the mixture, and allowing the mixture to cool slowly. Primer 62: 5'-GGGCCGGATCCGC-3' (SEQ ID NO: 76)

Primer 63: 3'-ATTCCCGGCCTAGGCGCCGG-5' (SEQ ID NO: 77)

The purified pShV2.1-amyEΔ vector and annealed oligonucleotides were ligated together and used to transform *E. coli* SURE competent cells according to the manufacturer's instructions. Chloramphenicol-resistant transformants were selected on
 5 LB plates supplemented with 30 μg of chloramphenicol per ml at 37°C. Plasmid DNA was isolated from one such transformant using the QIAGEN Plasmid Kit, and insertion of the *Bam*HI site was confirmed by digestion with *Bam*HI. This plasmid was designated pShV2.1-amyEΔB (Figure 26).

Plasmids pShV3 and pShV2.1-amyEΔB were digested with *Sall*/*Hind*III. A 7033
 10 bp vector fragment from pShV3 and a 1031 bp fragment bearing *amyE*Δ from pShV2.1-amyEΔ were gel-purified from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Purification Kit according to the manufacturer's instructions. The gel-purified fragments were ligated together and used to transform *E. coli* SURE cells according to the manufacturer's instructions. Ampicillin-resistant transformants were selected on 2X YT
 15 plates supplemented with 100 μg of ampicillin per ml. Plasmid DNA was isolated from one such transformant using the QIAGEN Plasmid Kit, and the plasmid was verified by digestion with *Sall* and *Hind*III. This plasmid was designated pShV3A (Figure 27).

Plasmid pMRT032 was digested with *Kpn*I/*Xba*I, filled with Klenow fragment DNA polymerase in the presence of dNTPs, and a fragment of approximately 1000 bp
 20 was isolated from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Purification Kit according to the manufacturer's instructions. This fragment was cloned into plasmid pShV3a digested with *Eco*RV, and transformed into *E. coli* XL1 Blue cells according to the manufacturer's instructions. Transformants were selected on 2X YT agar plates supplemented with 100 μg of ampicillin per ml after incubation at 37°C for 16 hours.
 25 Plasmid DNA from several transformants was isolated using QIAGEN tip-20 columns according to the manufacturer's instructions and verified on a 0.8% agarose-0.5X TBE gel by restriction analysis with *Sac*I/*Sph*I. The resulting plasmid was designated pMRT036 (Figure 28).

Plasmid pMRT036 was digested with *Sall*/*Hind*III, filled with Klenow fragment
 30 DNA polymerase in the presence of dNTPs, ligated and transformed into *E. coli* XL1 Blue cells according to the manufacturer's instructions. Transformants were selected on 2X YT-agar plates supplemented with 100 μg/ml ampicillin after incubation at 37°C for 16 hours. Plasmid DNA from several transformants was isolated using QIAGEN tip-20 columns according to the manufacturer's instructions and verified on a 0.8% agarose-
 35 0.5X TBE gel by restriction analysis with *Sac*I/*Xba*I, *Pst*I and *Nde*I. The resulting plasmid was designated pMRT037 (Figure 29).

The scBAN/*cry*IIIA stabilizer fragment from plasmid pDG268Δneo-

cryIIIAstab/Sav (U.S. Patent No. 5,955,310) was isolated from a 2% agarose-0.5X TBE gel as a *SfiI/SacI* fragment using a QIAquick DNA Purification Kit according to the manufacturer's instructions, ligated to plasmid pMRT037 digested with *SfiI/SacI*, and transformed into *E. coli* XL1 Blue cells. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml after incubation at 37°C for 16 hours. Plasmid DNA from several transformants was isolated using QIAGEN tip-20 columns according to the manufacturer's instructions and verified on a 0.8% agarose-0.5X TBE gel by restriction analysis with *PstI*. The resulting plasmid was designated pMRT041 (Figure 30).

Plasmids pMRT041 and pCJ791 were digested with *EcoRI/HindIII*. A fragment of approximately 1300 bp from pMRT041 and a fragment of approximately 4500 bp from pCJ791 were isolated from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Purification Kit according to the manufacturer's instructions, ligated, and transformed into *Bacillus subtilis* 168Δ4 competent cells. Transformants were selected on TBAB-agar plates supplemented with 1 µg of erythromycin and 25 µg of lincomycin per ml after incubation at 30°C for 24-48 hours. Plasmid DNA from several transformants was isolated using QIAGEN tip-20 columns according to the manufacturer's instructions and verified on a 0.8% agarose-0.5X TBE gel by restriction analysis with *SacI* and *EcoRI/HindIII*. The resulting plasmid was designated pMRT064.1 (Figure 31).

The *SacI* site at position 2666 in plasmid pMRT064.1 was deleted by SOE using primer pairs 64 and 65, and primer pairs 66 and 67 shown below. PCR amplification was conducted in 50 µl reactions composed of 1 ng of pMRT064.1 DNA, 0.4 µM of each primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM MgCl₂, and 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a RoboCycler 40 thermocycler programmed for 1 cycle at 95°C for 10 minutes; 25 cycles each at 95°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 7 minutes. The PCR product was visualized in a 0.8% agarose-0.5X TBE gel. The expected fragments were approximately 400 and 800 bp, respectively. The final fragment for cloning back into pMRT064.1 was amplified using primers 64 and 67. This fragment was cloned into pCR2.1 vector using the TA-TOPO Cloning Kit. Transformants were selected on 2X YT agar plates supplemented with 100 µg/ml ampicillin after incubation at 37°C for 16 hours. Transformants carrying the correct plasmid were verified by DNA sequencing using M13 forward and reverse primers, and primers 65, 67, and 68. This plasmid was designated pMRT068 (Figure 32), and was further transformed into *E. coli* DM1 cells (Stratagene, Inc., La Jolla, CA) according to the manufacturer's instructions. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml.

Primer 64: 5'-GGAAATTATCGTGATCAAC-3' (SEQ ID NO: 78)

Primer 65: 5'-GCACGAGCACTGATAAATATG-3' (SEQ ID NO: 79)

Primer 66: 5'-CATATTTATCAGTGCTCGTGC -3' (SEQ ID NO: 80)

Primer 67: 5'-TCGTAGACCTCATATGC-3' (SEQ ID NO: 81)

5 Primer 68: 5'-GTCGTAAACCGTGTGC-3' (SEQ ID NO: 82)

The *SacI* sites at positions 5463 and 6025 in plasmid pMRT064.1 were deleted using PCR amplification with primers 69 and 70, and using the PCR conditions described above. The resulting fragment was cloned into pCR2.1 vector using the TA-TOPO Cloning Kit (Invitrogen, Inc., Carlsbad, CA). Transformants were selected on 2X
10 YT-agar plates supplemented with 100 µg of ampicillin per ml after incubation at 37°C for 16 hours. Transformants carrying the correct plasmid were verified by DNA sequencing using M13 forward and reverse primers. This construct was designated pMRT069 (Figure 33).

Primer 69: 5'-CTAGAGGATCCCCGGGTACCGTGCTCTGCCTTTTAGTCC-3' (SEQ ID
15 NO: 83)

Primer 70: 5'-GTACATCGAATTCGTGCTCATTATTAATCTGTTCAGC-3' (SEQ ID NO:
84)

Plasmids pMRT068 and pMRT064.1 were digested with *BclI/AccI*. A fragment of approximately 1300 bp from pMRT068 and a fragment of approximately 3800 bp from
20 pMRT064.1 were isolated from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Purification Kit according to the manufacturer's instructions, ligated, and transformed into *Bacillus subtilis* 168Δ4 competent cells. Transformants were selected on TBAB-agar plates supplemented with 1 µg of erythromycin and 25 µg of lincomycin per ml after incubation at 30°C for 24-48 hours. Transformants carrying the correct plasmid were
25 identified on a 0.8% agarose-0.5X TBE gel by restriction analysis with *SacI* and *EcoRI/AvaI*. The resulting construct was designated pMRT071 (Figure 34).

Plasmids pMRT071 and pMRT069 were digested with *AvaI/EcoRI*. The 578 bp fragment from pMRT069 and the 4510 bp fragment from pMRT071 were isolated from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Purification Kit according to the
30 manufacturer's instructions, ligated, and transformed into *Bacillus subtilis* 168Δ4 competent cells. Transformants were selected on TBAB-agar plates supplemented with 1 µg of erythromycin and 25 µg of lincomycin per ml after incubation at 30°C for 24-48 hours. Transformants carrying the correct plasmid were identified on a 0.8% agarose-0.5X TBE gel by restriction analysis with *SacI*. The resulting construct was designated
35 pMRT074 (Figure 35).

Plasmid pMRT084 described in Example 11 was digested with *SacII/NdeI*, treated with T4 DNA polymerase, ligated, and transformed into *E. coli* XL1 Blue cells

according to the manufacturer's instructions. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml after incubation at 37°C for 16 hours. Transformants carrying the correct plasmid were identified on a 0.8% agarose-0.5X TBE gel by restriction analysis with *DraI*. The resulting plasmid was named
5 pMRT120 (Figure 36).

Plasmid pMRT074 was digested with *HindIII*, treated with Klenow fragment DNA polymerase, and digested with *EcoRI*. Plasmid pMRT120 was digested with *EcoRI/Ec/136II*. A fragment of approximately 600 bp from pMRT120 and a fragment of approximately 4300 bp from pMRT074 were isolated from a 0.8% agarose-0.5X TBE gel
10 using a QIAquick DNA Purification Kit according to the manufacturer's instructions, ligated, and transformed into *Bacillus subtilis* 168Δ4 competent cells. Transformants were selected on TBAB-agar plates supplemented with 1 µg of erythromycin and 25 µg of lincomycin per ml after incubation at 30°C for 24-48 hours. Transformants carrying the correct plasmid were identified on a 0.8% agarose-0.5X TBE gel by restriction
15 analysis with *SspI*. The resulting construct was designated pMRT122 (Figure 37).

Plasmid pMRT122 was transformed into *Bacillus subtilis* A164Δ5 competent cells. Transformants were selected on TBAB-agar plates supplemented with 1 µg of erythromycin and 25 µg of lincomycin per ml after incubation at 30°C for 24-48 hours. The plasmid was introduced into the chromosome of *Bacillus subtilis* A164Δ5 via
20 homologous recombination into the *cypX* locus by incubating a freshly streaked plate of *Bacillus subtilis* A164Δ5 (pMRT086) cells at 45°C for 16 hours and selecting for healthy growing colonies. Genomic DNA was isolated from this strain using a QIAGEN tip-20 column according to the manufacturer's instructions and used to transform *Bacillus subtilis* RB187 (Example 9). Transformants were selected on TBAB plates
25 supplemented with 1 µg of erythromycin and 25 µg of lincomycin per ml after incubation at 45°C for 16 hours. At this temperature, the pE194 replicon is unable to replicate. Cells are able to maintain erythromycin resistance only by maintaining the plasmid in the bacterial chromosome.

The plasmid was removed from the chromosome via homologous recombination
30 resulting in the deletion of a portion of the *cypX* gene on the chromosome by growing the transformants in Luria-Bertani (LB) medium without selection at the permissive temperature of 34°C for many generations. At this temperature the pE194 origin of replication is active and actually promotes the excision of the plasmid from the chromosome (*Molecular Biological Methods for Bacillus*, edited by C.R. Harwood and
35 S.M. Cutting, 1990, John Wiley and Sons Ltd.).

After several generations of outgrowth the cells were plated on non-selective LB agar plates and colonies which had lost the plasmid and were now *cypX*-deleted and

producing hyaluronic acid were identified as follows: (1) cell patches were "wet" when plated on minimal plates indicating production of hyaluronic acid, (2) erythromycin sensitivity indicated loss of the pE194-based plasmid, and (3) PCR confirmed the presence of the 800 bp *cypX* deletion in the strain of interest by using primers 34 and 45.

Chromosomal DNA from potential *cypX* deletants was isolated using the REDextract-N-Amp™ Plant PCR kits as follows: Single *Bacillus* colonies were inoculated into 100 µl of Extraction Solution, incubated at 95°C for 10 minutes, and then diluted with an equal volume of Dilution Solution. PCR was performed using 4 µl of extracted DNA in conjunction with the REDextract-N-Amp™ PCR Reaction Mix and the desired primers according to the manufacturer's instructions, using PCR cycling conditions as described in Example 5. PCR reaction products were visualized using a 0.8% agarose-0.5X TBE gel. The verified strain was designated *Bacillus subtilis* RB197.

Example 13: Construction of *Bacillus subtilis* RB200

The *cypX* gene of *Bacillus subtilis* RB192 was deleted using the same methods described in Example 9 for *Bacillus subtilis* RB187. The resultant strain was designated *Bacillus subtilis* RB200.

Example 14: Construction of *Bacillus subtilis* RB202

Bacillus subtilis A164Δ5Δ*cypX* was constructed as follows: Plasmid pMRT122 (Example 12) was transformed into *Bacillus subtilis* A164Δ5 competent cells. Transformants were selected on TBAB-agar plates supplemented with 1 µg of erythromycin and 25 µg of lincomycin per ml after incubation at 30°C for 24-48 hours. The plasmid was introduced into the chromosome of *Bacillus subtilis* A164Δ5 via homologous recombination into the *cypX* locus by incubating a freshly streaked plate of *Bacillus subtilis* A164Δ5 (pMRT086) cells at 45°C for 16 hours and selecting for healthy growing colonies. The plasmid was removed from the chromosome via homologous recombination resulting in the deletion of a portion of the *cypX* gene on the chromosome by growing the transformants in Luria-Bertani (LB) medium without selection at the permissive temperature of 34°C for many generations. At this temperature the pE194 origin of replication is active and actually promotes the excision of the plasmid from the chromosome (*Molecular Biological Methods for Bacillus*, edited by C.R. Harwood and S.M. Cutting, 1990, John Wiley and Sons Ltd.). After several generations of outgrowth the cells were plated on non-selective LB agar plates and colonies which had lost the plasmid and were now *cypX*-deleted were identified as follows: (1) erythromycin sensitivity indicated loss of the pE194-based plasmid, and (2) PCR confirmed the

presence of the 800 bp *cypX* deletion in the strain of interest by using primers 34 and 45 as described above. The verified strain was designated *Bacillus subtilis* A164 Δ 5 Δ *cypX*.

Bacillus subtilis A164 Δ 5 Δ *cypX* was made competent and transformed with *Bacillus subtilis* TH1 genomic DNA (Example 7) isolated using a QIAGEN tip-20 column according to the manufacturer's instructions. Transformants were selected on TBAB plates containing 5 μ g of chloramphenicol per ml at 37°C. The *Bacillus subtilis* A164 Δ 5 Δ *cypX* *hasA/hasB/hasC/hasD* integrant was identified by its "wet" phenotype and designated *Bacillus subtilis* RB201. The *cat* gene was deleted from *Bacillus subtilis* RB201 using the same method described in Example 9. The resultant strain was designated *Bacillus subtilis* RB202.

Example 15: Construction of *Bacillus subtilis* MF002 (*tuaD/gtaB*)

Plasmid pHA3 (Example 2, Figure 9) was digested with *Asp*718. The digested plasmid was then blunted by first inactivating the restriction enzyme at 85°C for 30 minutes. Blunting was performed by adding 0.5 μ l of 10 mM each dNTPs, 1 μ l of 1 U/ μ l T4 polymerase and incubating at 11°C for 10 minutes. Finally the polymerase was inactivated by incubating the reaction at 75°C for 10 minutes. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's instructions and finally digested with *Not*I. The smallest plasmid fragment of approximately 2522 bp was then gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered DNA insert (*tuaD/gtaB*) was then ligated with the vector DNA described below.

Plasmid pDG268MCS Δ neo/scBAN/Sav (U.S. Patent No. 5,955,310) was digested with *Ecl*136II. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's instructions, and finally digested with *Not*I. The largest plasmid fragment of approximately 6800 bp was gel-purified from a 0.8% agarose-0.5X TBE gel using a QIAquick DNA Gel Extraction Kit according to the manufacturer's instructions.

The recovered vector and DNA insert were ligated using the Rapid DNA Cloning Kit according to the manufacturer's instructions. Prior to transformation in *Bacillus subtilis*, the ligation described above was linearized using *Sca*I to ensure double cross-over integration in the chromosome rather than single cross-over integration in the chromosome. *Bacillus subtilis* 168 Δ 4 competent cells were transformed with the ligation digested with the restriction enzyme *Sca*I.

Bacillus subtilis chloramphenicol-resistant transformants were selected on TBAB plates supplemented with 5 μ g of chloramphenicol per ml. To screen for integration of

the plasmid by double cross-over at the *amyE* locus, *Bacillus subtilis* primary transformants were patched on TBAB plates supplemented with 6 µg of neomycin per ml and on TBAB plates supplemented with 5 µg of chloramphenicol per ml to isolate chloramphenicol resistant and neomycin sensitive transformants were isolated.

5 Chromosomal DNA from chloramphenicol resistant and neomycin sensitive *Bacillus subtilis* 168Δ4 transformants was isolated using the REDextract-N-Amp™ Plant PCR kits (Sigma Chemical Company, St. Louis, MO) as follows: Single *Bacillus* colonies were inoculated into 100 µl of Extraction Solution, incubated at 95°C for 10 minutes, and then diluted with an equal volume of Dilution Solution. PCR was
10 performed using 4 µl of extracted DNA in conjunction with the REDextract-N-Amp PCR Reaction Mix and the desired primers according to the manufacturer's instructions, with PCR cycling conditions described in Example 5.

PCR amplifications were performed on these transformants using the synthetic oligonucleotides described below to confirm the absence/presence and integrity of the
15 genes *hasA*, *gtaB*, and *tuaD* of the operon of the *Bacillus subtilis* transformants. Primers 3 and 8 were used to confirm the absence of the *hasA* gene, primer 71 and primer 15 to confirm the presence of the *tuaD* gene, and primers 20 and 71 to confirm the presence of the *gtaB* gene. PCR reaction products were visualized in a 0.8% agarose-0.5X TBE gel. The verified strain, a *Bacillus subtilis* 168Δ4 *hasA/tuaD/gtaB*
20 integrant, was designated *Bacillus subtilis* RB176.

Primer 71: 5'-AACTATTGCCGATGATAAGC-3' (binds upstream of *tuaD*) (SEQ ID NO: 85)

Genomic DNA was isolated from the chloramphenicol resistant, and neomycin sensitive *Bacillus subtilis* RB176 transformants using a QIAGEN tip-20 column
25 according to the manufacturer's instructions. The *Bacillus subtilis* RB176 genomic DNA was used to transform competent *Bacillus subtilis* A164Δ5. Transformants were selected on TBAB plates containing 5 µg of chloramphenicol per ml, and grown at 37°C. A *Bacillus subtilis* A164Δ5 *tuaD/gtaB* integrant was designated *Bacillus subtilis* RB177.

The *cat* gene was deleted in strain *Bacillus subtilis* RB177 using the method
30 described in Example 9. The resultant strain was designated *Bacillus subtilis* MF002.

Example 16: Construction of the *pel* integration plasmid pRB162

Plasmid pDG268MCSΔneo/scBAN/Sav (U.S. Patent No. 5,955,310) was double-digested with *SacI* and *AatII*. The largest plasmid fragment of approximately 6193bp
35 was gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered vector DNA was then ligated with the DNA insert described below.

The 5' and 3' fragments of a *Bacillus subtilis* pectate lyase gene (*pel*, accession number BG10840, SEQ ID NOs. 86 [DNA sequence] and 87 [deduced amino acid sequence]) was PCR amplified from *Bacillus subtilis* 168 (BGSC 1A1, Bacillus Genetic Stock Center, Columbus, OH) using primers 72 (introduces 5' *SpeI* restriction site) and 73 (introduces 3' *SacI* restriction site) for the 5' *pel* fragment and primers 74 (introduces 5' *SacI/BamHI* restriction sites) and 75 (introduces 3' *NotI/AatII* restriction sites) for the 3' *pel* fragment:

Primer 72:

5'-ACTAGTAATGATGGCTGGGGCGCGTA-3' (SEQ ID NO: 88)

10 Primer 73:

5'-GTCGACATGTTGTCGTATTGTGAGTT-3' (SEQ ID NO: 89)

Primer 74:

5'-GAGCTCTACAACGCTTATGGATCCGCGGCCGCGGCACACACATCTGGAT-3' (SEQ ID NO: 90)

15 Primer 75:

5'-GACGTCAGCCCGTTTGCAGCCGATGC-3' (SEQ ID NO: 91)

PCR amplifications were carried out in triplicate in 30 μ l reactions composed of 50 ng of *Bacillus subtilis* 168 chromosomal DNA, 0.4 μ M each of primer pair 72/73 for the 5' *pel* fragment or primer pair 74/75 for the 3' *pel* fragment, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1X PCR Buffer II with 2.5 mM $MgCl_2$, and 2.5 units of AmpliTaq Gold™ DNA polymerase. The reactions were performed in a RoboCycler 40 thermacycler programmed for 1 cycle at 95°C for 9 minutes; 3 cycles each at 95°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute; 27 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and 1 cycle at 72°C for 5 minutes. The PCR products were visualized using a 0.8% agarose-0.5X TBE gel. The expected fragments were approximately 530 bp for the 5' *pel* fragment and 530 bp for the 3' *pel* fragment.

The 530 bp 5' *pel* and 530 bp 3' *pel* PCR fragments were cloned into pCR2.1 using the TA-TOPO Cloning Kit and transformed into *E. coli* OneShot™ competent cells according to the manufacturers' instructions. Transformants were selected on 2X YT agar plates supplemented with 100 μ g of ampicillin per ml incubated at 37°C. Plasmid DNA from these transformants was purified using a QIAGEN robot according to the manufacturer's instructions and the DNA sequence of the inserts confirmed by DNA sequencing using the primers described above (primers 72 and 73 for 5' *pel* and primers 74 and 75 for 3' *pel*). The plasmids harboring the 530 bp and the 530 bp PCR fragments were designated pCR2.1-*pel* 5' and pCR2.1-*pel*3', respectively (Figures 38 and 39, respectively).

Plasmid pCR2.1-*pel*3' was double-digested with *SacI* and *AatII*. The smallest

plasmid fragment of approximately 530 bp was gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions.

5 The recovered vector (pDG268MCS Δ neo/scBAN) and DNA insert (3' *pel*) were ligated using the Rapid DNA Cloning Kit according to the manufacturer's instructions. The ligation mix was transformed into *E. coli* SURE competent cells (Stratagene, Inc., La Jolla, CA). Transformants were selected on 2X YT agar plates supplemented with 100 μ g of ampicillin per ml at 37°C.

10 Plasmid DNA was purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *Sac*I and *Aat*II digestion on a 0.8% agarose gel using 0.5X TBE buffer. The correct plasmid was identified by the presence of an approximately 530 bp *Sac*I/*Aat*II 3' *pel* fragment and was designated pRB161 (Figure 40).

15 Plasmid pRB161 was double-digested with *Spe*I and *Sal*I. The largest plasmid fragment of approximately 5346 bp was gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered vector DNA was then ligated with the DNA insert described below.

20 Plasmid pCR2.1-*pel*5' was double-digested with *Spe*I and *Sal*I. The smallest plasmid fragment of approximately 530 bp was gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions.

25 The recovered vector (pDG268MCS Δ neo/scBAN/*pel* 3') and insert (*pel* 5') DNA were ligated using the Rapid DNA Cloning Kit according to the manufacturer's instructions. The ligation mix was transformed into *E. coli* SURE competent cells (Stratagene, Inc., La Jolla, CA). Transformants were selected on 2X YT agar plates supplemented with 100 μ g of ampicillin per ml.

30 Plasmid DNA was purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *Spe*I and *Sal*I digestion on a 0.8% agarose gel using 0.5X TBE buffer. The correct plasmid was identified by the presence of an approximately 530 bp *Spe*I/*Sal*I *pel* 5' fragment and was designated pRB162 (Figure 41).

Example 17: Construction of pRB156

35 Plasmid pHA7 (Example 4, Figure 13) was digested with *Hpa*I. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's instructions and finally digested with *Asp*718. The double-digested

plasmid was then blunted by first inactivating the restriction enzyme at 85°C for 30 minutes. Blunting was performed by adding 0.5 µl of 10 mM each dNTPs and 1 µl of 1 U/µl of T4 polymerase and incubating at 11°C for 10 minutes. The polymerase was then inactivated by incubating the reaction at 75°C for 10 minutes. The largest plasmid
5 fragment of approximately 8600 bp was then gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered DNA insert (pDG268Δneo-cryIIIAstab/sehasA) was then re-ligated using the Rapid DNA Cloning Kit according to the manufacturer's instructions.

The ligation mix was transformed into *E. coli* SURE competent cells (Stratagene,
10 Inc., La Jolla, CA). Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml at 37°C. Plasmid DNA was purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *ScaI* digestion on a 0.8% agarose gel using 0.5X TBE buffer. The correct plasmid was identified by the presence of an approximately 8,755 bp fragment and was
15 designated pRB156 (Figure 42).

Example 18: Construction of *Bacillus subtilis* MF009

The *hasA* gene under control of the scBAN promoter was introduced into the pectate lyase gene (*pel*) locus of *Bacillus subtilis* MF002 to generate *Bacillus subtilis*
20 MF009.

Plasmid pRB156 was digested with *SacI*. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's instructions, and finally digested with *NotI*. The smallest plasmid fragment of approximately 1,377 bp was gel-purified using a QIAquick DNA Gel Extraction Kit from a
25 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered DNA insert was then ligated with the vector DNA described below.

Plasmid pRB162 (Example 16, Figure 41) was digested with *NotI*. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's instructions, and finally digested with *SacI*. The largest plasmid
30 fragment of approximately 5850 bp was gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered vector DNA was then ligated with the DNA insert described above.

The ligation mixture was transformed directly in *Bacillus subtilis* 168Δ4
35 competent cells. *Bacillus subtilis* chloramphenicol-resistant transformants were selected on TBAB plates supplemented with 5 µg of chloramphenicol per ml at 37°C. To screen for integration of the plasmid by double cross-over at the *pel* locus, *Bacillus subtilis*

primary transformants were patched on TBAB plates supplemented with 6 µg of neomycin per ml and on TBAB plates supplemented with 5 µg of chloramphenicol per ml. Integration of the plasmid by double cross-over at the *peI* locus does not incorporate the neomycin resistance gene and therefore renders the strain neomycin sensitive.

5 Using this plate screen, chloramphenicol resistant and neomycin sensitive transformants were isolated.

Genomic DNA was isolated from the chloramphenicol resistant and neomycin sensitive *Bacillus subtilis* 168Δ4 transformants using a QIAGEN tip-20 column according to the manufacturer's instructions. This genomic DNA was used to transform competent

10 *Bacillus subtilis* MF002 (Example 15). Transformants were selected on TBAB plates containing 5 µg of chloramphenicol per ml and grown at 37°C. The *Bacillus subtilis* A164Δ5 *hasA* and *tuaD/gtaB* integrant was identified by its "wet" phenotype and designated *Bacillus subtilis* MF009.

15 **Example 19: Construction of *Bacillus subtilis* MF010**

Plasmid pDG268MCSΔneo/BAN/Sav (U.S. Patent No. 5,955,310) was digested with *NotI*. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's instructions, and finally digested with *SfiI*. The smallest plasmid fragment of approximately 185 bp was gel-purified using a QIAquick DNA Gel

20 Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered DNA insert was then ligated with the vector DNA described below.

Plasmid pRB162 (Example 16, Figure 41) was digested with *NotI*. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the

25 manufacturer's instructions, and finally digested with *SfiI*. The largest plasmid fragment of approximately 5747 bp was gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered vector DNA was then ligated with the DNA insert described above.

The recovered vector and DNA insert were ligated using the Rapid DNA Cloning

30 Kit according to the manufacturer's instructions. The ligation mix was transformed into *E. coli* XLI Blue competent cells. Transformants were selected on 2X YT agar plates supplemented with 100 µg of ampicillin per ml.

Plasmid DNA was purified from several transformants using a QIAGEN robot according to the manufacturer's instructions and analyzed by *Bam*HI digestion on a

35 0.8% agarose gel using 0.5X TBE buffer. The correct plasmid was identified by the linearization of the plasmid which provides an approximately 7,156 bp fragment and was designated pRB164 (Figure 43).

Plasmid pRB156 (Example 17, Figure 42) was digested with *SacI*. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's instructions, and finally digested with *NotI*. The smallest plasmid fragment of approximately 1377 bp was gel-purified using a QIAquick DNA Gel
 5 Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered DNA insert was then ligated with the vector DNA described below.

Plasmid pRB164 was digested with *NotI*. The digested plasmid was then purified using a QIAquick DNA Purification Kit according to the manufacturer's
 10 instructions, and finally digested with *SacI*. The largest plasmid fragment of approximately 5922 bp was gel-purified using a QIAquick DNA Gel Extraction Kit from a 0.8% agarose-0.5X TBE gel according to the manufacturer's instructions. The recovered vector DNA was then ligated with the DNA insert described above.

This ligation mix was transformed directly in *Bacillus subtilis* 168 Δ 4 competent
 15 cells. *Bacillus subtilis* chloramphenicol-resistant transformants were selected on TBAB plates supplemented with 5 μ g of chloramphenicol per ml at 37°C. To screen for integration of the plasmid by double cross-over at the *amyE* locus, *Bacillus subtilis* primary transformants were patched on TBAB plates supplemented with 6 μ g of neomycin per ml and on TBAB plates supplemented with 5 μ g of chloramphenicol per
 20 ml. Integration of the plasmid by double cross-over at the *amyE* locus does not incorporate the neomycin resistance gene and therefore renders the strain neomycin sensitive. Using this plate screen, chloramphenicol resistant and neomycin sensitive transformants were isolated.

Genomic DNA was isolated from the chloramphenicol resistant and neomycin
 25 sensitive *Bacillus subtilis* 168 Δ 4 transformants using a QIAGEN tip-20 column according to the manufacturer's instructions. This genomic DNA was used to transform competent *Bacillus subtilis* MF002 (Example 15). Transformants were selected on minimal plates containing 5 μ g of chloramphenicol per ml and grown at 37°C for 16 hours. A *Bacillus subtilis* A164 Δ 5 BAN/*hasA* and scBAN/*tuaD/gtaB* integrant was identified by its "wet"
 30 phenotype and designated *Bacillus subtilis* MF010.

Example 20: Fermentations

The ability of the *Bacillus subtilis* strains listed in Table 1 to produce hyaluronic acid was evaluated under various growth conditions.

35

Table 1

<u><i>B. subtilis</i> Strain</u>	<u>promoter/gene complement</u>	<u><i>catA</i></u>
<i>cvnXA</i>		

	RB161		scBAN/hasA/tuaD/gtaB		no
		no			
	RB163		scBAN/hasA/tuaD/gcaD		no
		no			
5	TH-1		scBANhasA/hasB/hasC/hasD		no
		no			
	RB184		scBAN/hasA/tuaD	no	no
	RB187		scBAN/hasA/tuaD/gtaB		yes
		no			
10	RB192		scBAN/hasA/tuaD	yes	no
	RB194		scBAN/hasA/tuaD/gtaB		yes
		yes			
	RB197		scBAN/hasA/tuaD/gtaB		yes
		yes			
15	RB200		scBAN/hasA/tuaD	yes	yes
	RB202		scBAN/hasA/hasB/hasC/hasD		yes
		yes			
	MF009		scBAN/tuaD/gtaB		no
		no			
20	MF010		scBAN/hasA scBAN/tuaD/gtaB		no
		no			
			BAN/hasA		

25 The *Bacillus subtilis* strains were fermented in standard small fermenters in a medium composed per liter of 6.5 g of KH_2PO_4 , 4.5 g of Na_2HPO_4 , 3.0 g of $(\text{NH}_4)_2\text{SO}_4$, 2.0 g of $\text{Na}_3\text{-citrate}\cdot 2\text{H}_2\text{O}$, 3.0 g of $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 6.0 ml of Mikrosoy-2, 0.15 mg of biotin (1 ml of 0.15 mg/ml ethanol), 15.0 g of sucrose, 1.0 ml of SB 2066, 2.0 ml of P2000, 0.5 g of $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$. The medium was pH 6.3 to 6.4 (unadjusted) prior to autoclaving. The
30 $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ was added after autoclaving.

The seed medium used was B-3, *i.e.*, Agar-3 without agar, or "S/S-1" medium. The Agar-3 medium was composed per liter of 4.0 g of nutrient broth, 7.5 g of hydrolyzed protein, 3.0 g of yeast extract, 1.0 g of glucose, and 2% agar. The pH was not adjusted; pH before autoclaving was approximately 6.8; after autoclaving
35 approximately pH 7.7.

The sucrose/soy seed flask medium (S/S-1) was composed per liter of 65 g of sucrose, 35 g of soy flour, 2 g of $\text{Na}_3\text{-citrate}\cdot 2\text{H}_2\text{O}$, 4 g of KH_2PO_4 , 5 g of Na_2HPO_4 , and 6 ml of trace elements. The medium was adjusted pH to about 7 with NaOH; after dispensing the medium to flasks, 0.2% vegetable oil was added to suppress foaming.
40 Trace elements was composed per liter of 100 g of citric acid- H_2O , 20 g of $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 5 g of $\text{MnSO}_4\cdot \text{H}_2\text{O}$, 2 g of $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, and 2 g of ZnCl_2 .

The pH was adjusted to 6.8 – 7.0 with ammonia before inoculation, and controlled thereafter at $\text{pH } 7.0 \pm 0.2$ with ammonia and H_3PO_4 . The temperature was maintained at 37°C. Agitation was at a maximum of 1300 RPM using two 6-bladed
45 rushton impellers of 6 cm diameter in 3 liter tank with initial volume of 1.5 liters. The

aeration had a maximum of 1.5 VVM.

For feed, a simple sucrose solution was used. Feed started at about 4 hours after inoculation, when dissolved oxygen (D.O.) was still being driven down (*i.e.*, before sucrose depletion). The feed rate was ramped linearly from 0 to approximately 6 g sucrose/ L_0 -hr over a 7 hour time span. A lower feed rate, ramped linearly from 0 to approximately 2 g sucrose/ L_0 -hr, was also used in some fermentations.

Viscosity was noticeable by about 10 hours and by 24 hours viscosity was very high, causing the D.O. to bottom-out. End-point viscosity reached 3,220 cP. Cell mass development reached a near maximum (12 to 15 g/liter) by 20 hours. Cells were removed by diluting 1 part culture with 3 parts water, mixing well and centrifuging at about 30,000 x *g* to produce a clear supernatant and cell pellet, which can be washed and dried.

Assays of hyaluronic acid concentration were performed using the ELISA method, based on a hyaluronan binding protein (protein and kits commercially available from Seikagaku America, Falmouth, MA).

Bacillus subtilis RB 161 and RB163 were cultured in batch and fed-batch fermentations. In the fed-batch processes, the feed rate was varied between cultures of *Bacillus subtilis* strains RB163 and RB161. Assays of hyaluronic acid concentrations were again performed using the ELISA method. The results are provided in Table 2.

Table 2

Strain and Growth Conditions	HA (relative yield) ELISA method
RB-161 (hasA/tuaD/gtaB) simple batch	0.7±0.1
RB-163 (hasA/tuaD/gcaD) fed batch ~ 6 g sucrose/ L_0 -hr	0.9 ± 0.1
RB161 (hasA/tuaD/gtaB) fed batch ~ 6 g sucrose/ L_0 -hr	0.9 ± 0.1
RB-163 (hasA/tuaD/gcaD) fed batch ~ 2 g sucrose/ L_0 -hr	1.0 ± 0.2

RB161 (hasA/tuaD/gtaB) fed batch ~ 2 g sucrose/L ₀ -hr	1.0 ± 0.1
--	-----------

The results of the culture assays for the same strain at a fed batch rate of 2 g/L sucrose/L₀-hr compared to 6 g/L sucrose/L₀-hr demonstrated that a faster sucrose feed rate did not significantly improve titers.

5 A summary of the *Bacillus* strains run under same conditions (fed batch at approximately 2 g sucrose/L₀-hr, 37°C) is shown in Figure 44. In Figure 44, ± values indicate standard deviation of data from multiple runs under the same conditions. Data without ± values are from single runs. Hyaluronic acid concentrations were determined using the modified carbazole method (Bitter and Muir, 1962, *Anal Biochem.* 4: 330-334).

10 A summary of peak hyaluronic acid weight average molecular weights (MDa) obtained from fermentation of the recombinant *Bacillus subtilis* strains under the same conditions (fed batch at approximately 2 g sucrose/L₀-hr, 37°C) is shown in Figure 45. Molecular weights were determined using a GPC MALLS assay. Data was gathered from GPC MALLS assays using the following procedure. GPC-MALLS (gel permeation
15 or size-exclusion) chromatography coupled with multi-angle laser light scattering) is widely used to characterize high molecular weight (MW) polymers. Separation of polymers is achieved by GPC, based on the differential partitioning of molecules of different MW between eluent and resin. The average molecular weight of an individual polymer is determined by MALLS based the differential scattering extent/angle of
20 molecules of different MW. Principles of GPC-MALLS and protocols suited for hyaluronic acid are described by Ueno *et al.*, 1988, *Chem. Pharm. Bull.* 36, 4971-4975; Wyatt, 1993, *Anal. Chim. Acta* 272: 1-40; and Wyatt Technologies, 1999, "Light Scattering University DAWN Course Manual" and "DAWN EOS Manual" Wyatt Technology Corporation, Santa Barbara, California). An Agilent 1100 isocratic HPLC, a
25 Tosoh Biosep G6000 PWxl column for the GPC, and a Wyatt Down EOS for the MALLS were used. An Agilent G1362A refractive index detector was linked downstream from the MALLS for eluate concentration determination. Various commercial hyaluronic acid products with known molecular weights served as standards.

30

Deposit of Biological Material

The following biological material has been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604, and

given the following accession number:

Deposit	Accession Number	Date of Deposit
<i>E. coli</i> XL 10 Gold kan (pMRT106)	NRRL 8-30536	December 12, 2001

5 The deposit represents a substantially pure culture of the deposited strain. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

10

 The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of the appended claims. Indeed, various modifications of the invention
15 in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

WO 03/054163

PCT/US02/41067

Applicant's or agent's file
reference number 10241.204-WOInternational application No
To be assigned PCT/US02/41067

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 *bis*)A. The indications made below relate to the microorganism referred to in the description
on page 72, line 3.

B. IDENTIFICATION OF

Further deposits are identified on an additional sheet ☐0

Name of depository institution

Agricultural Research Service Patent Culture Collection (NRRL)

Address of depository institution (*including postal code and country*)Northern Regional Research Center
1815 University Street
Peoria, IL 61604, US

Date of deposit

December 12, 2001

Accession Number

B-30536

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*)This information is continued on an additional sheet ☐0

In respect of those designations in which a European and/or Australia Patent is sought, during the pendency of the patent application, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC/Regulation 3.25 of Australia Statutory Rule 1991 No. 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indication listed below will be submitted to the International Bureau Later (*specify the general nature of the indications e.g. "Accession Number of Deposit"*)

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Claims

What is claimed is:

1. A method for producing a hyaluronic acid, comprising:

(a) cultivating a *Bacillus* host cell under conditions suitable for production of the hyaluronic acid, wherein the *Bacillus* host cell comprises an artificial operon comprising a short "consensus" *amyQ* promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region operably linked to a hyaluronan synthase encoding sequence, a UDP-glucose 6-dehydrogenase encoding sequence, and a UDP-glucose pyrophosphorylase encoding sequence,

wherein the hyaluronan synthase encoding sequence is (a) a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the full length of SEQ ID NO: 2; or (b) a nucleic acid sequence which hybridizes under high stringency conditions to the complement of SEQ ID NO: 1, wherein high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and 50% formamide and washing three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C;

wherein the UDP-glucose 6-dehydrogenase encoding sequence is (a) a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the full length of SEQ ID NO: 12; or (b) a nucleic acid sequence which hybridizes under high stringency conditions to the complement of SEQ ID NO: 11, wherein high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and 50% formamide and washing three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C ; and

wherein the UDP-glucose pyrophosphorylase encoding sequence is (a) a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the full length of SEQ ID NO: 22; or (b) a nucleic acid sequence which hybridizes under high stringency conditions to the complement of SEQ ID NO: 21, wherein high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and 50% formamide and washing three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C; and

- (b) recovering the hyaluronic acid from the cultivation medium.
2. The method of claim 1, wherein the hyaluronan synthase encoding sequence encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or a polypeptide fragment thereof having hyaluronan synthase activity.
3. The method of claim 1, wherein the UDP-glucose 6-dehydrogenase encoding sequence encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 12 or a polypeptide fragment thereof having UDP-glucose 6-dehydrogenase activity.
4. The method of claim 1, wherein the artificial operon further comprises one or more additional genes encoding enzymes in the biosynthesis of a precursor sugar of the hyaluronic acid or the *Bacillus* host cell further comprises one or more nucleic acid constructs comprising one or more additional genes encoding enzymes in the biosynthesis of a precursor sugar of the hyaluronic acid.
5. The method of claim 4, wherein the one or more additional genes encoding enzymes in the biosynthesis of a precursor sugar of the hyaluronic acid are selected from the group consisting of a UDP-N-acetylglucosamine pyrophosphorylase gene, glucose-6-phosphate isomerase gene, hexokinase gene, phosphoglucomutase gene, amidotransferase gene, mutase gene and acetyl transferase gene.
6. The method of claim 1, wherein the UDP-glucose pyrophosphorylase encoding sequence encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 22 or a polypeptide fragment thereof having UDP-glucose pyrophosphorylase activity.
7. The method of claim 5, wherein the UDP-N-acetylglucosamine phosphorylase encoding sequence is (a) a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the full length of SEQ ID NO: 30; or (b) a nucleic acid sequence which hybridizes under high stringency conditions to the complement of SEQ ID NO: 29, wherein high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and 50% formamide and washing three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C.
8. The method of claim 7, wherein the UDP-N-acetylglucosamine phosphorylase

encoding sequence encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 30 or a polypeptide fragment thereof having UDP-N-acetylglucosamine pyrophosphorylase activity.

9. The method of claim 5, wherein the glucose-6-phosphate isomerase encoding sequence is (a) a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence having at least 95% sequence identity to the full length of SEQ ID NO: 101; or (b) a nucleic acid sequence which hybridizes under high stringency conditions to the complement of SEQ ID NO: 100, wherein high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and 50% formamide and washing three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C.

10. The method of claim 9, wherein the glucose-6-phosphate isomerase encoding sequence encodes a polypeptide comprising the amino acid sequence to SEQ ID NO: 101, or a polypeptide fragment thereof having glucose-6-phosphate isomerase activity.

11. The method of claim 5, wherein the one or more additional genes selected from the group consisting of UDP-N-acetylglucosamine pyrophosphorylase gene, glucose-6-phosphate isomerase gene, hexokinase gene, phosphoglucosmutase gene, amidotransferase gene, mutase gene and acetyl transferase gene are under the control of the same or a different promoter(s) as the hyaluronan synthase encoding sequence.

12. The method of claim 1, wherein the artificial operon further comprises an mRNA processing/stabilizing sequence located downstream of the short "consensus" *amyQ* promoter operably linked to the hyaluronan synthase encoding sequence, the UDP-glucose 6-dehydrogenase encoding sequence, and the UDP-glucose pyrophosphorylase encoding sequence and upstream of the hyaluronan synthase encoding sequence, the UDP-glucose 6-dehydrogenase encoding sequence, and the UDP-glucose pyrophosphorylase encoding sequence.

13. The method of claim 1, wherein the artificial operon further comprises a selectable marker gene.

14. The method of claim 1, wherein the *Bacillus* host cell is selected from the group consisting of *Bacillus agaradherens*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*,

Bacillus brevis, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis* and *Bacillus thuringiensis*.

15. The method of claim 1, wherein the *Bacillus* host cell is unmarked with a selectable marker.

16. The method of claim 1, wherein the artificial operon is integrated into the chromosome of the *Bacillus* host cell.

17. The method of claim 1, wherein the *Bacillus* host cell is a *Bacillus licheniformis* cell.

18. The method of claim 1, wherein the *Bacillus* host cell is a *Bacillus subtilis* cell.

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Hyaluronic acid

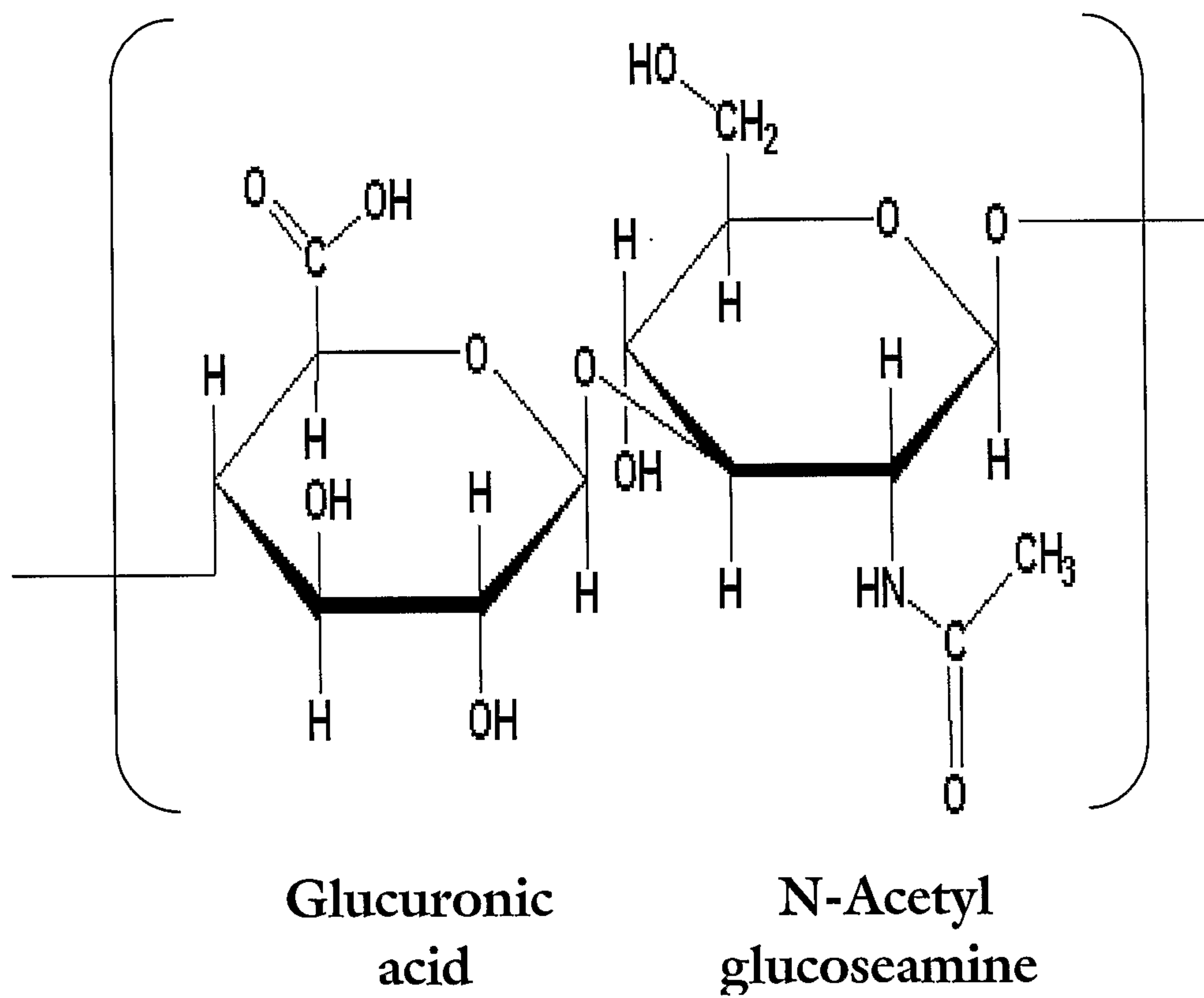


Fig. 1

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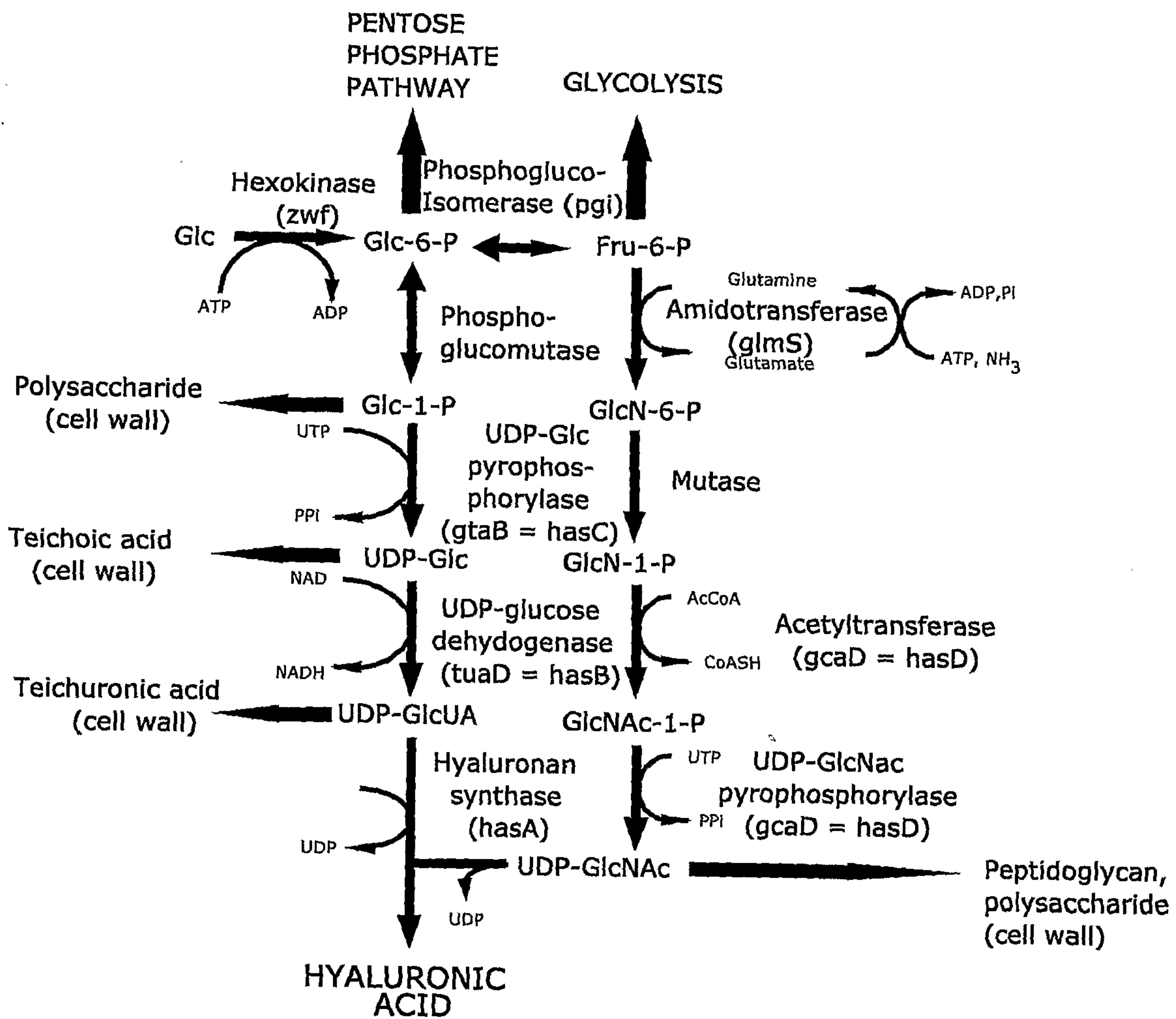


Fig. 2

3/45

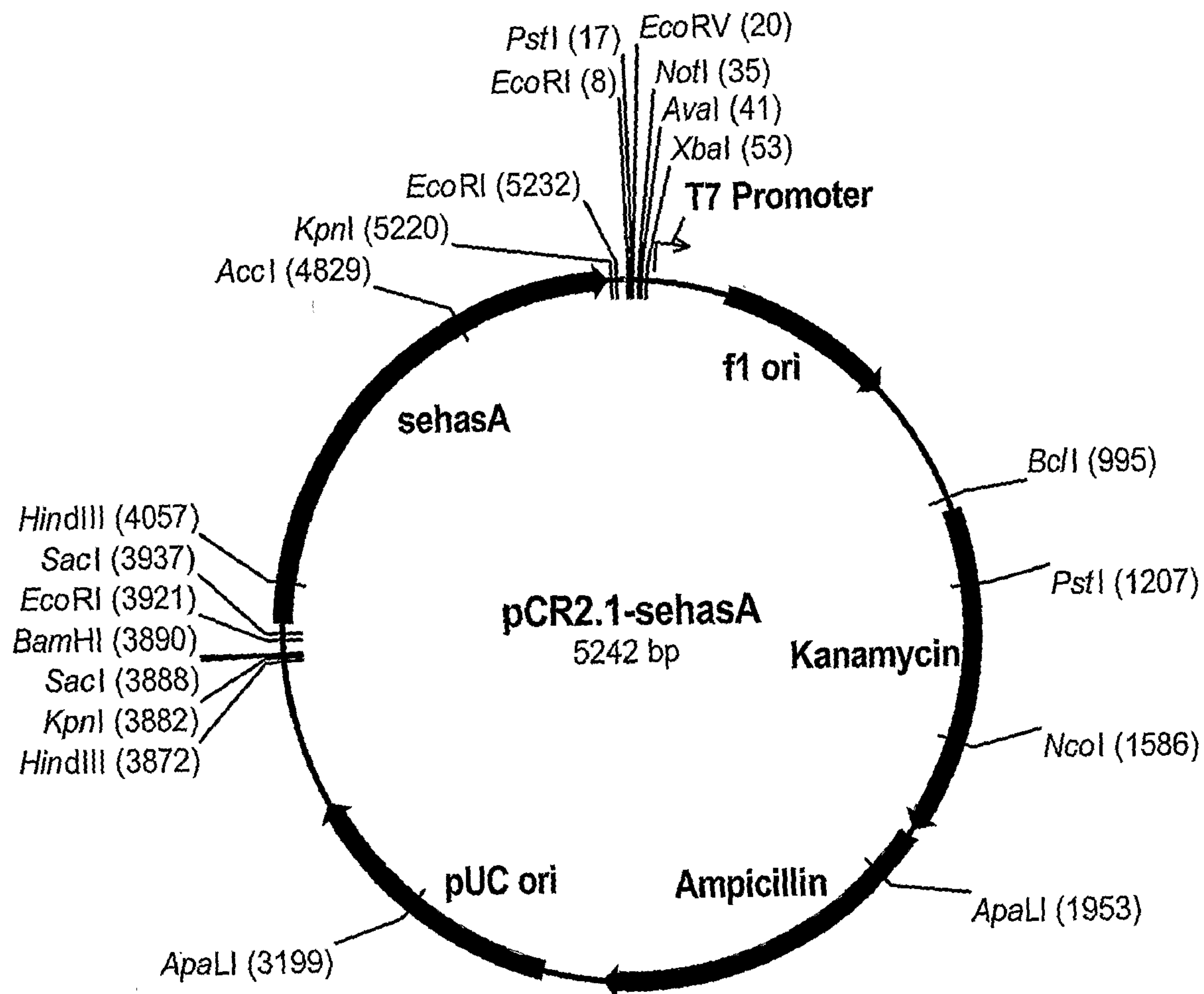


Fig. 3

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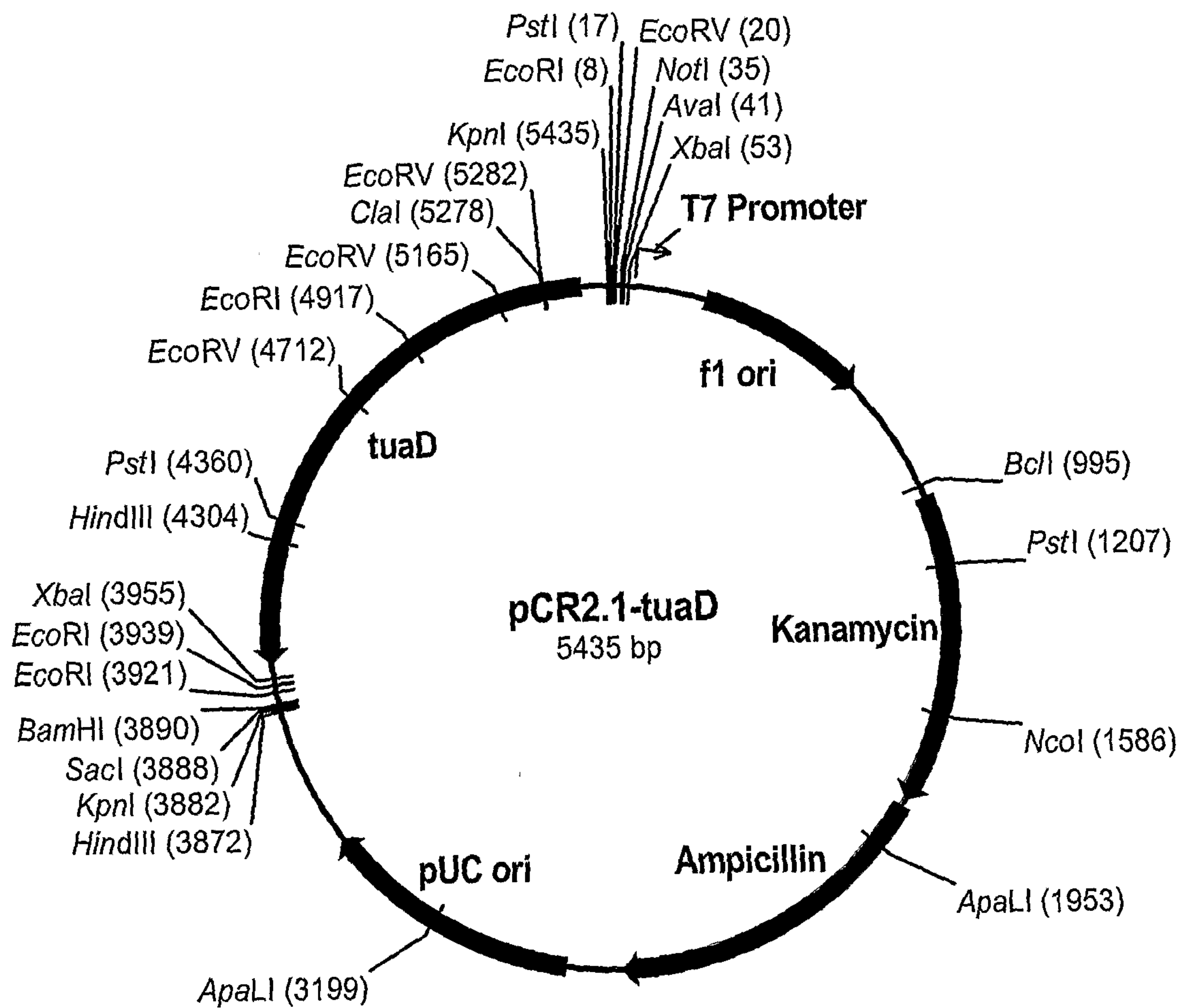


Fig. 4

5/45

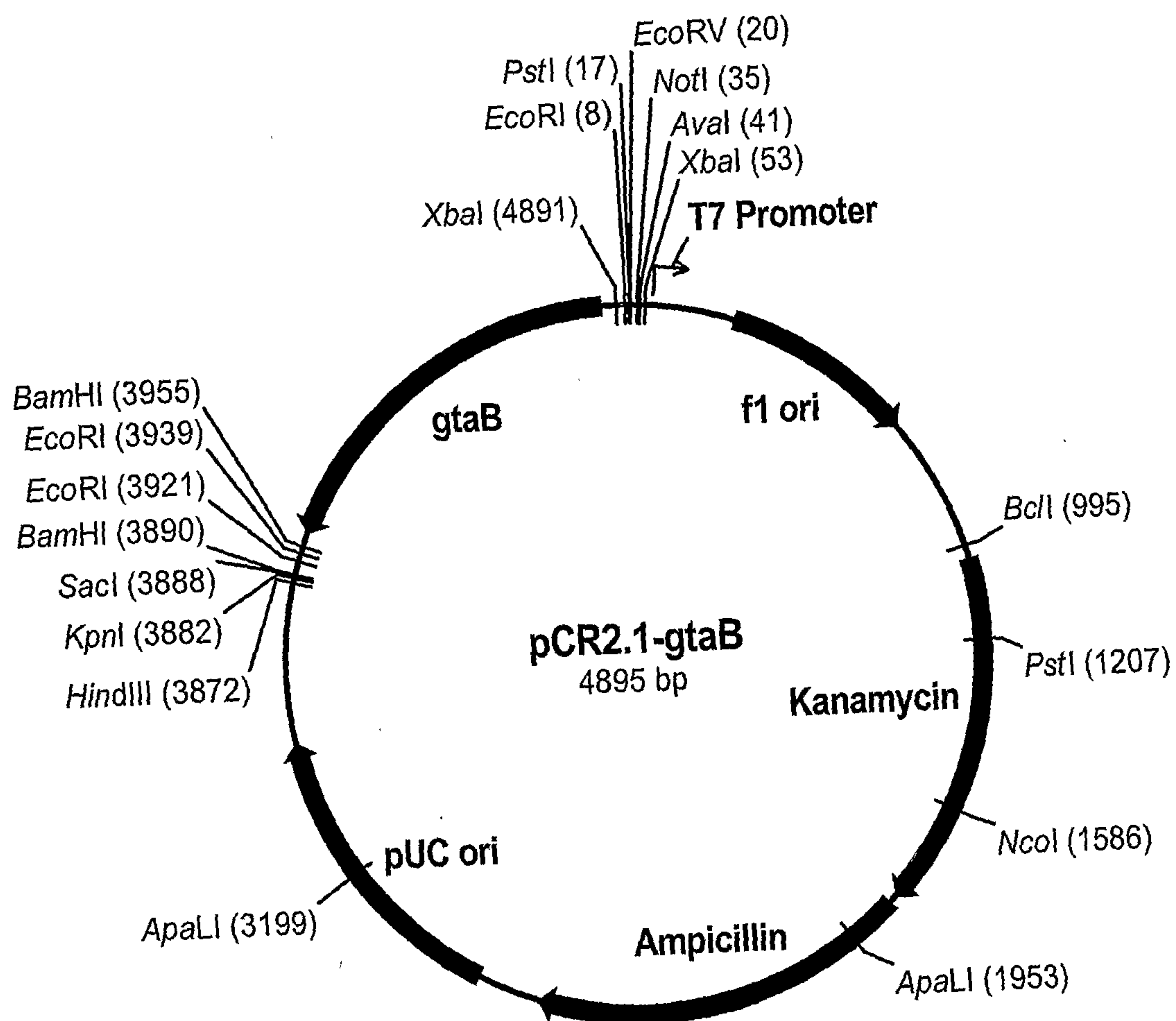


Fig. 5

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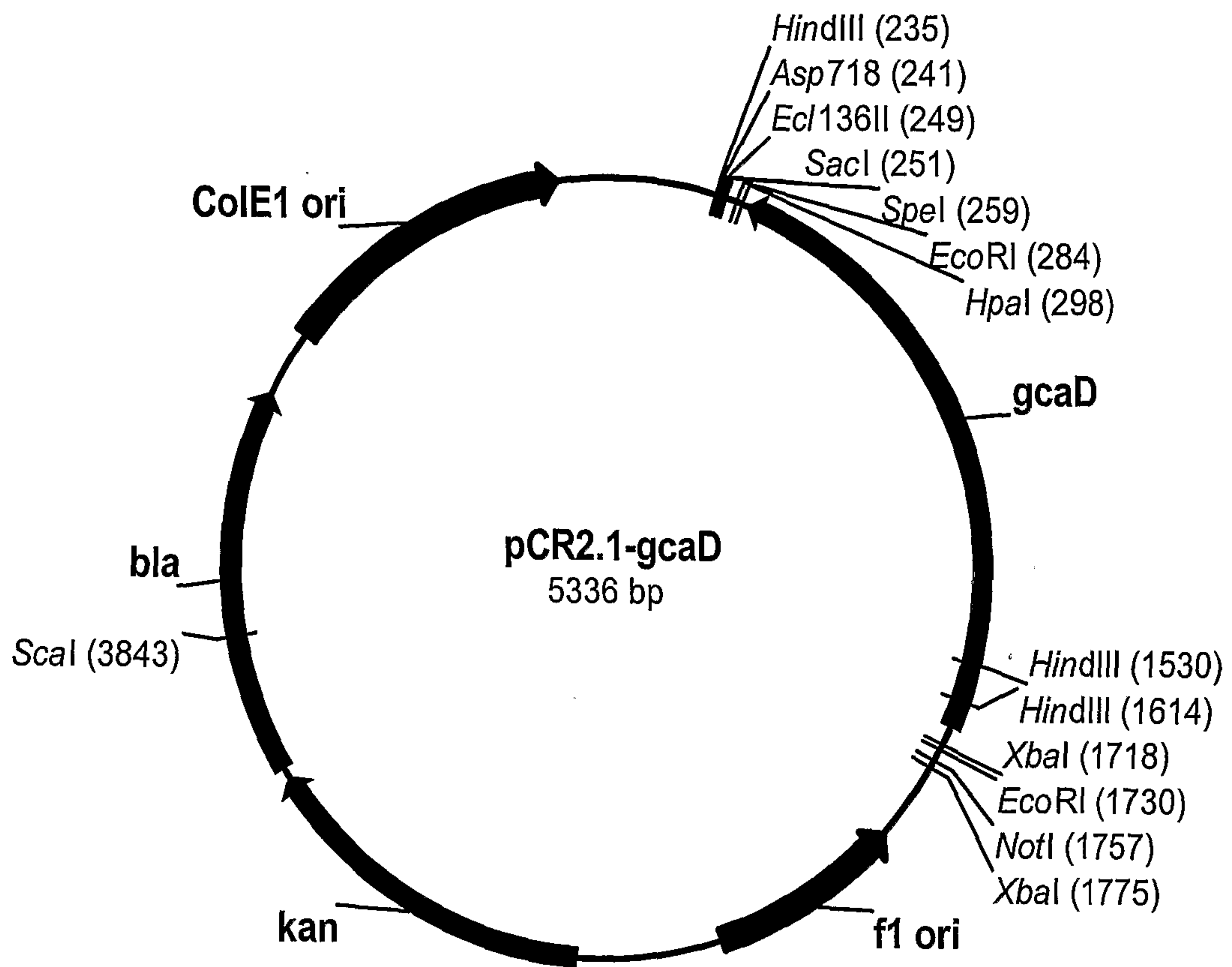


Fig. 6

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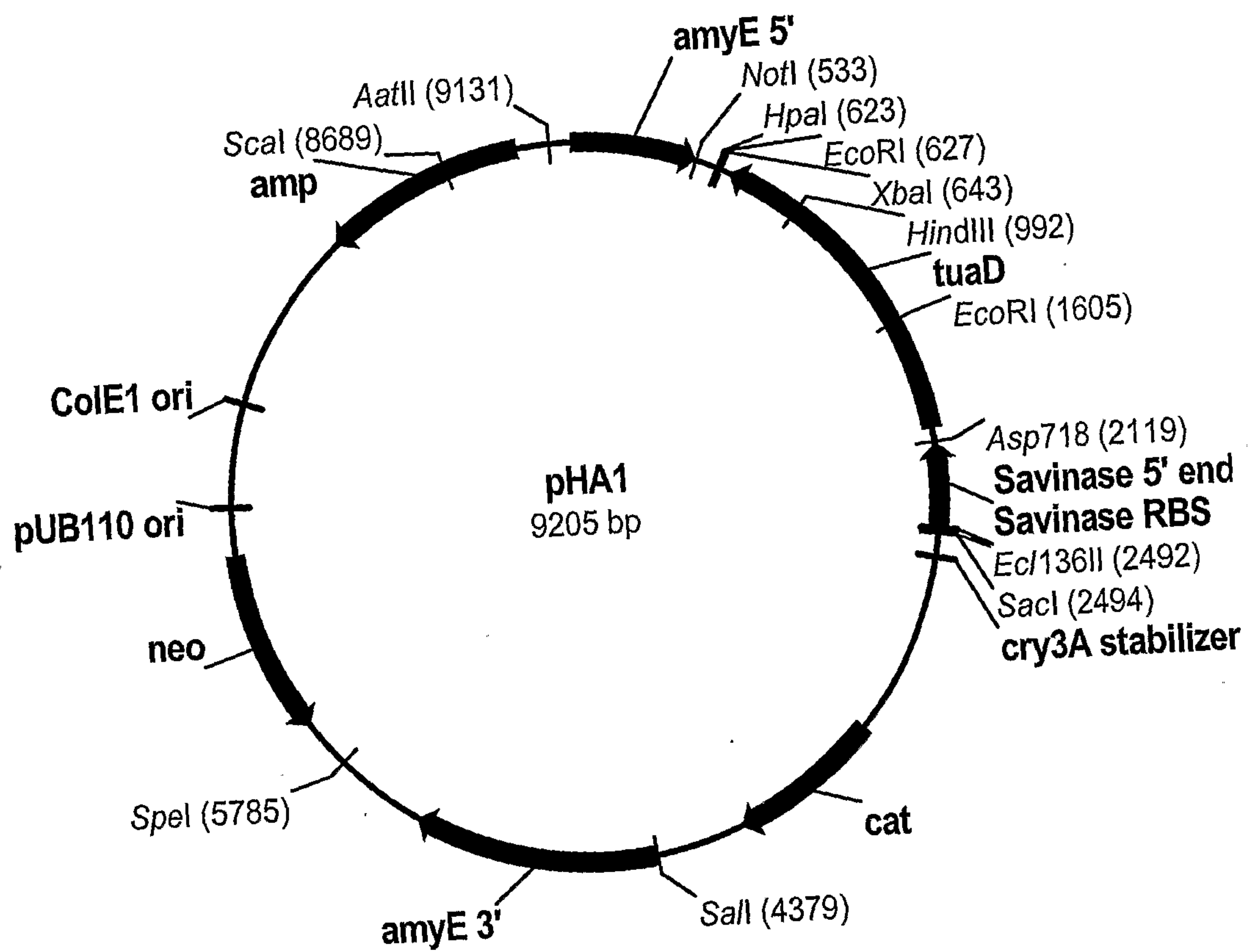


Fig. 7

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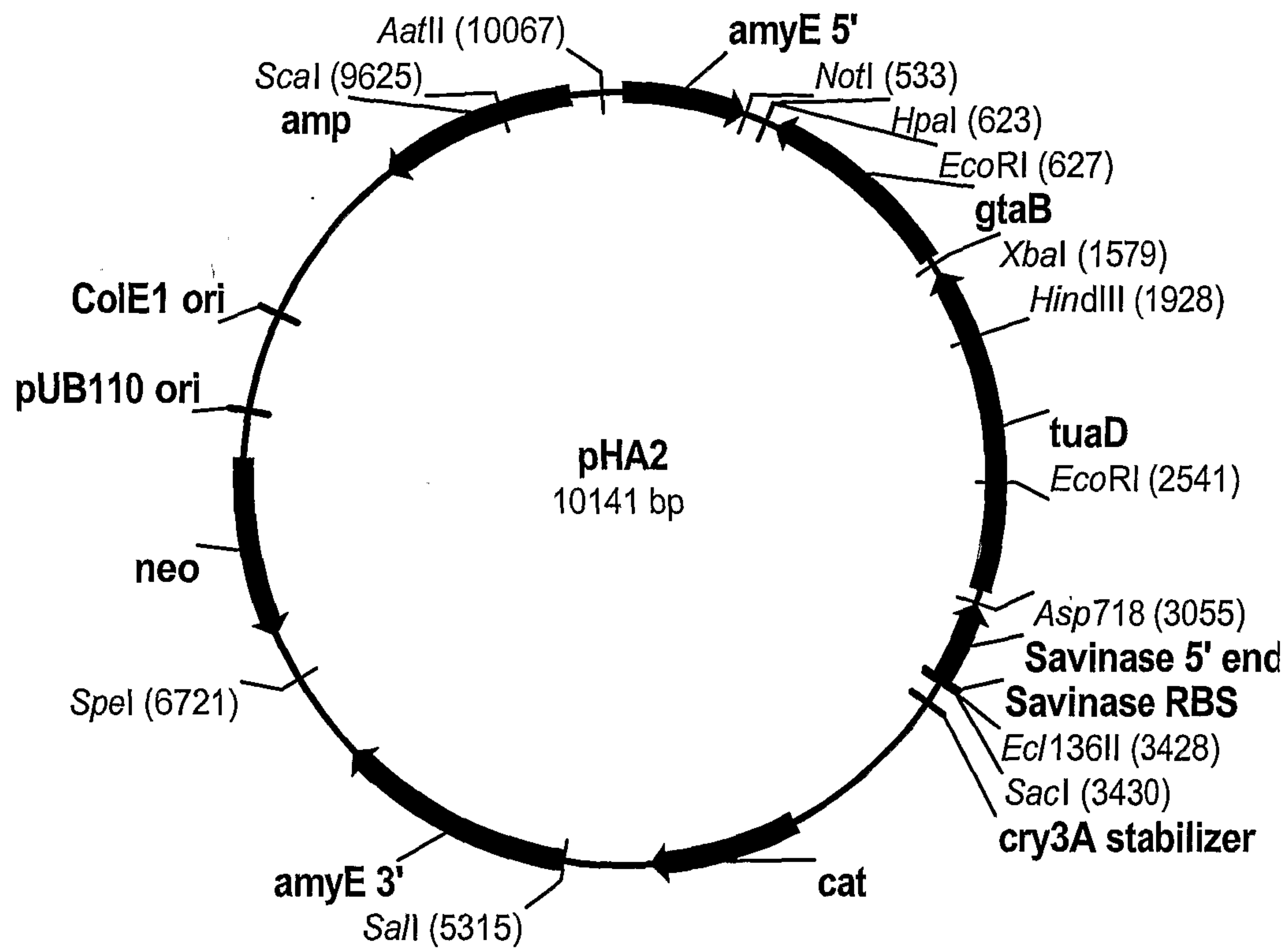


Fig. 8

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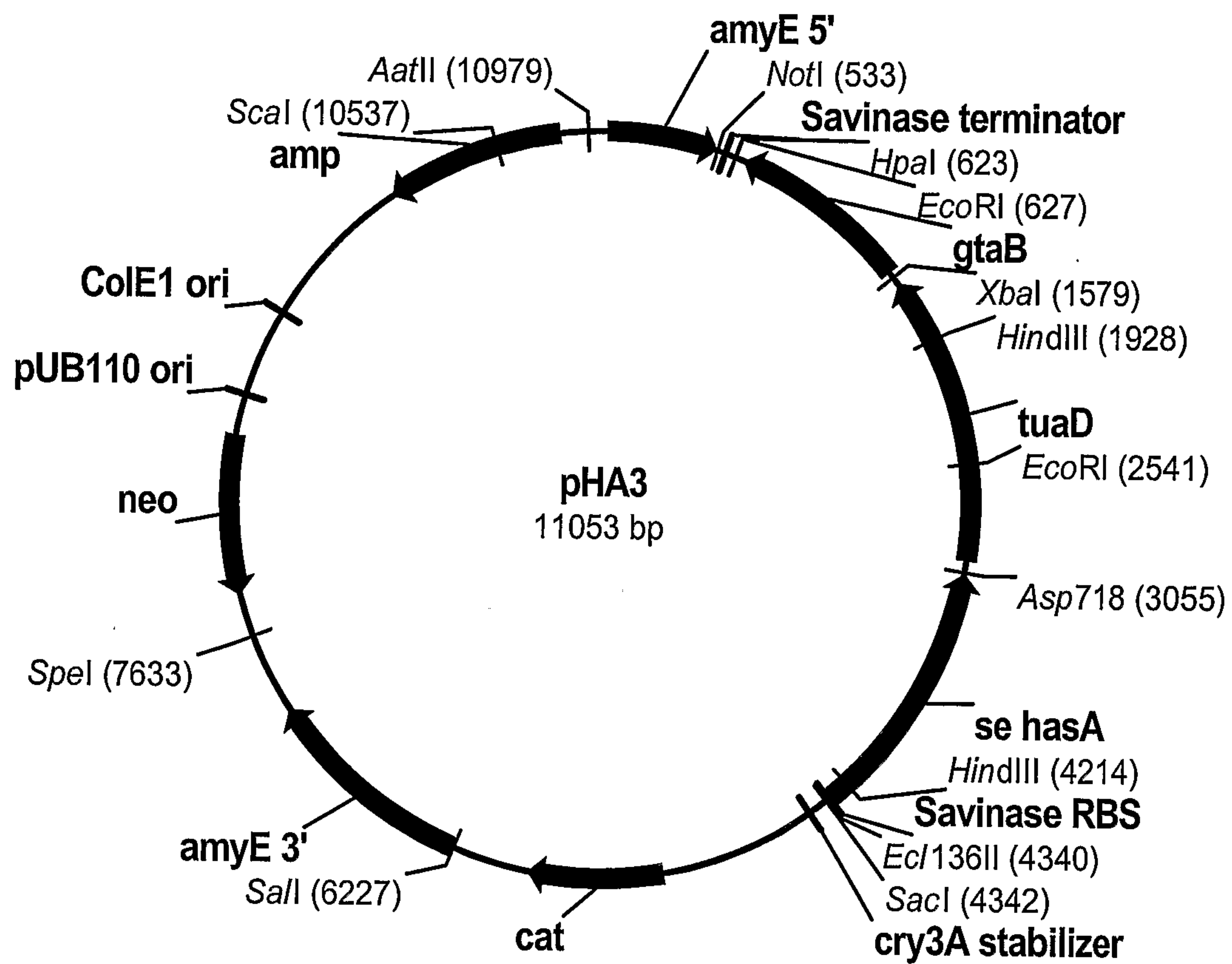


Fig. 9

10/45

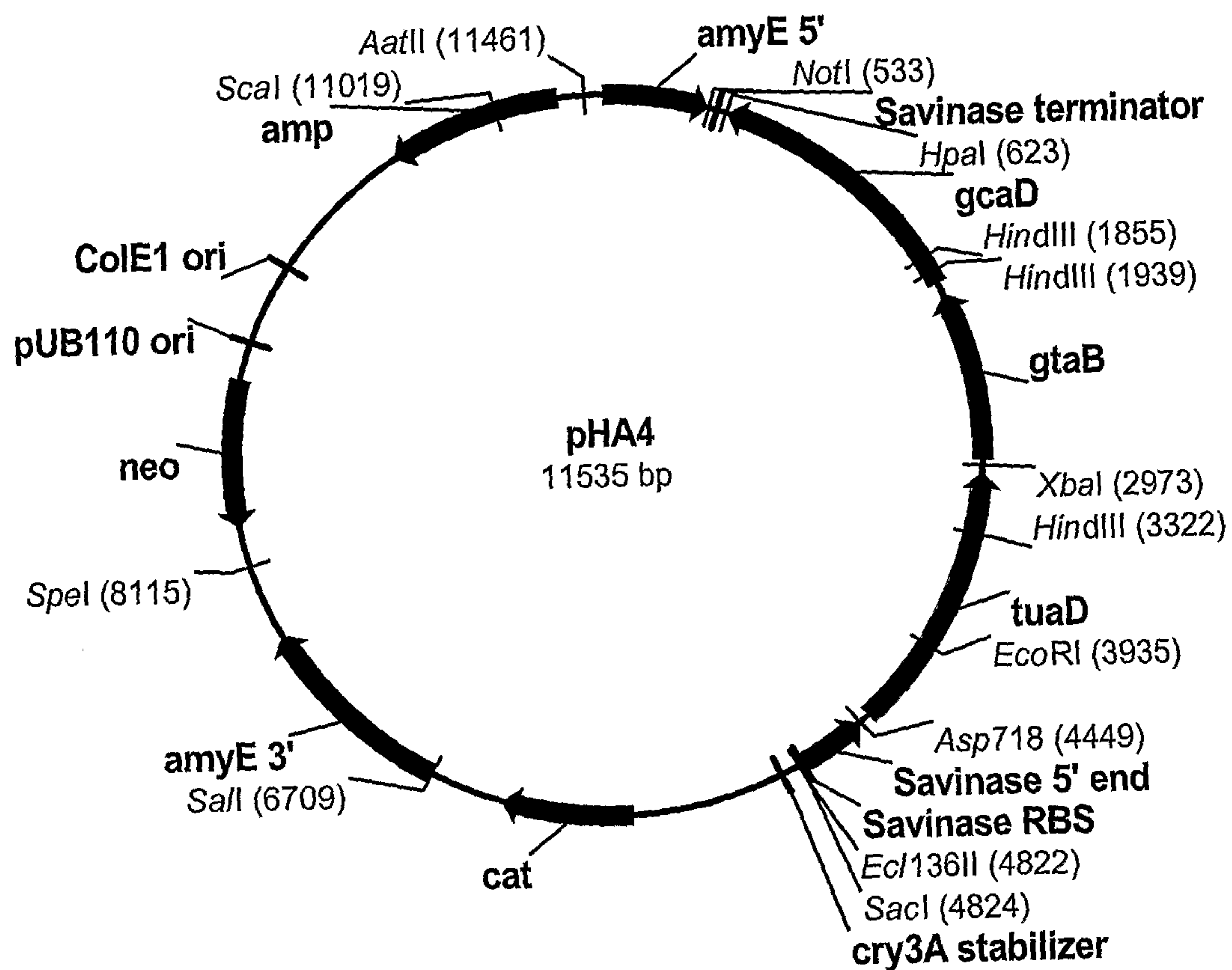


Fig. 10

11/45

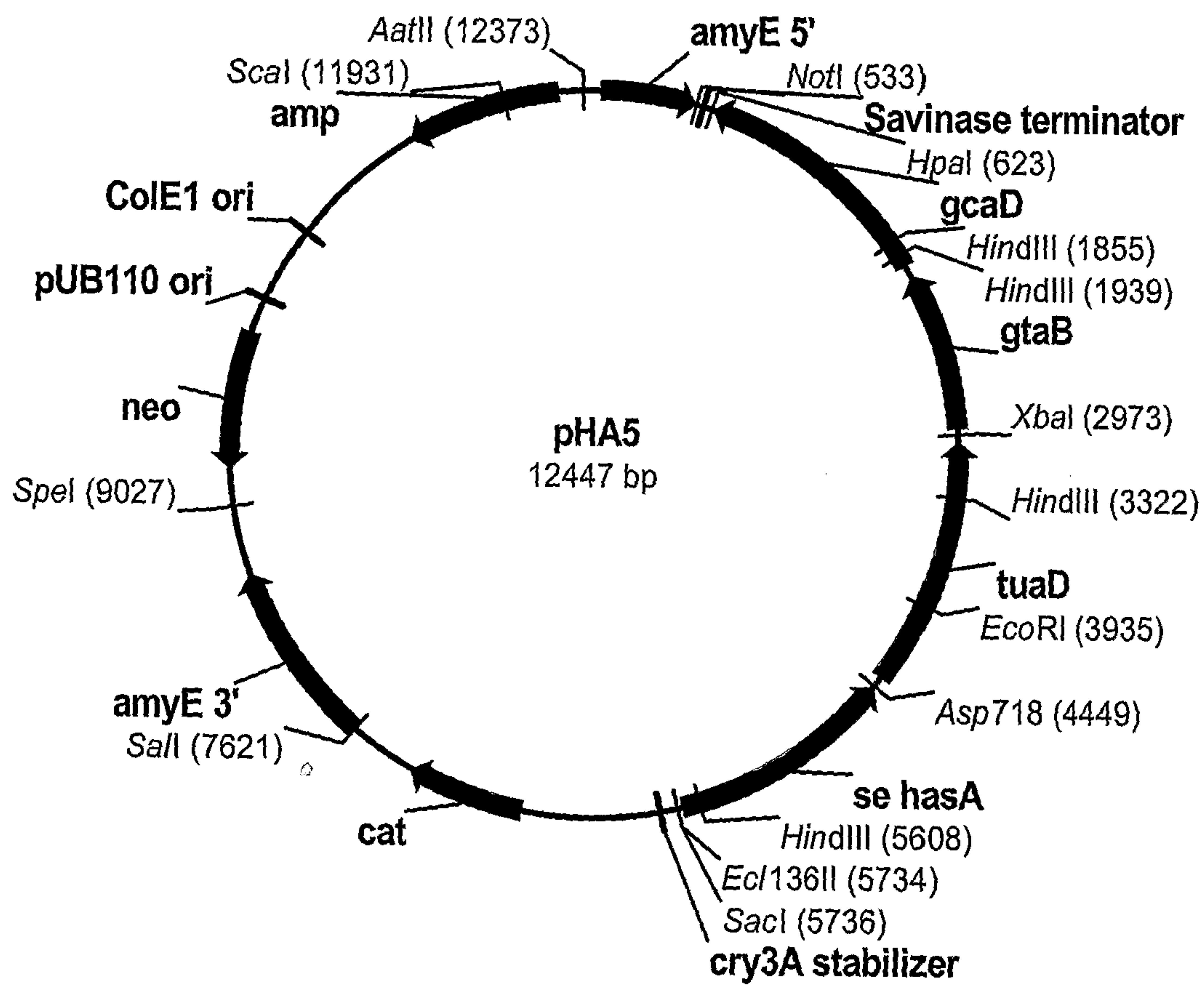


Fig. 11

12/45

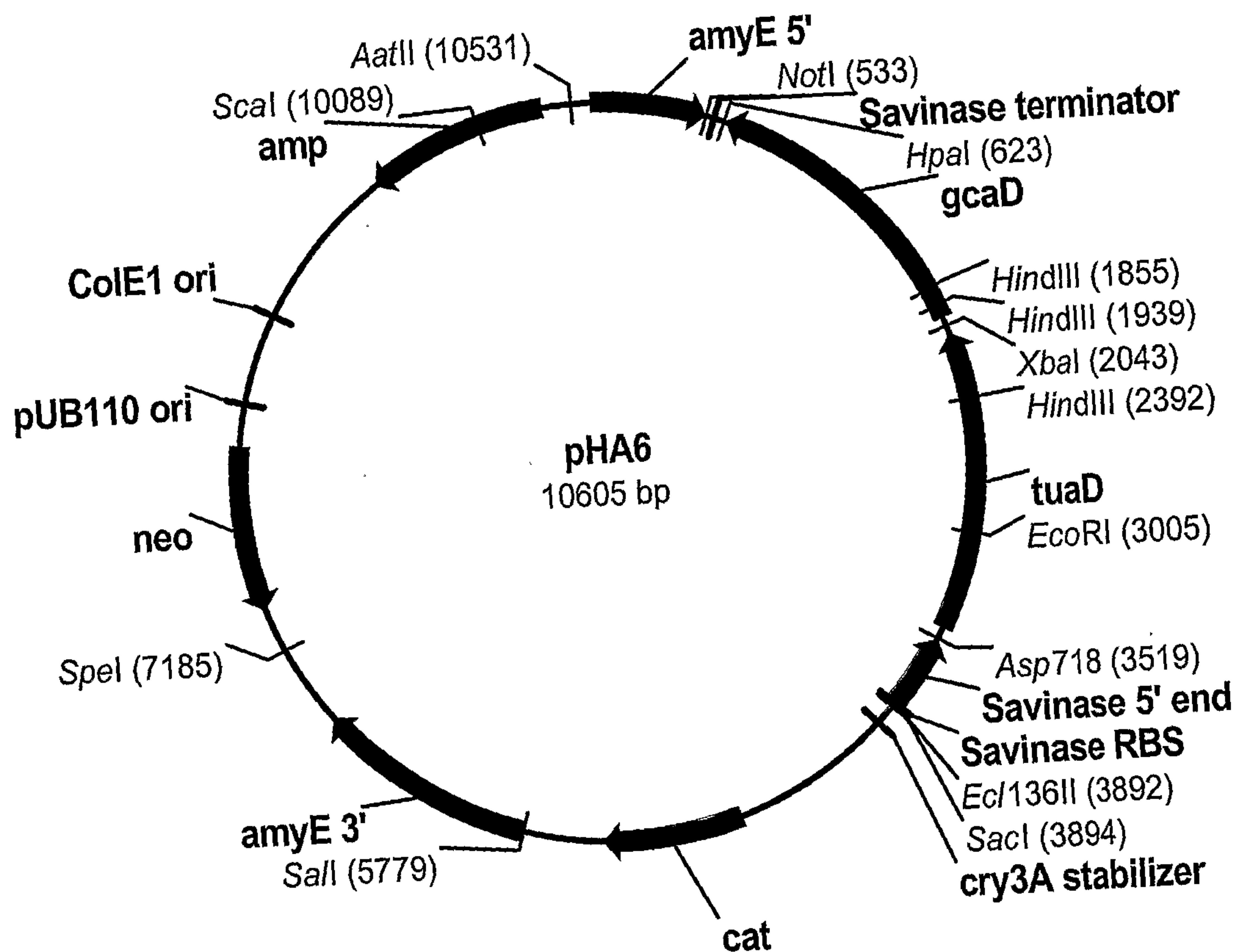


Fig. 12

13/45

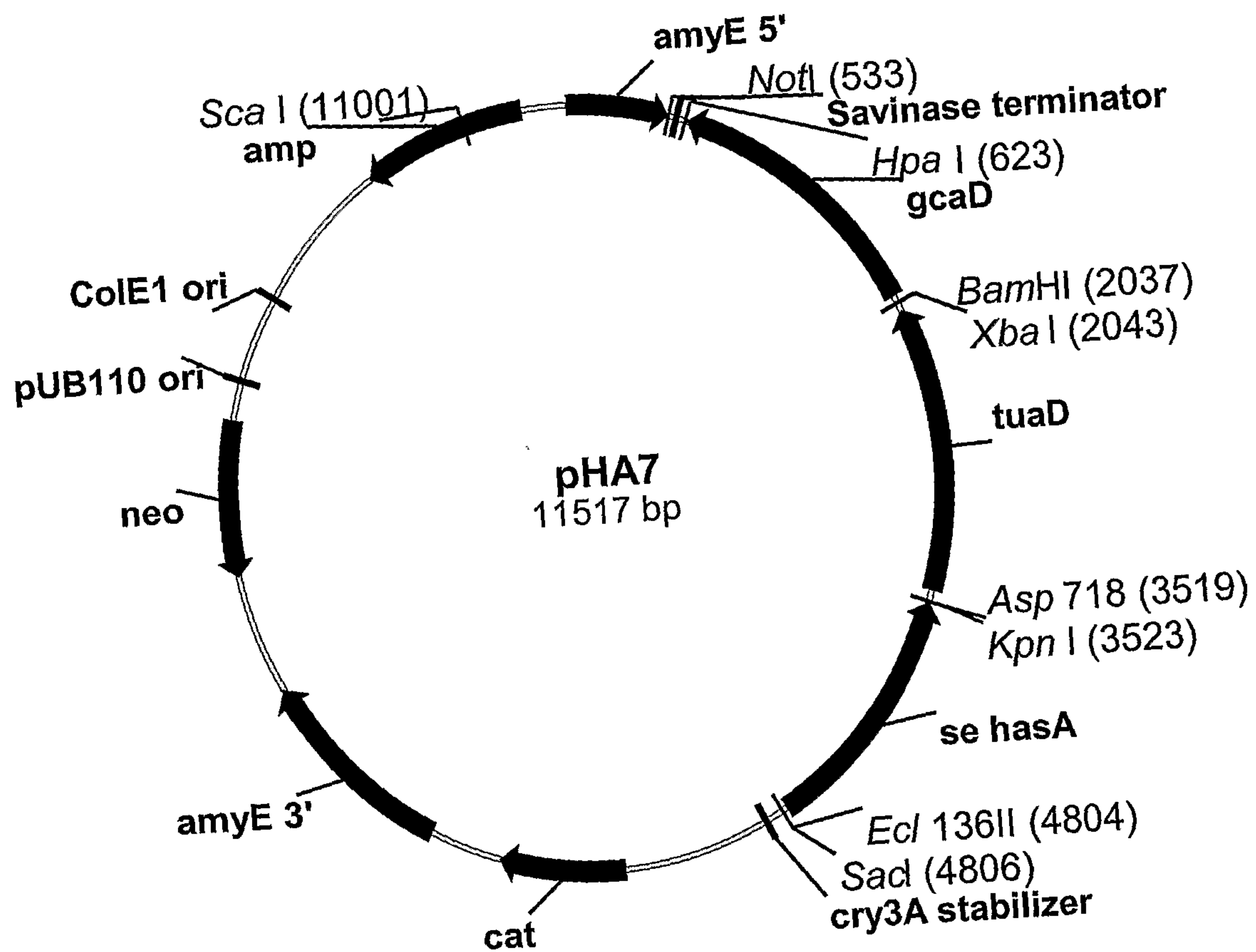


Fig. 13

14/45

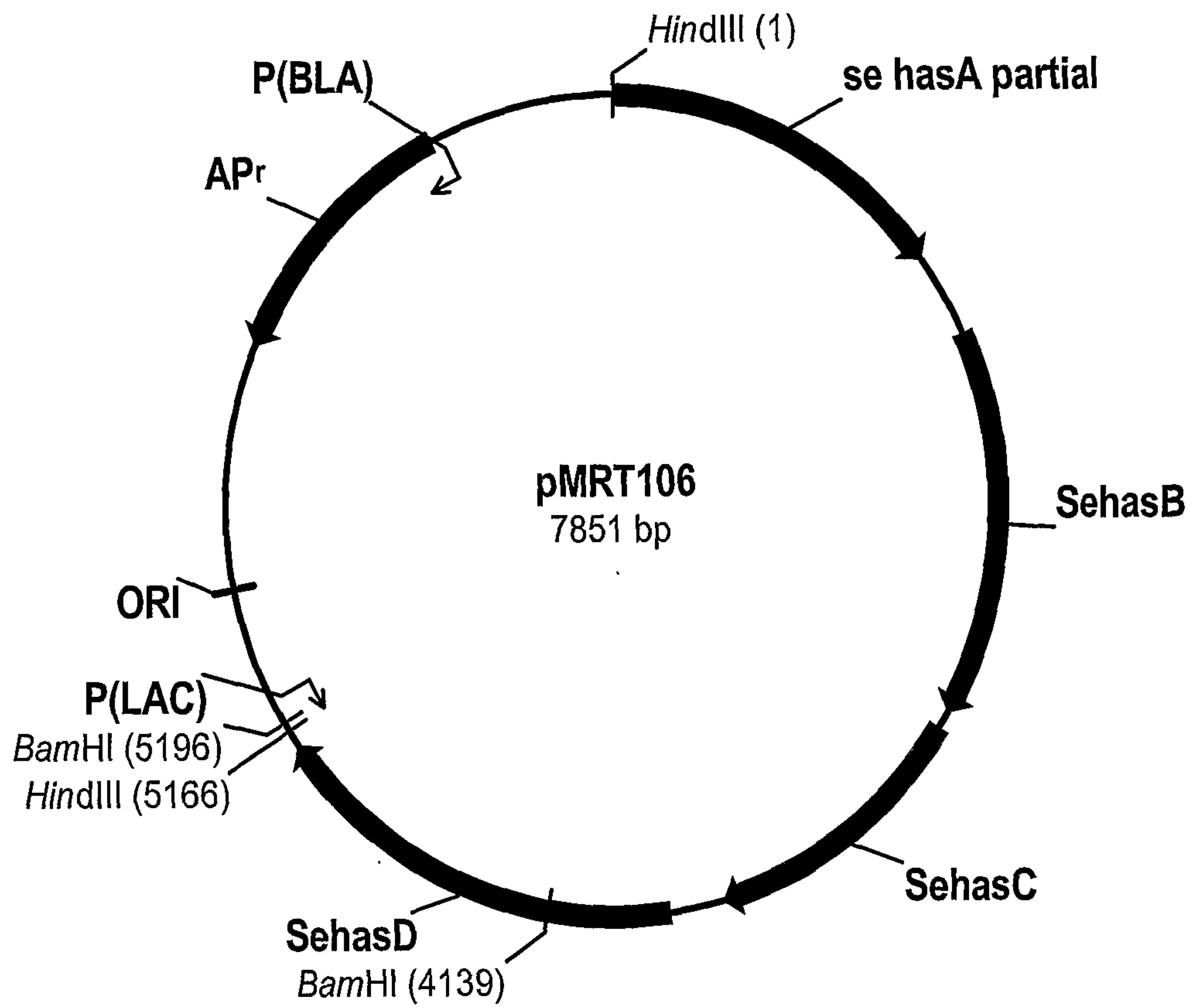


Fig. 14

15/45

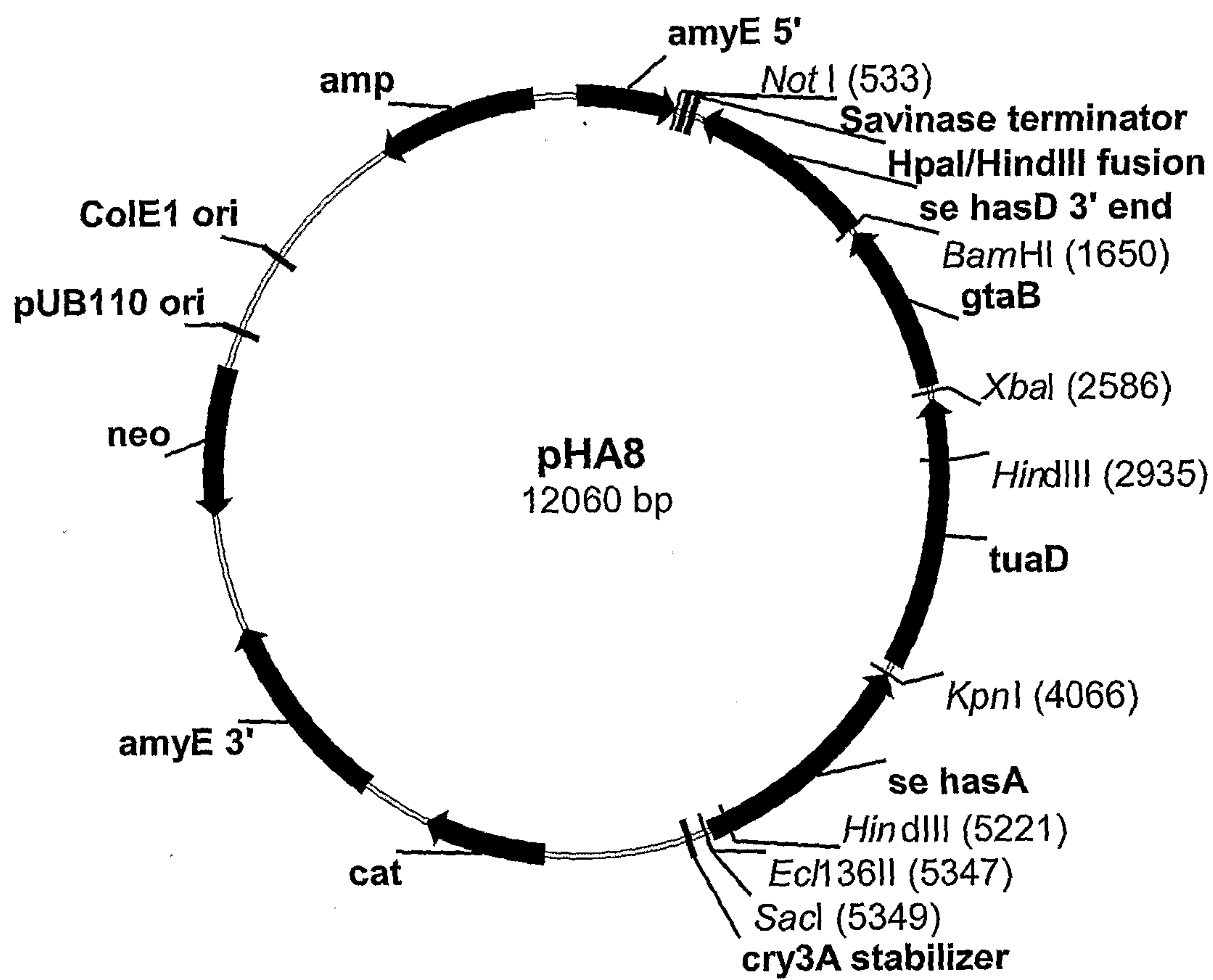


Fig. 15

16/45

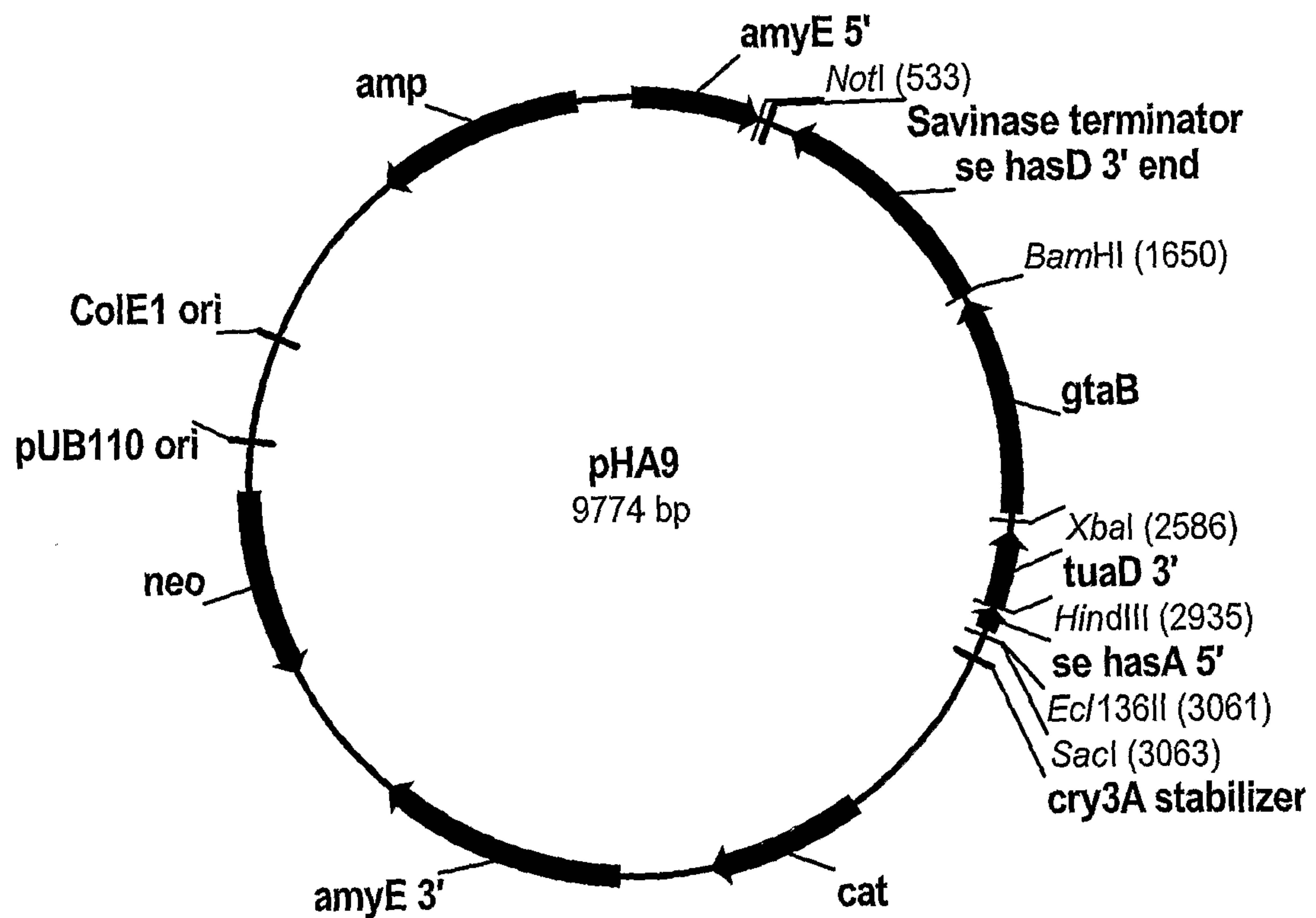


Fig. 16

17/45

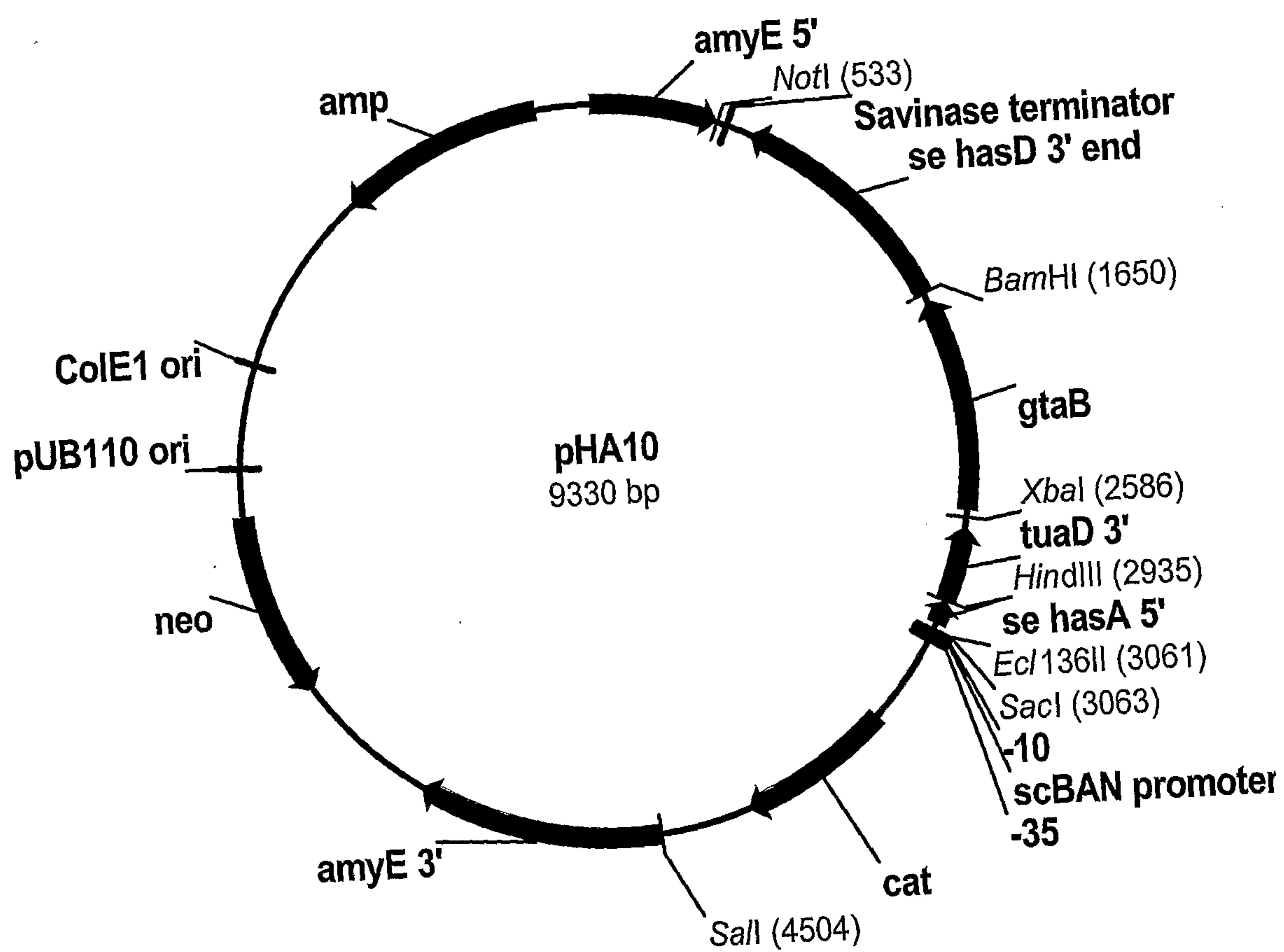


Fig. 17

18/45

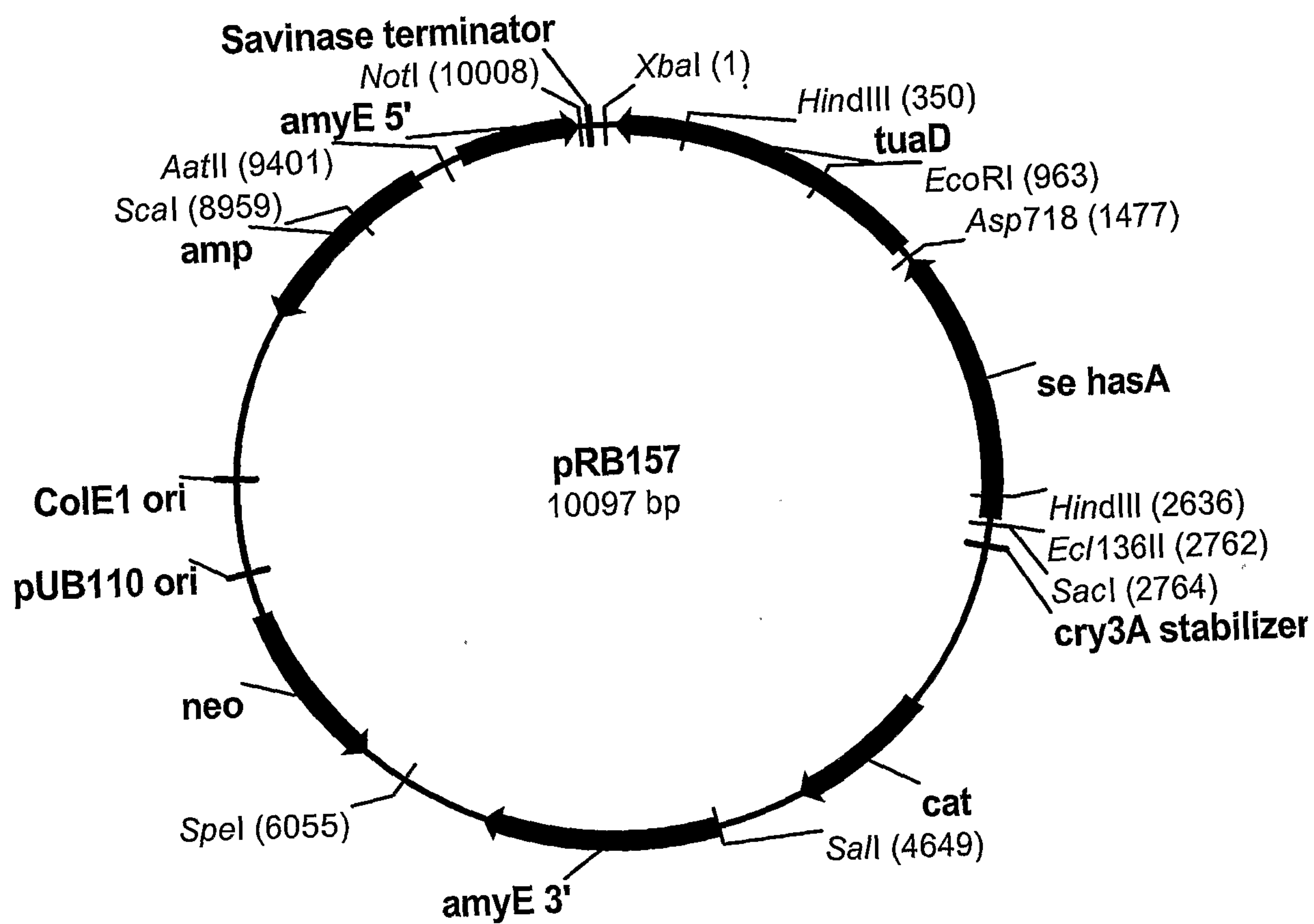


Fig. 18

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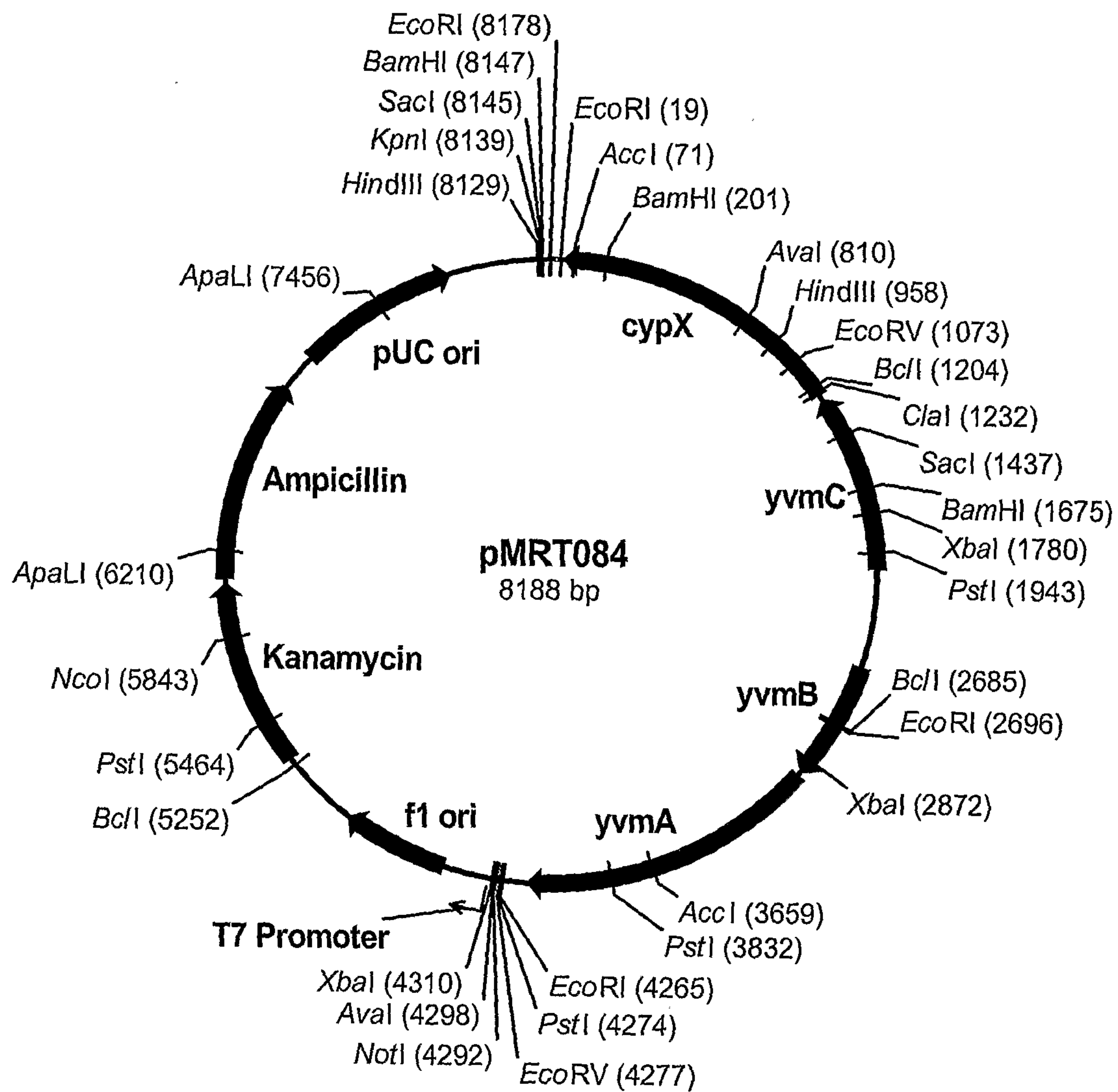


Fig. 19

20/45

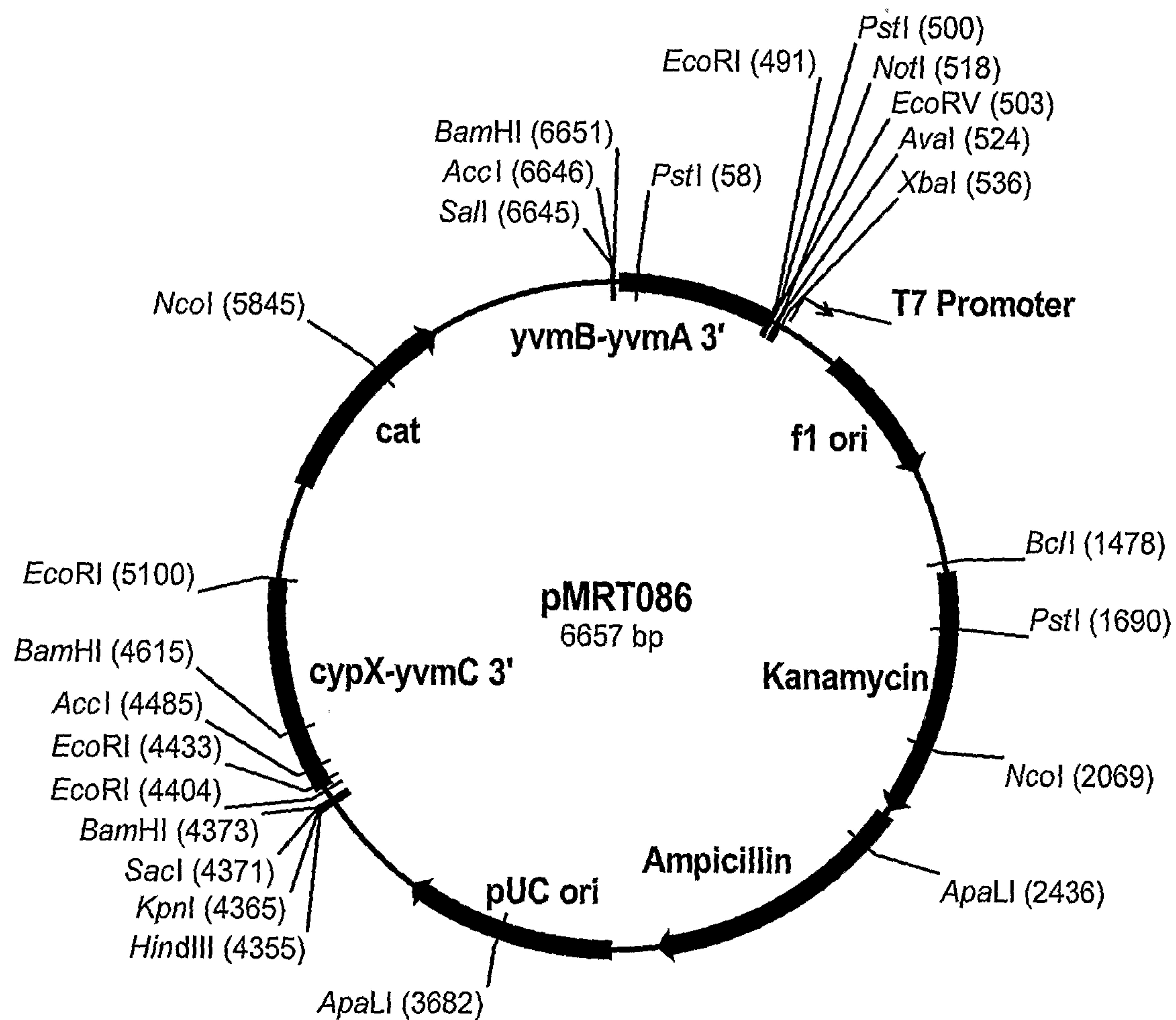


Fig. 20

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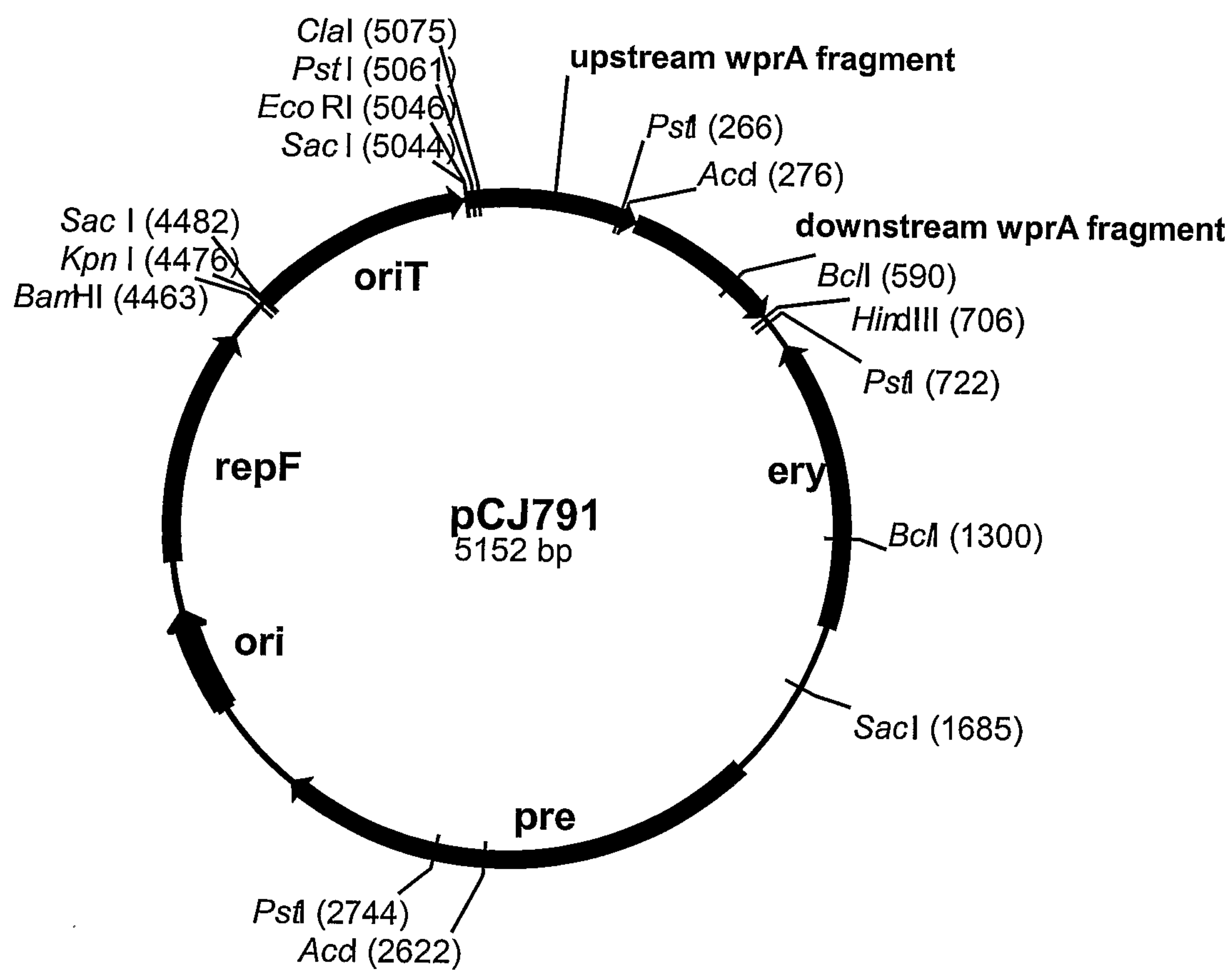


Fig. 21

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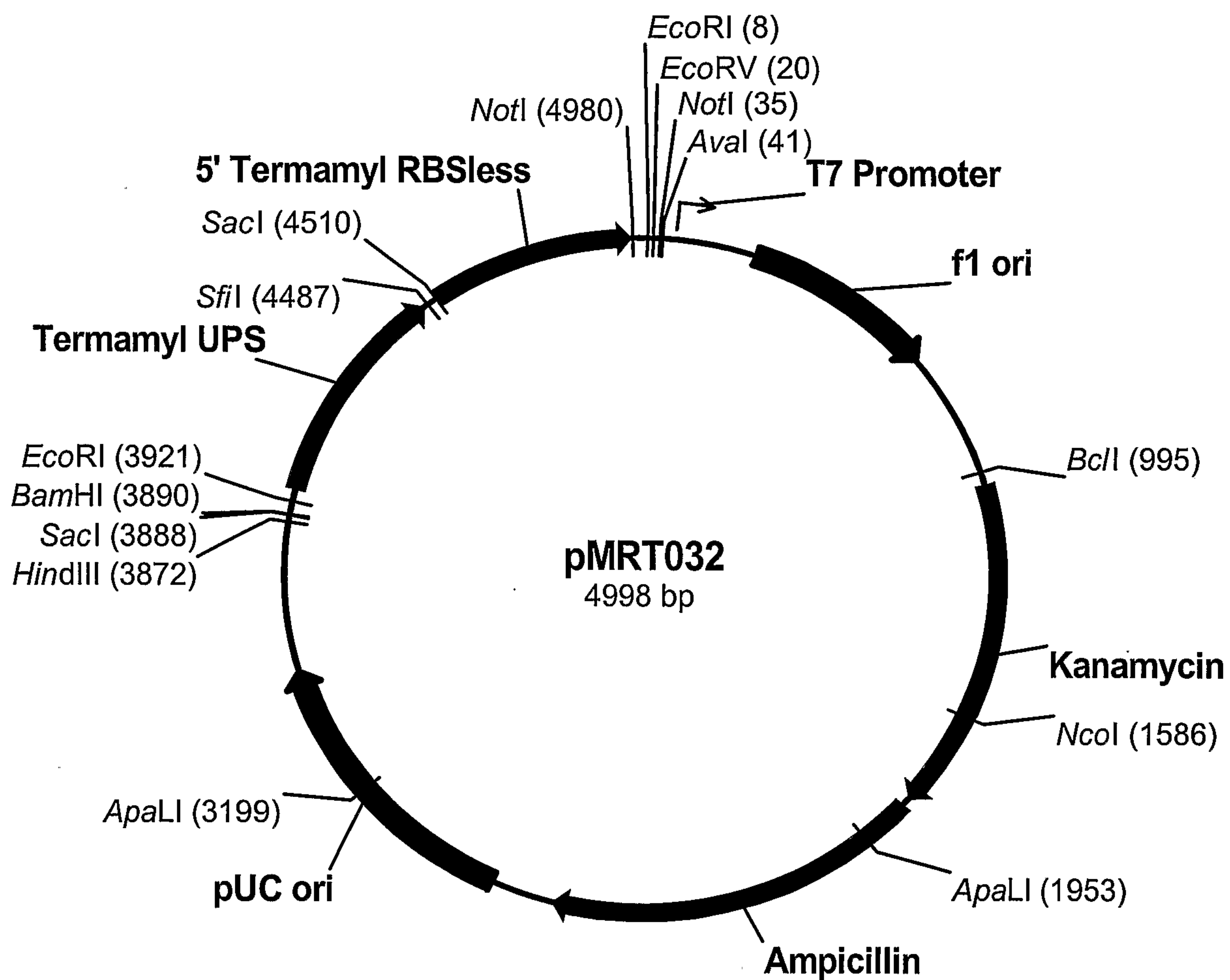


Fig. 22

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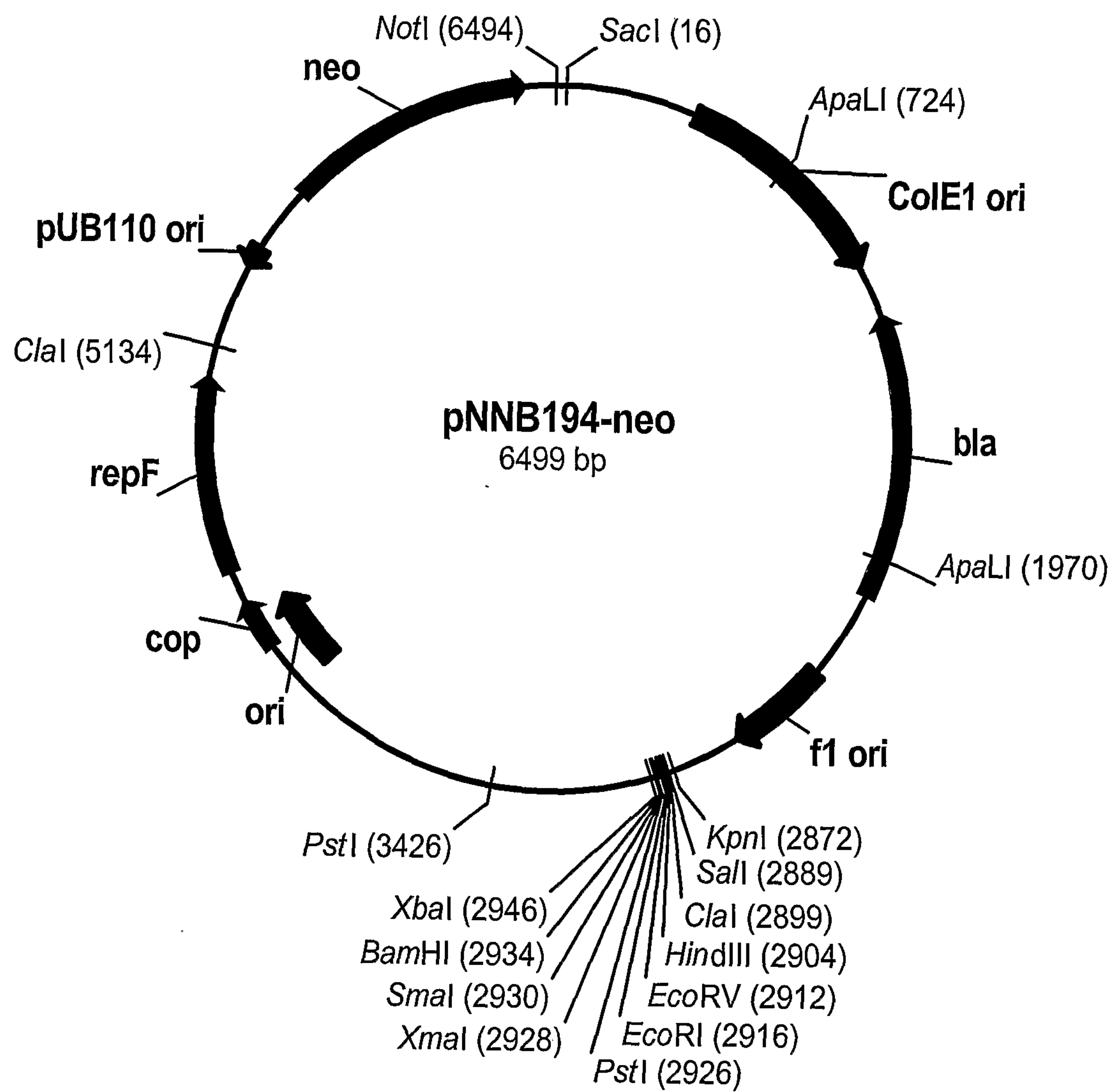


Fig. 23

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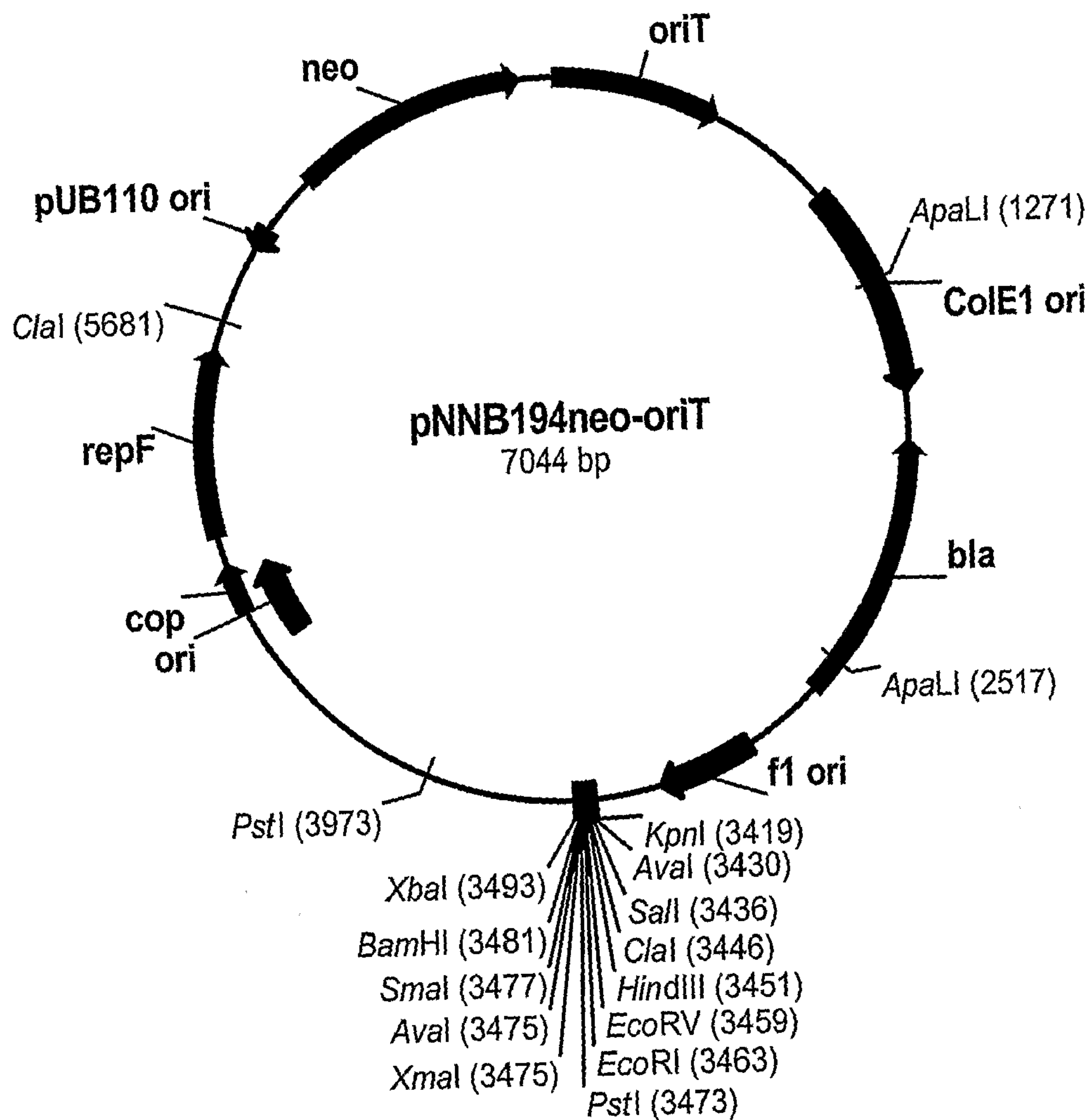


Fig. 24

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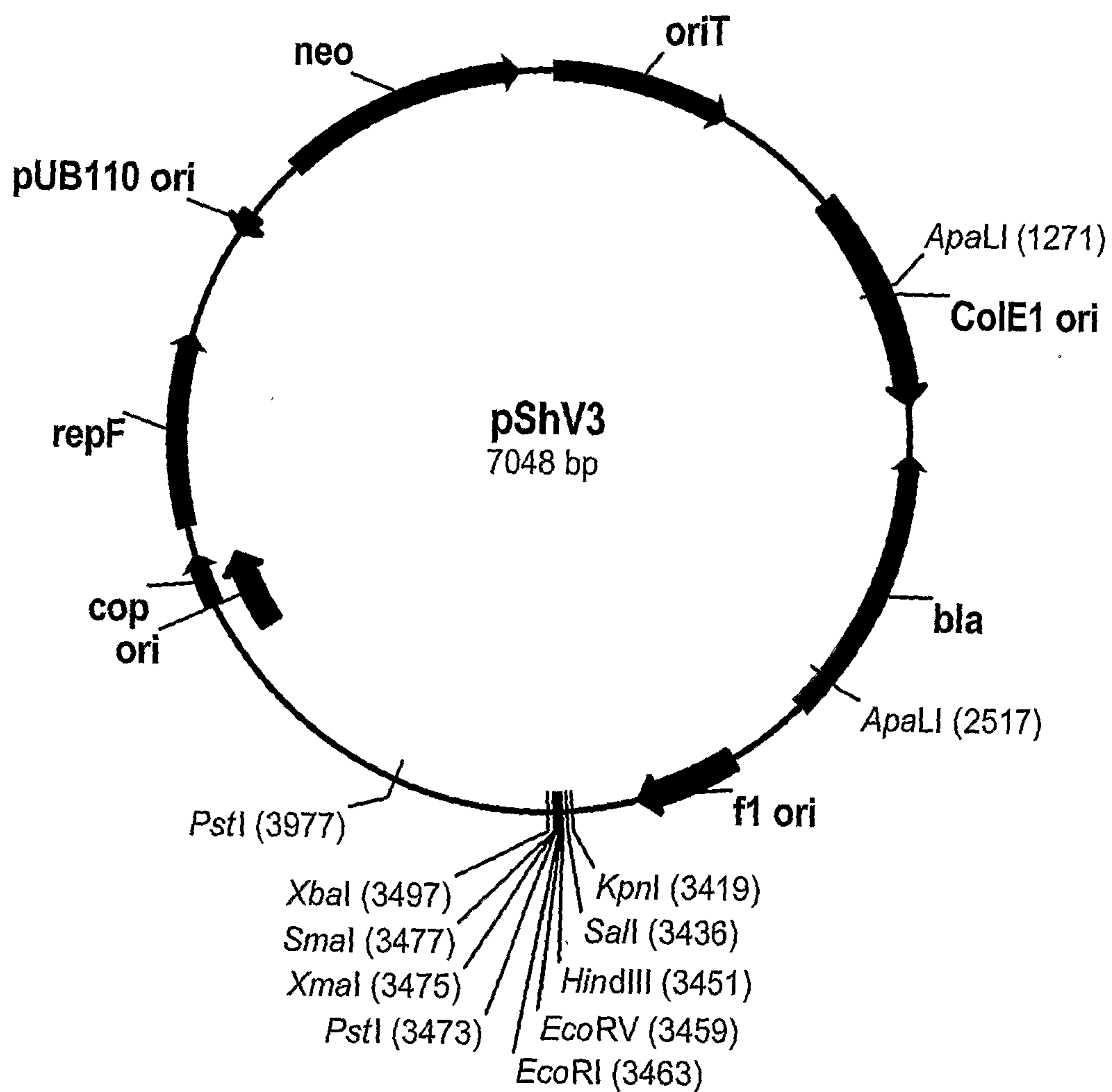


Fig. 25

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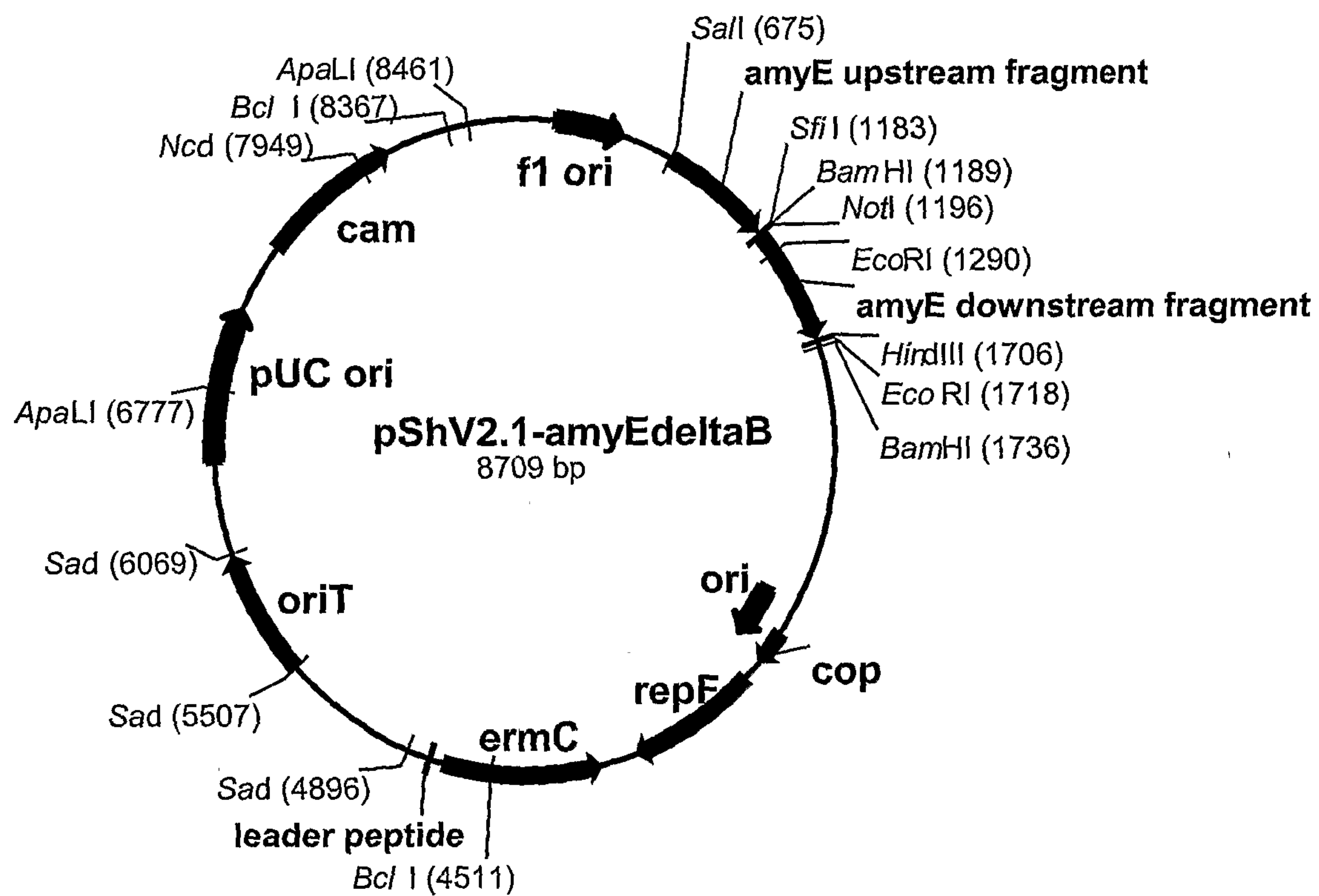


Fig. 26

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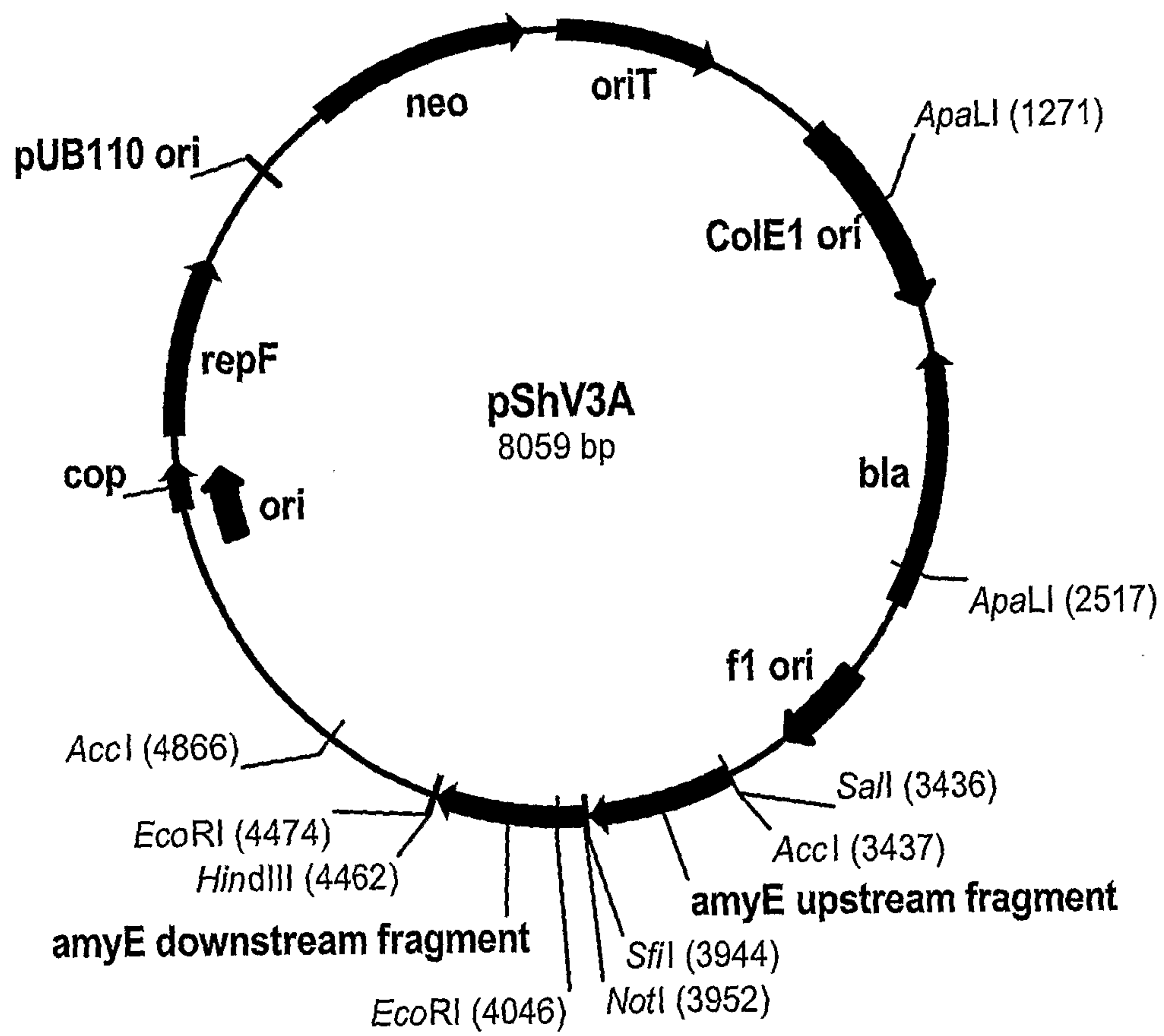


Fig. 27

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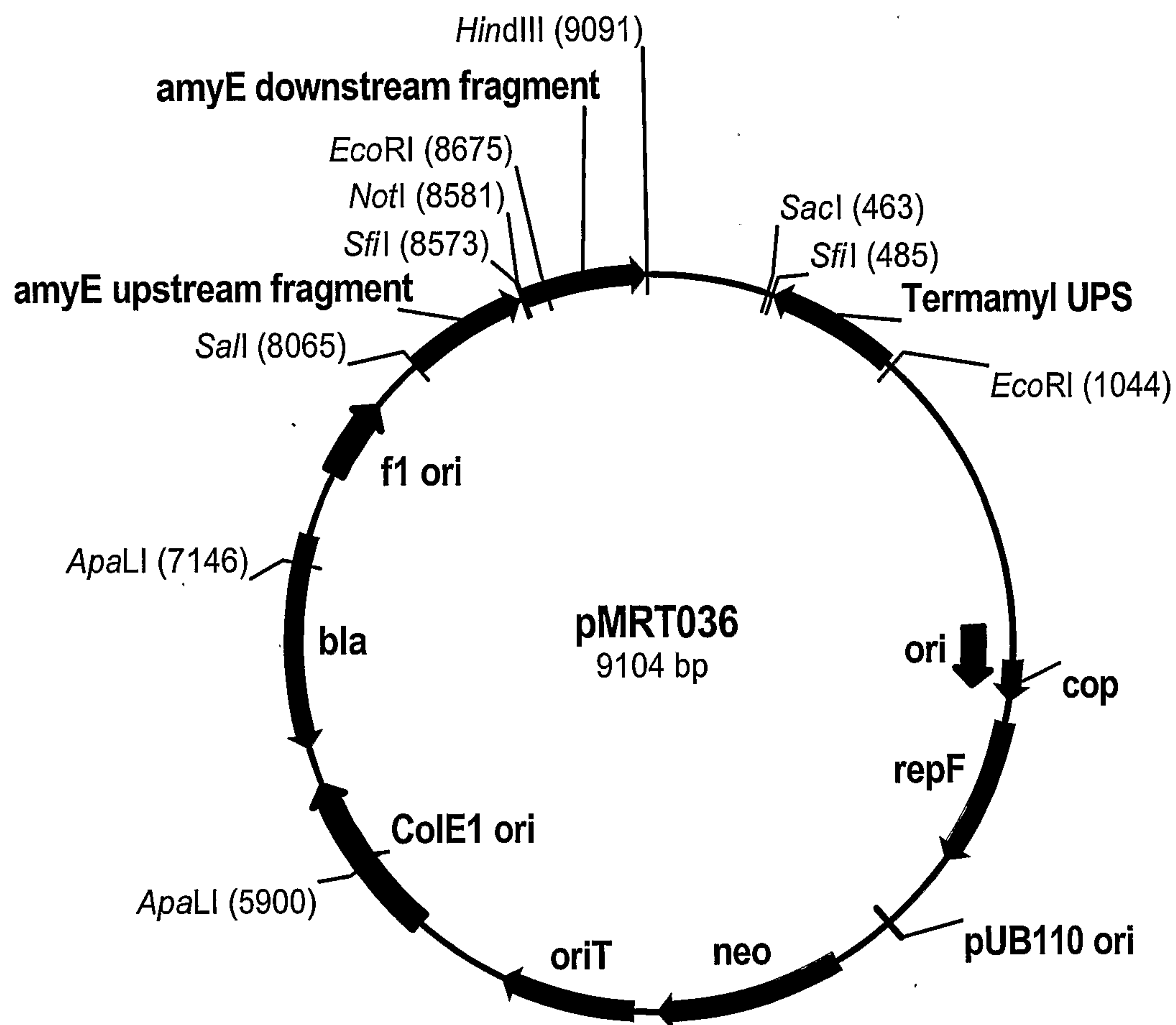


Fig. 28

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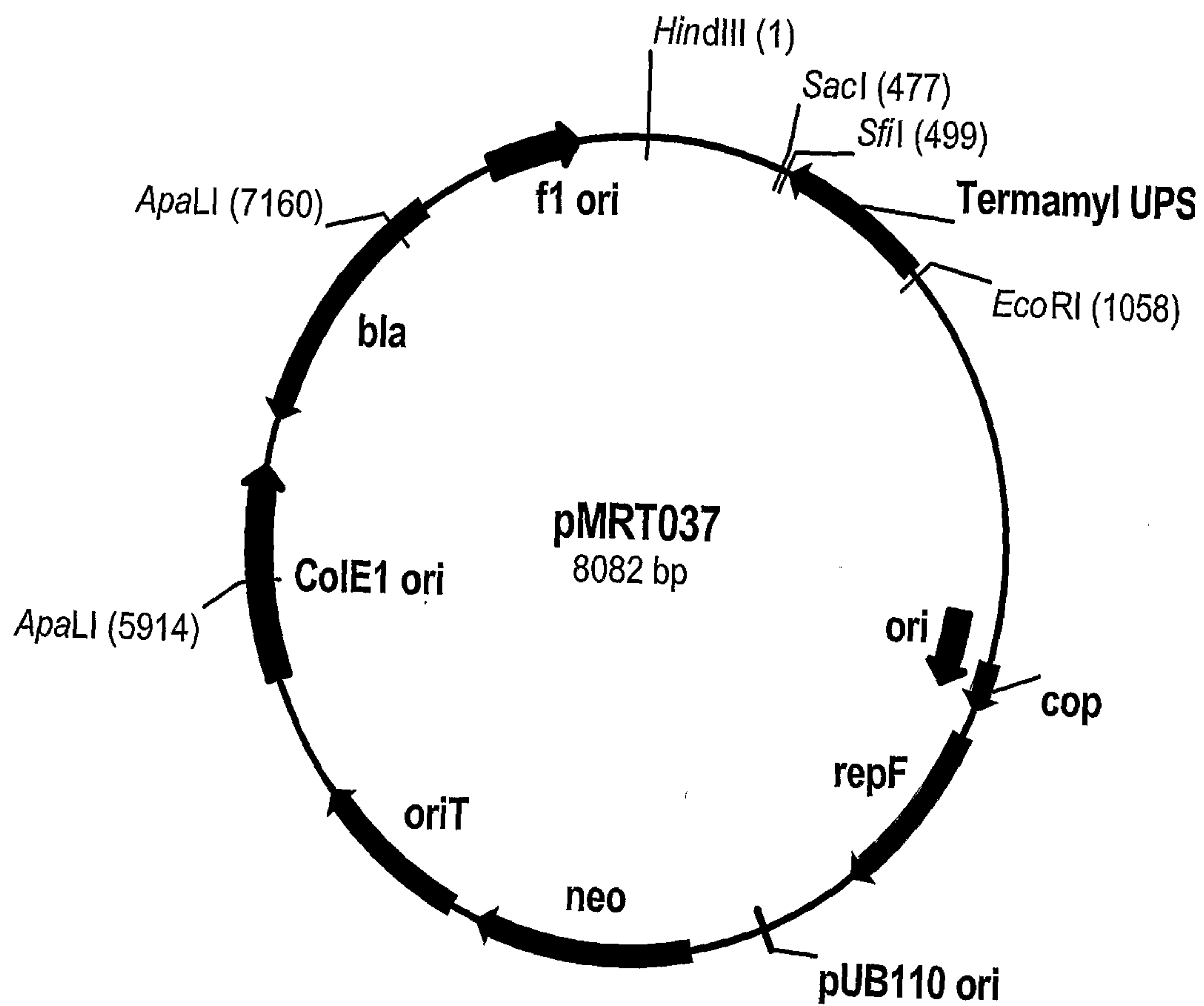


Fig. 29

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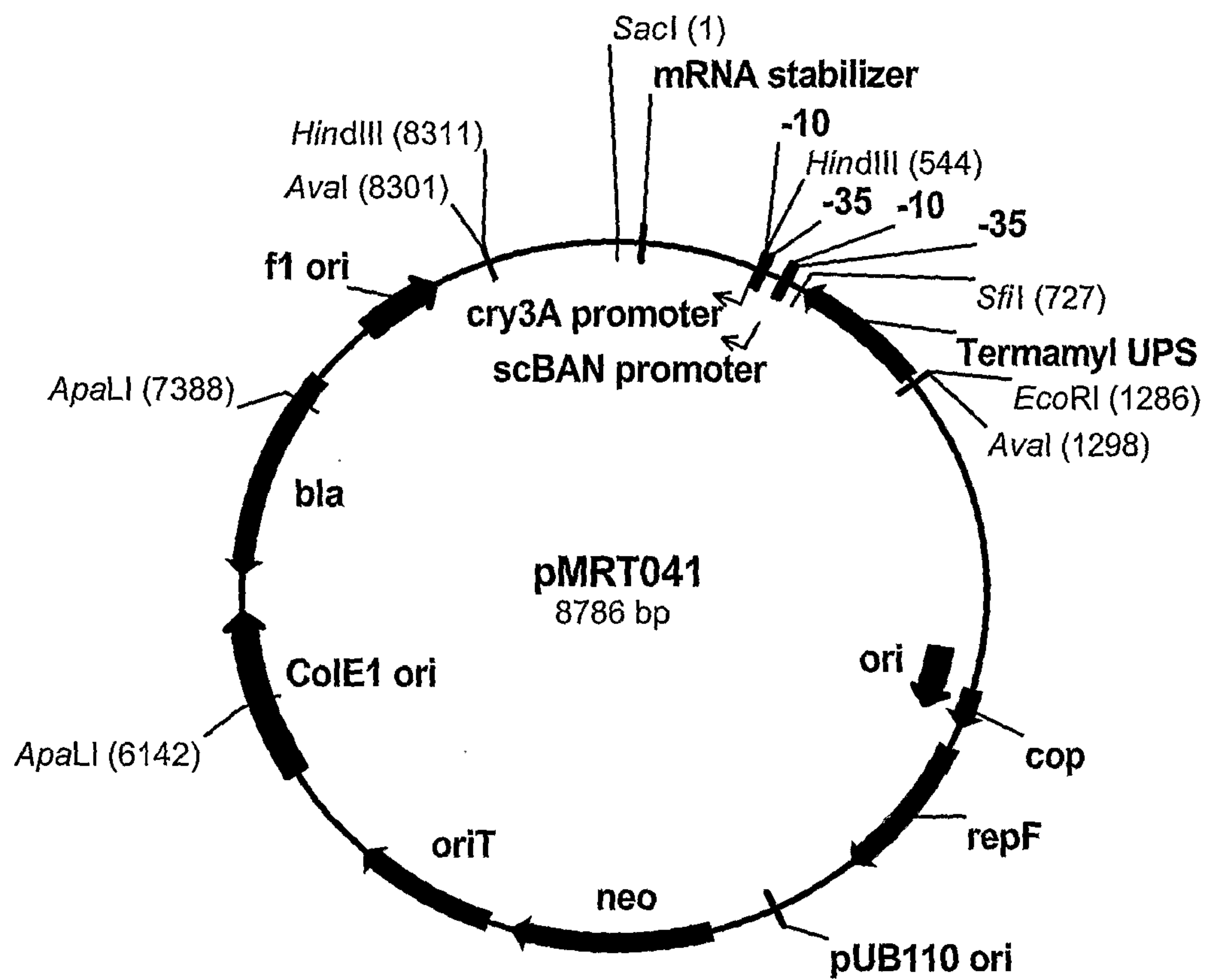


Fig. 30

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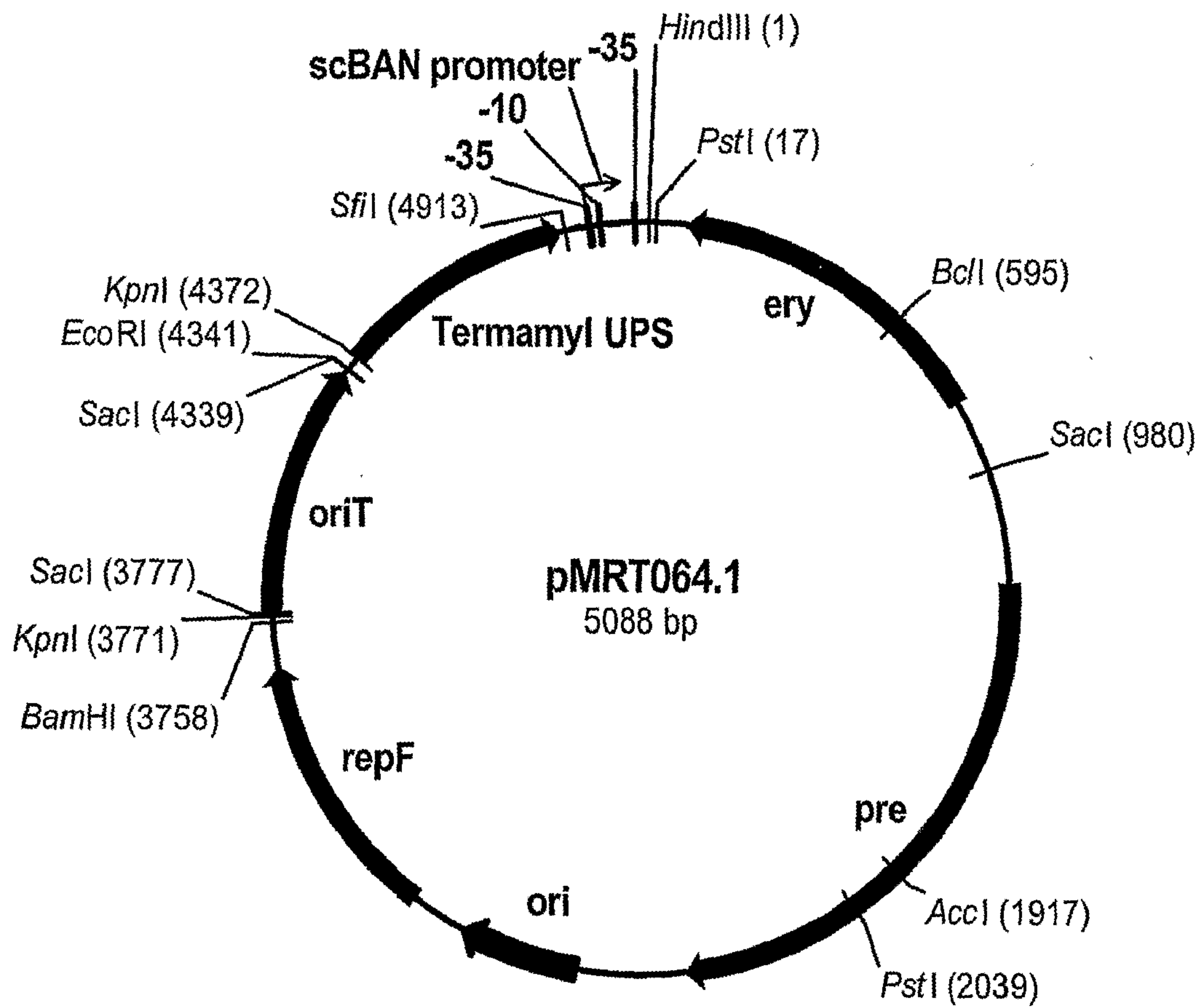


Fig. 31

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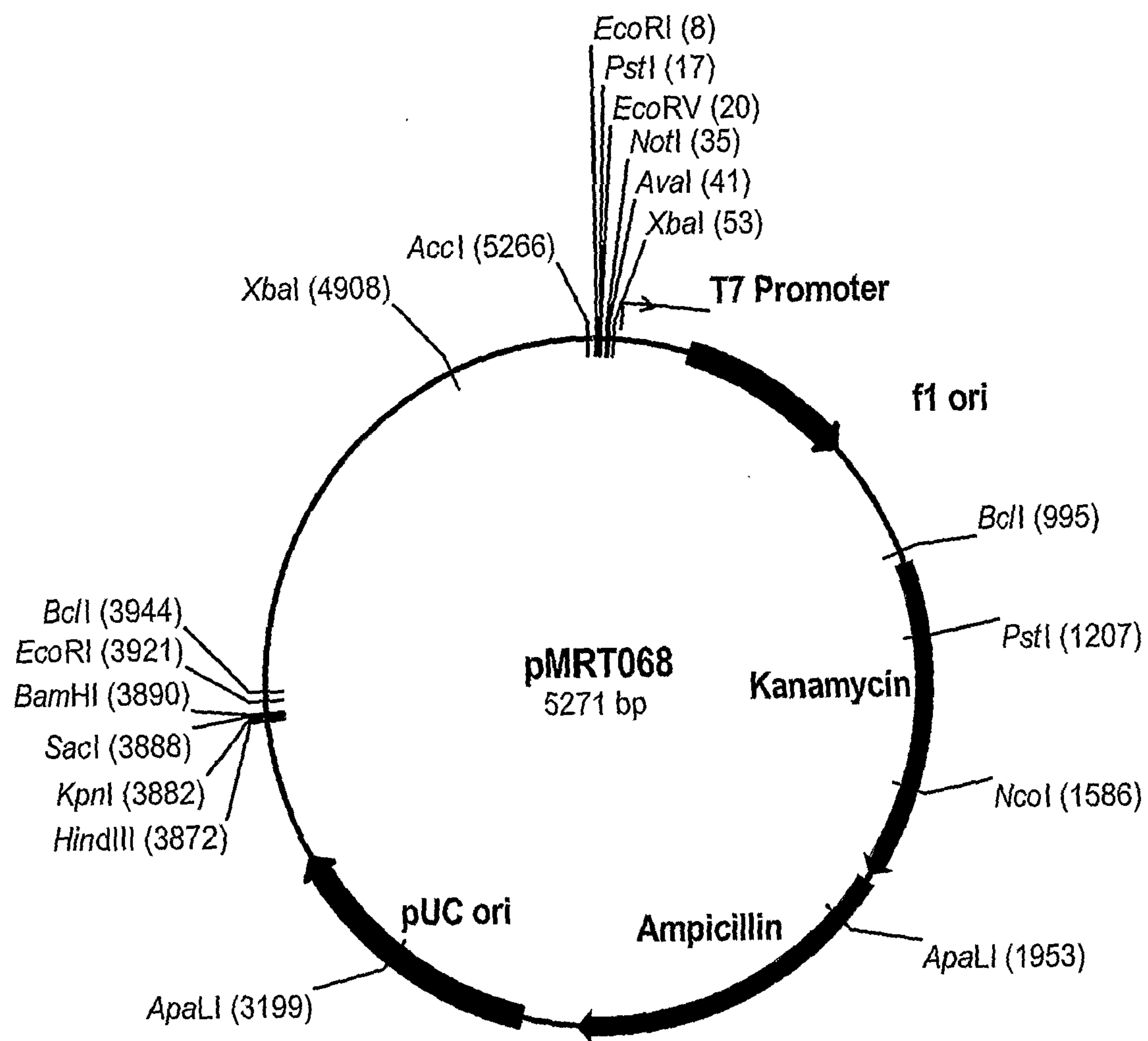


Fig. 32

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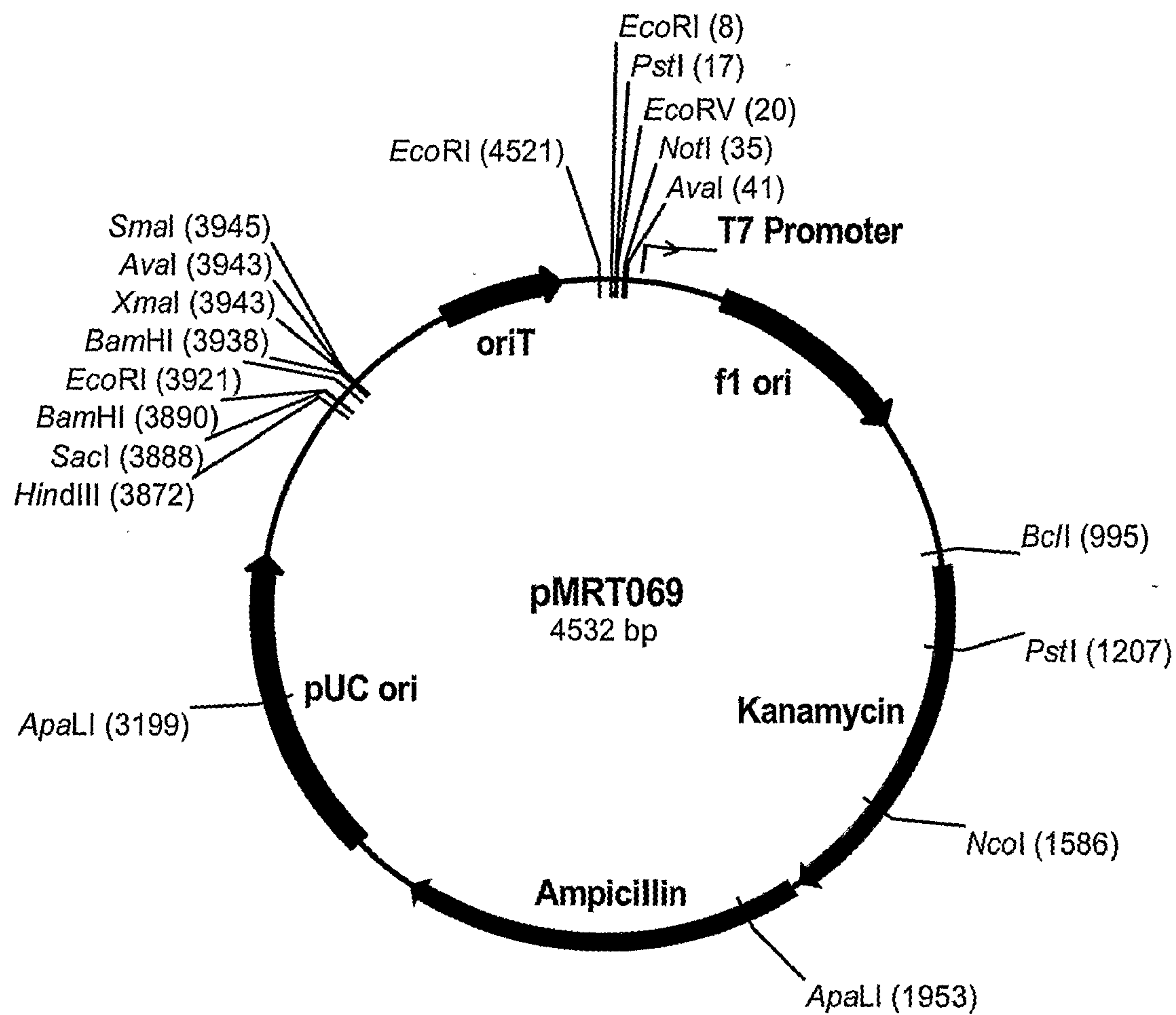


Fig. 33

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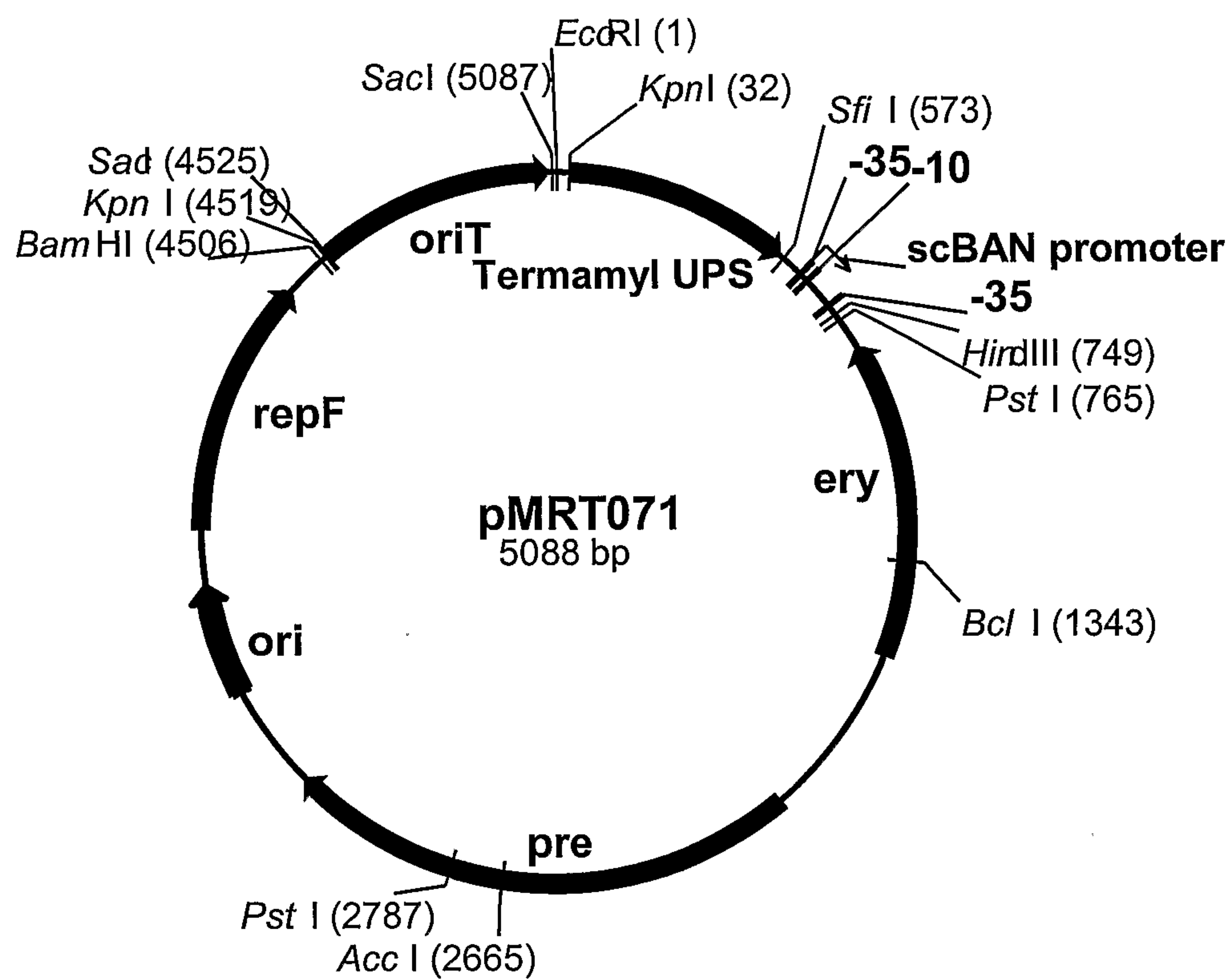


Fig. 34

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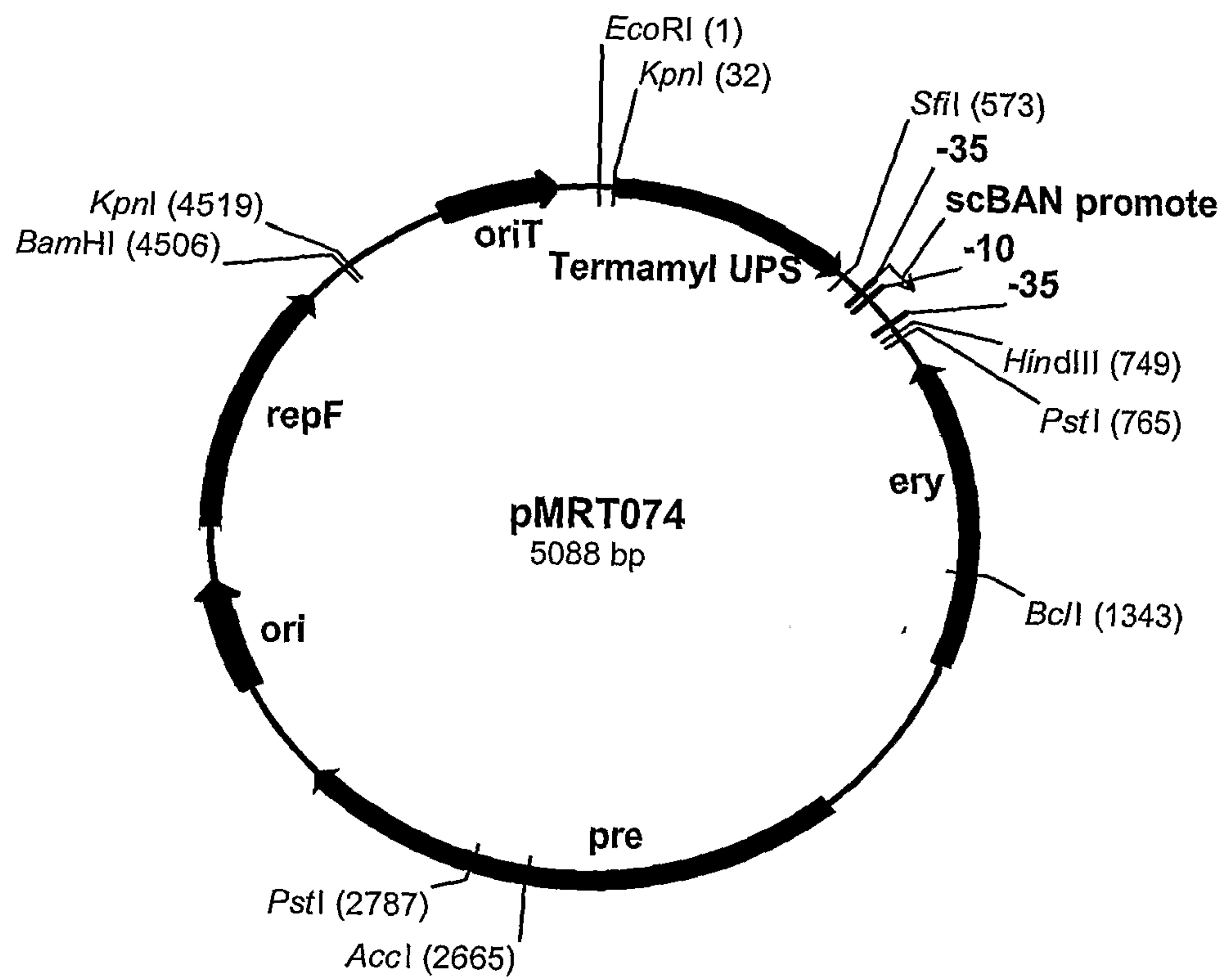


Fig. 35

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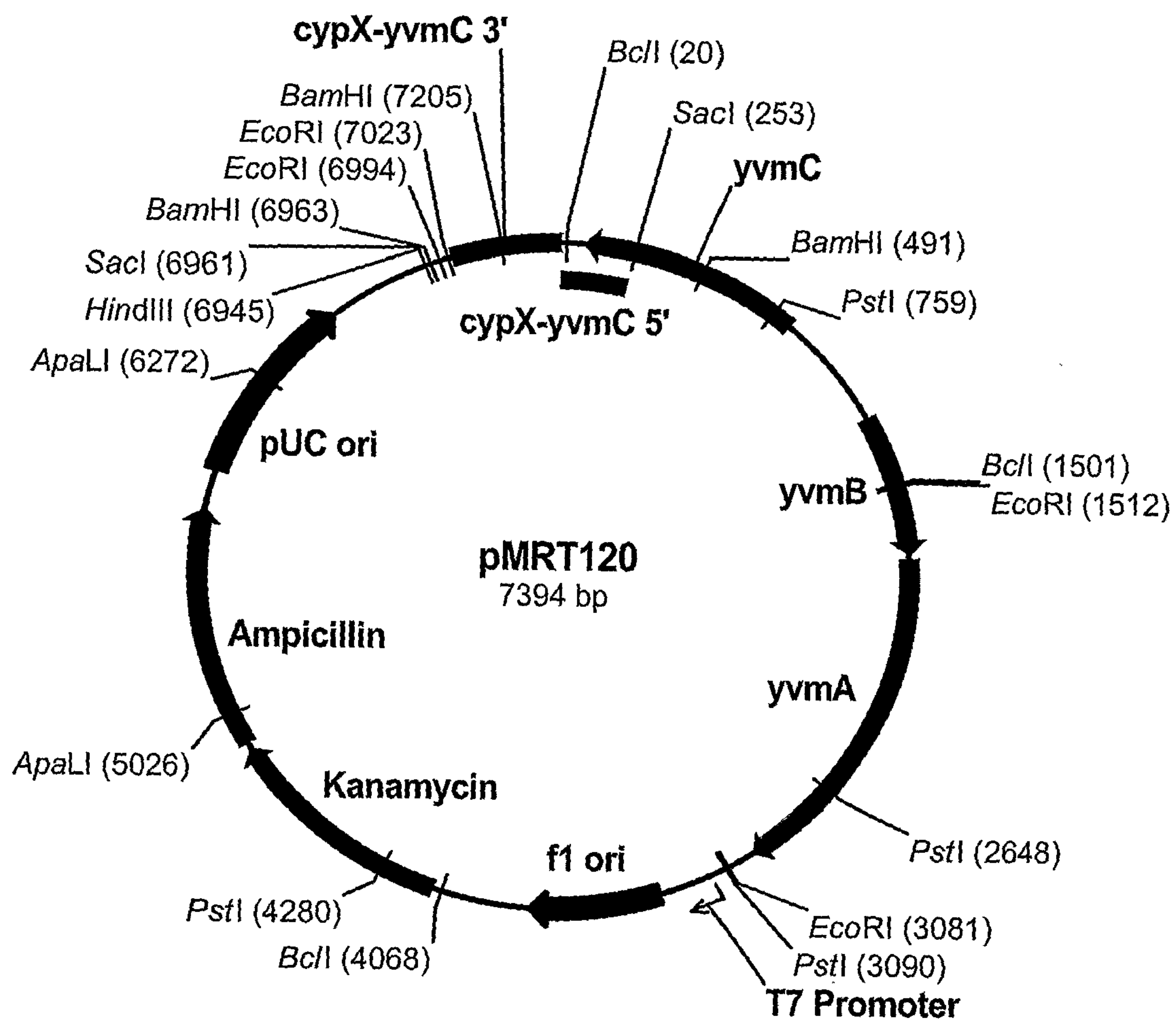


Fig. 36

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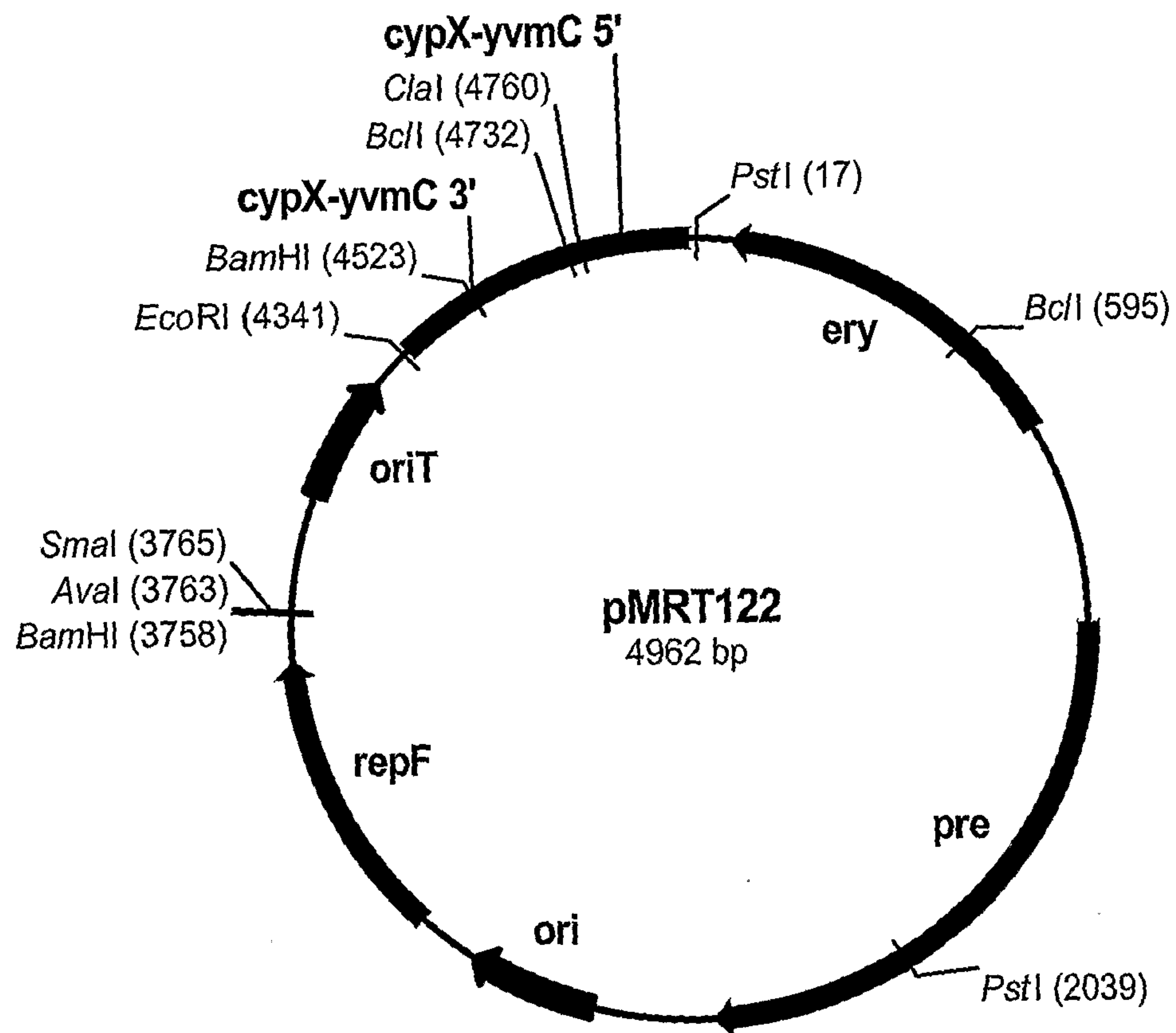


Fig. 37

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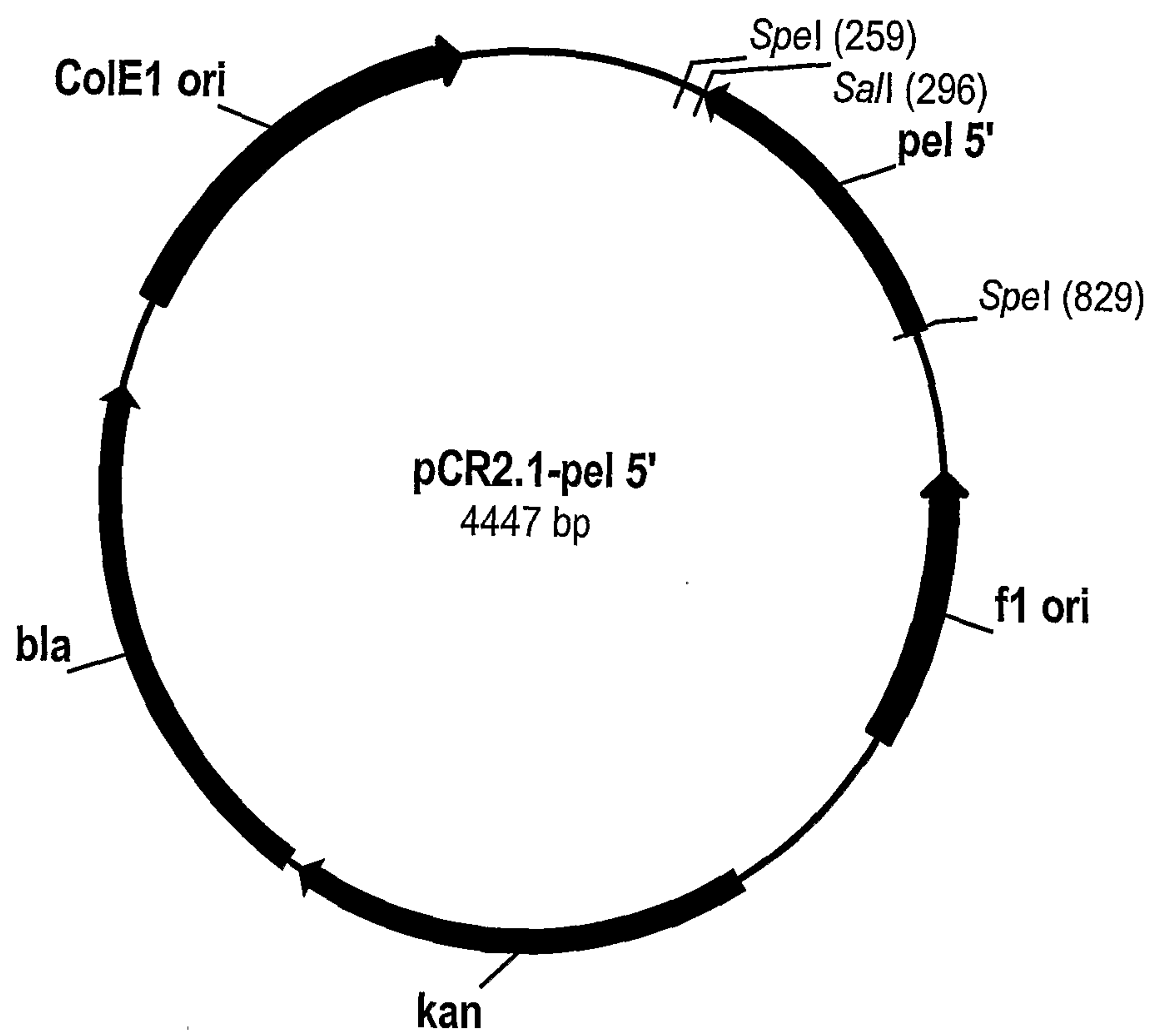


Fig. 38

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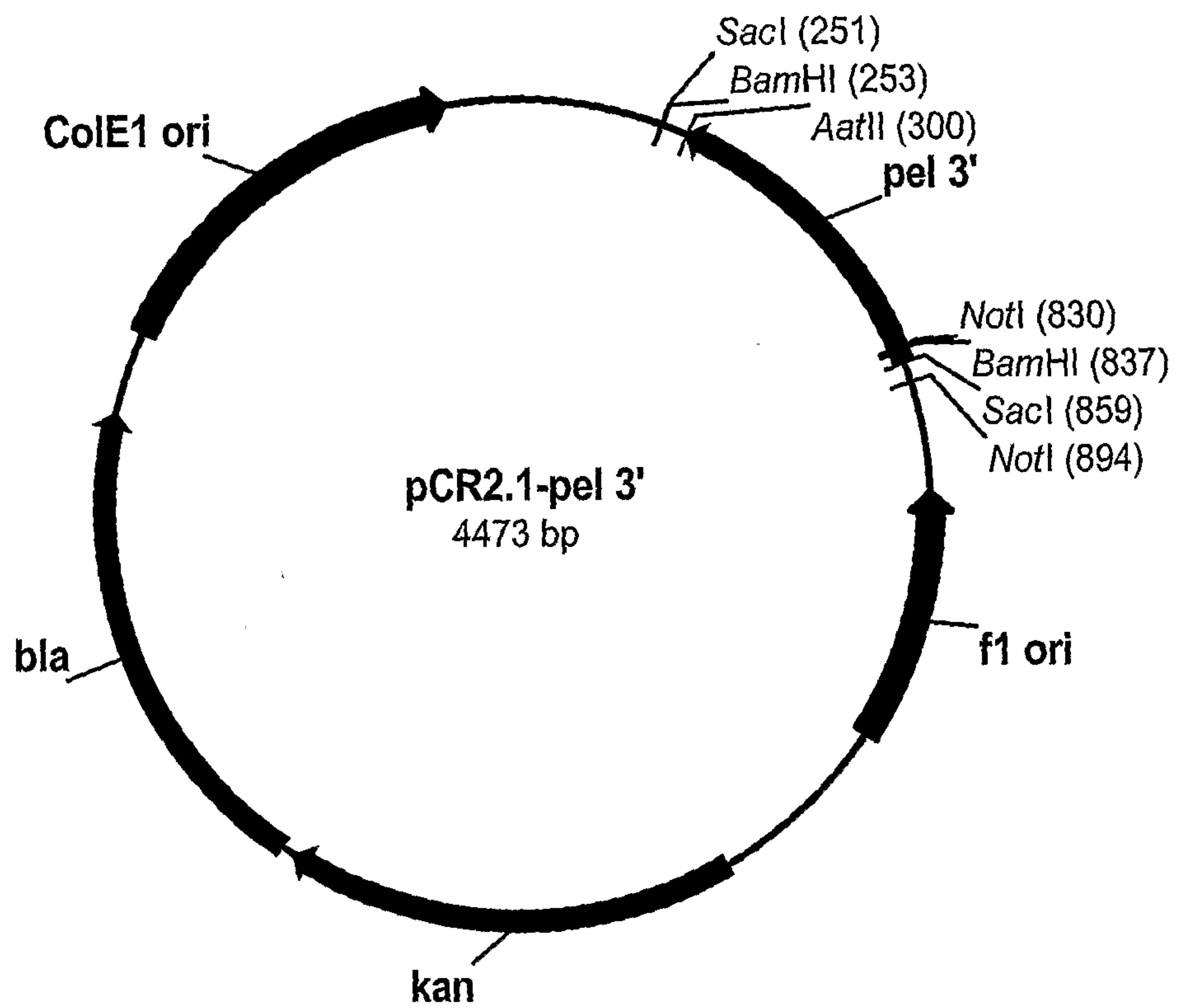


Fig. 39

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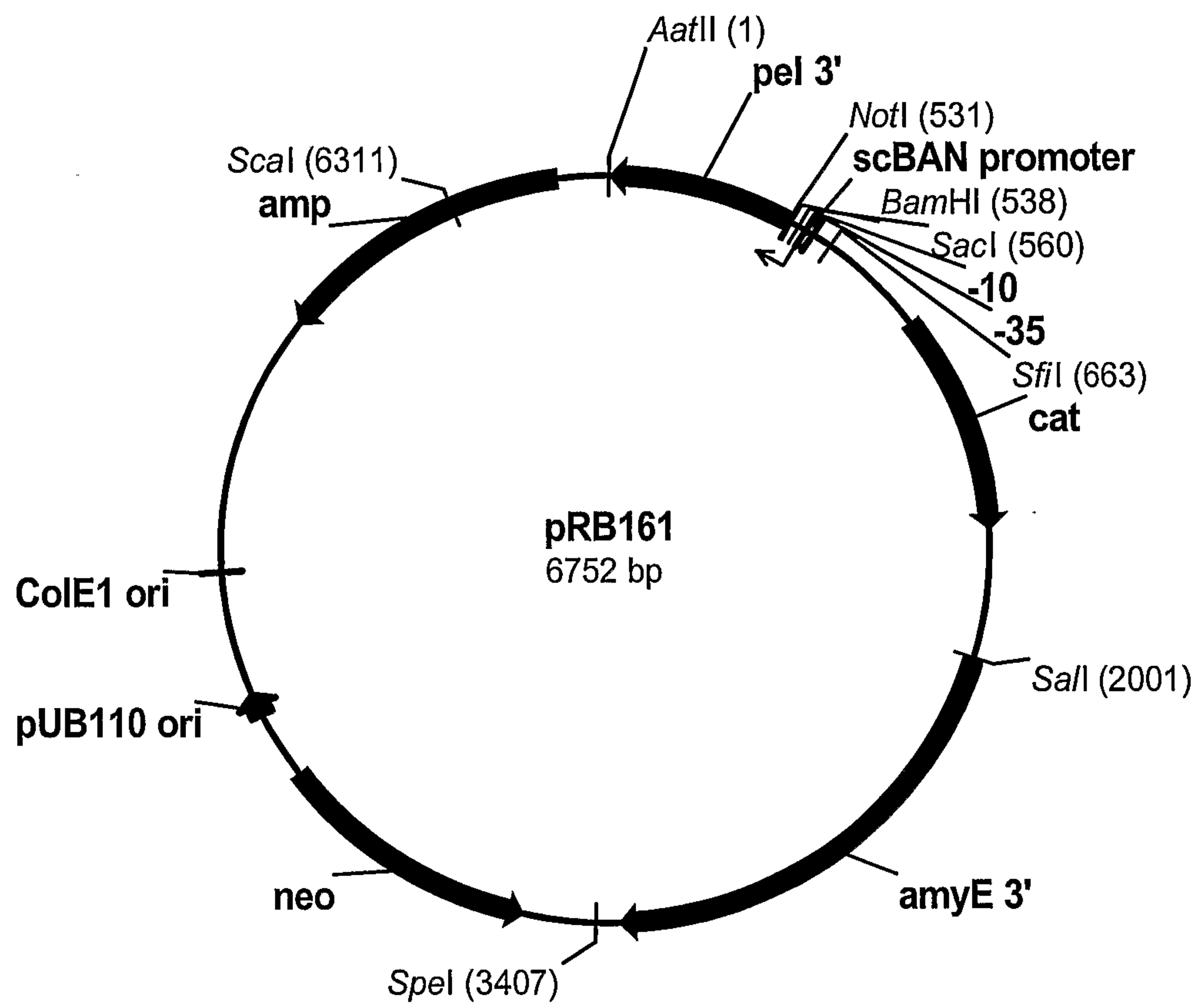


Fig. 40

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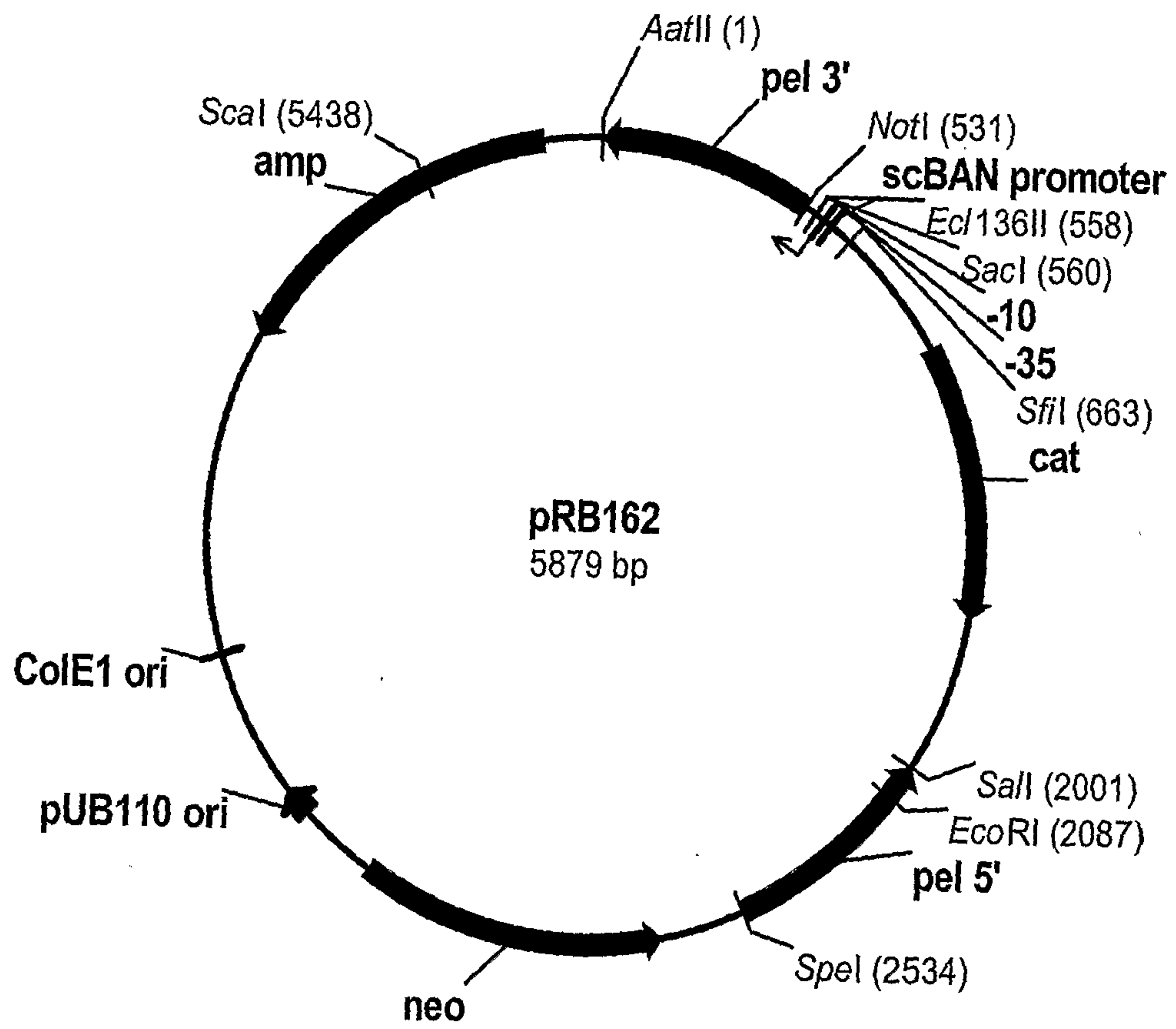


Fig. 41

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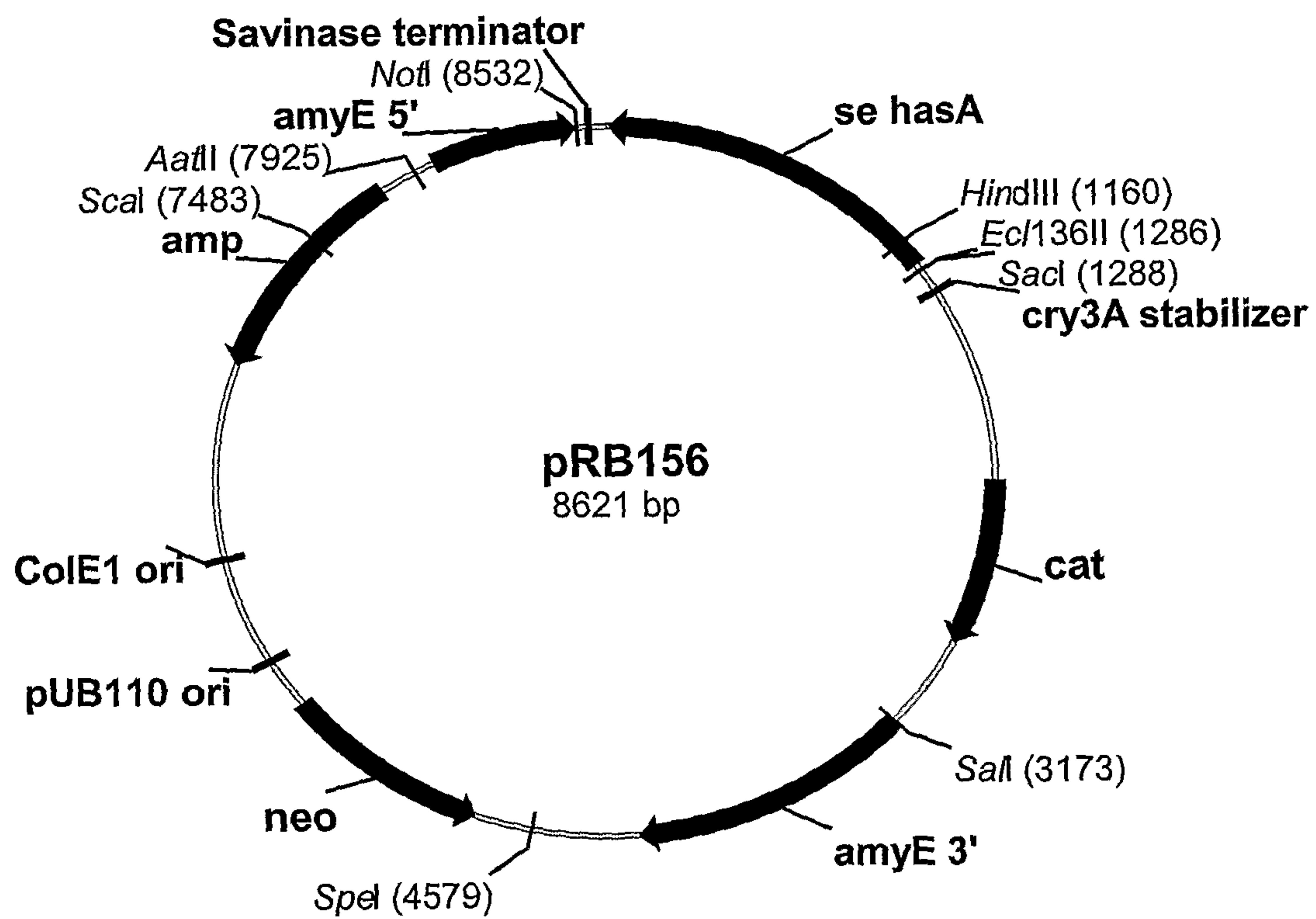


Fig. 42

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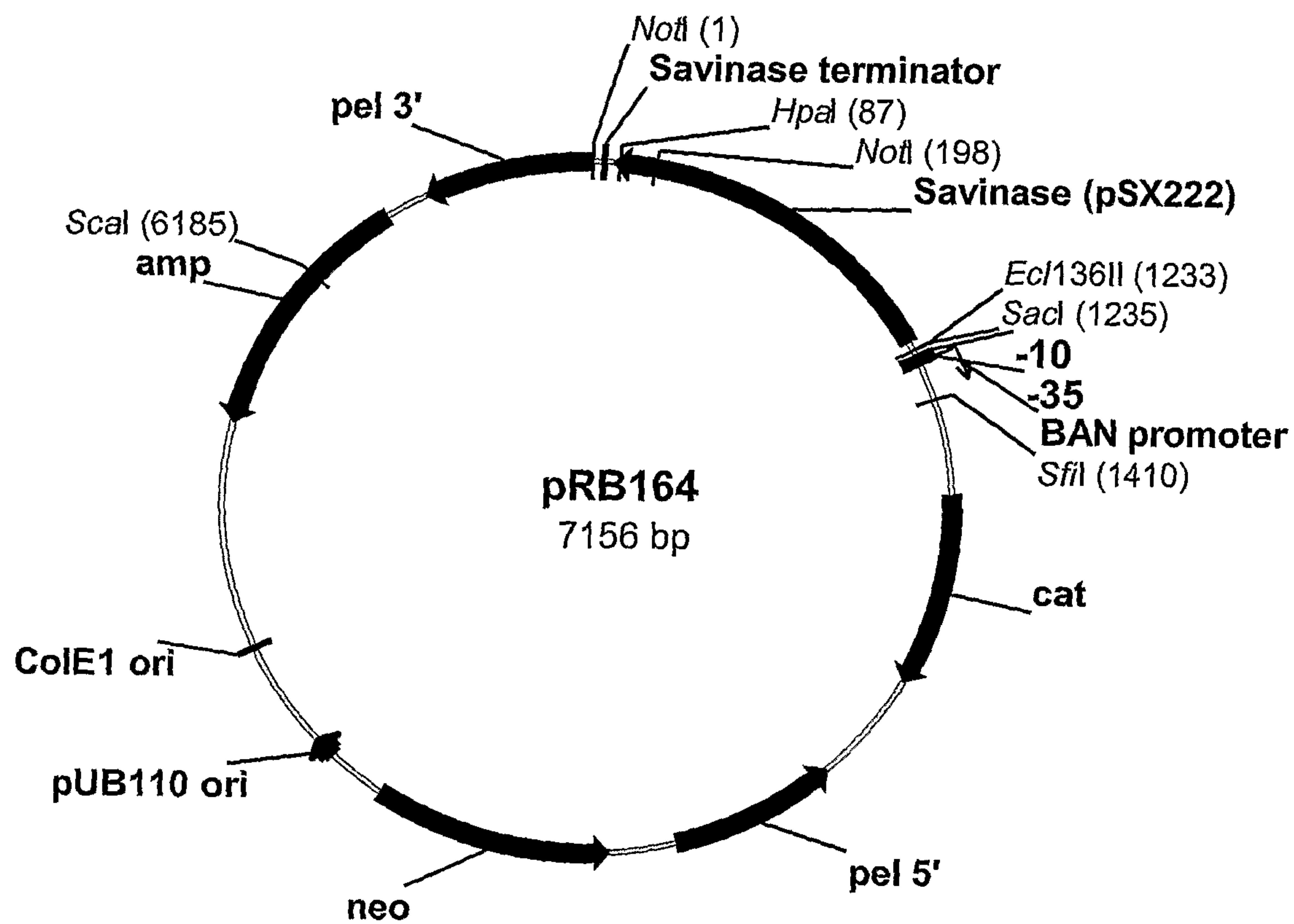


Fig. 43

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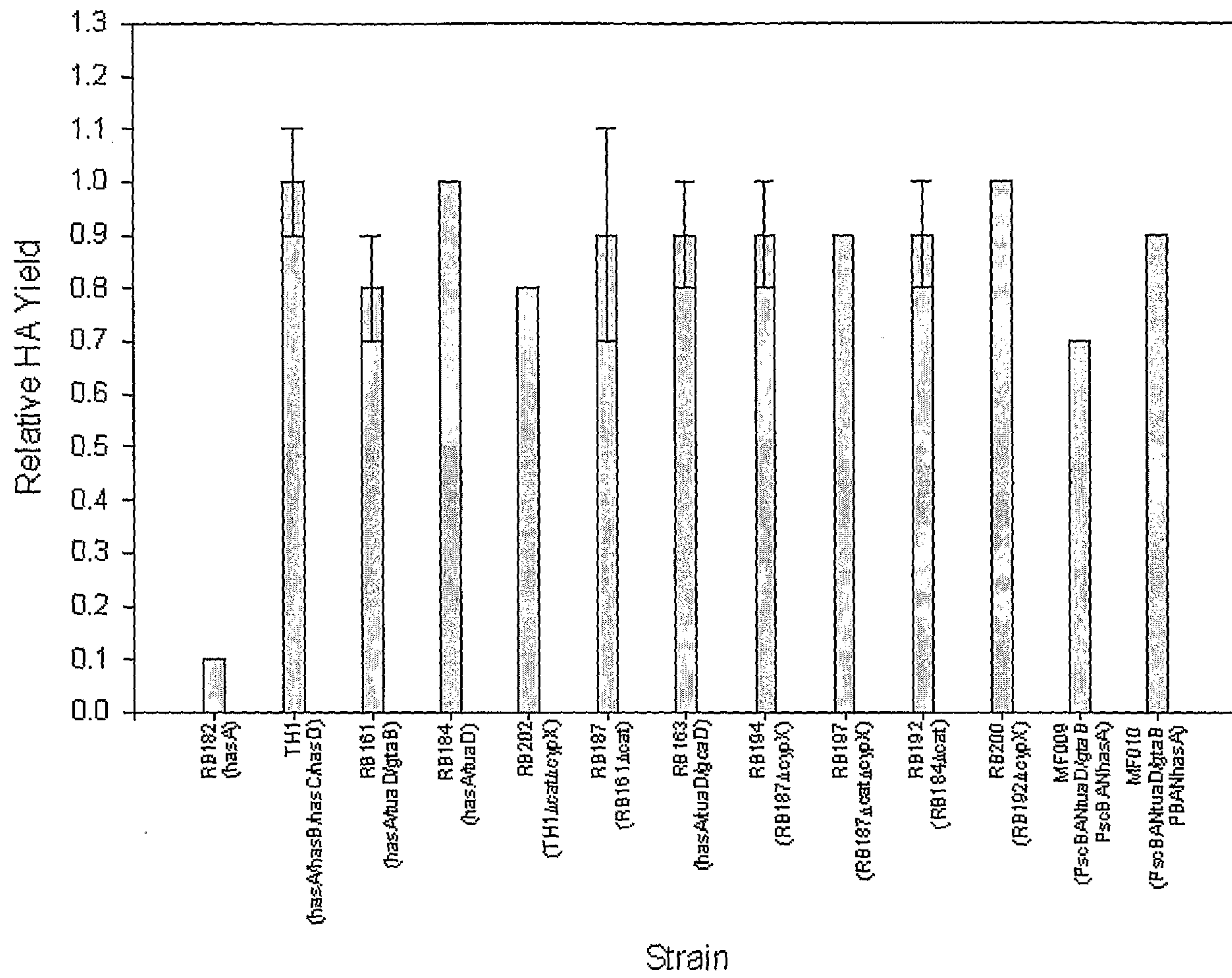


Fig. 44

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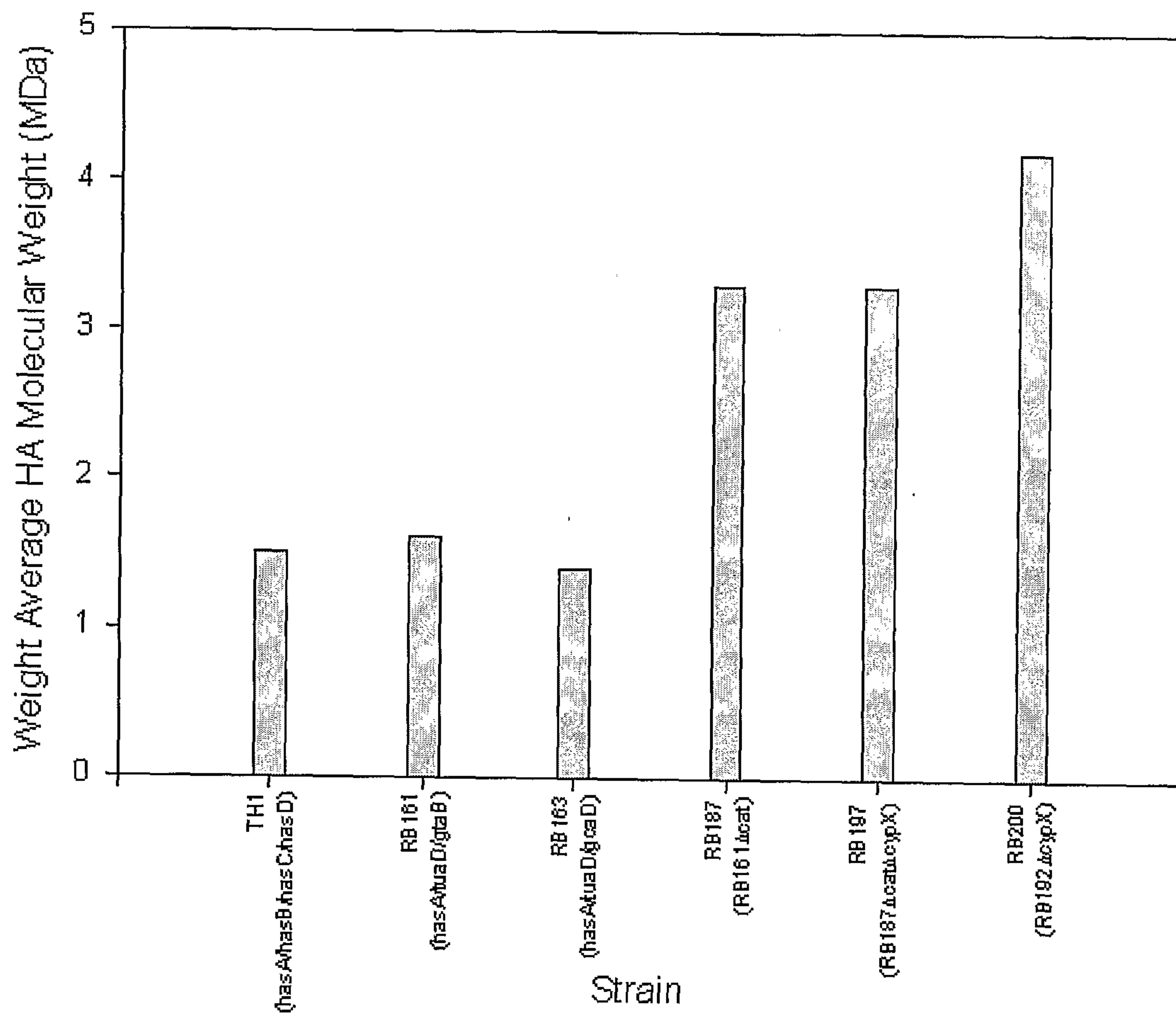


Fig. 45