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(71) Applicant: SOLIDAGO AG [CH/CH]; Hallwylstrasse 21, CH–3005 Bern (CH).

(72) Inventors: BRUNKER, Peter; Luegislandstrasse 163, CH–8051 Zürich (CH). MINAS, Wolfgang; Regensdorferstrasse 5, CH–8049 Zürich (CH). KALLIO, Pauli; Haderlistrasse 6, CH–8964 Friedlisberg (CH). BAILEY, James, E.; Winkelwiese 6, CH–8001 Zürich (CH).

(74) Agent: MICHELI, Michel, P.; Kirker & Cie S.A., 122, rue de Genève, Case postale 324, CH–1226 Thônex (CH).

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(57) Abstract

Methods and compositions for increasing the production of erythromycins are provided. In particular, the invention relates to the engineering of erythromycin–producing organisms to express a heterologous oxygen–binding protein.

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ENHANCEMENT OF ERYTHROMYCIN PRODUCTION BY EXPRESSION OF A CLONED OXYGEN-BINDING PROTEIN

Field of the Invention

Methods and compositions for increasing the production of erythromycins are provided. In particular, the invention relates to the engineering of erythromycin-producing organisms to express a heterologous oxygen-binding protein.

Background to the Invention

Erythromycin is a potent antibiotic with current annual production of some 2,200 tons. This clinically useful, broad-spectrum macrolide antibiotic is naturally produced by the actinomycetes *Saccharopolyspora erythraea*. In spite of extensive classical strain development efforts, classical mutagenesis and selection, volumetric yields of erythromycin in fermentation processes remain rather low at 8 to 9 g/l, as compared to 5-10 fold higher yields in the case of penicillins. Hence, production costs remain high.

Genetic engineering is one approach to overcome the limitations of classical strain improvement programs (*see*, for review, Lal *et al.*, 1996, Crit. Rev. Microbiol. 22(4):201-255). Thus far, recombinant DNA techniques with erythromycin-producing strains of *S. erythraea* have been attempted on only a few strains. The approach generally used has been to identify and clone the genes encoding antibiotic biosynthetic proteins, so as to modify or amplify them.

For example, Hanel *et al.* attempted to achieve higher levels of erythromycin A production in *S. erythraea* by inserting into the chromosome an extra copy of *ery*C1, a gene presumably involved in directly regulating expression of erythromycin biosynthetic genes. Hanel *et al.*, 1993, Biotech. Lett. 15:105-110. In the presence of thiostrepton, the selectable marker gene used for transformation, erythromycin A production of the transformants was two to three fold higher than the non-transformed strain. However, this increased production was lost in the absence of thiostrepton.

Other approaches that have been advanced to increase production levels of erythromycin have been to clone and amplify the biosynthetic erythromycin gene cluster (Lal *et al. supra.*), or to transfer the erythromycin biosynthetic gene cluster

into another organism for potentially higher production. Kao *et al.*, 1994, Science 265:509-512.

Production of some antibiotics is highly dependent on the amount of oxygen available during culture conditions. Clark *et al.*, 1995, Microbiology 141:663-669. Accordingly, one way of increasing production of these types of antibiotics has been to engineer the microbial host to express the *Vitreoscilla* hemoglobin gene (VHb). Such a metabolic engineering strategy has been shown effective in increasing actinorhodin and cephalosporin C production in *Streptomyces coelicolor* and *Acremonium chrysogenum*, respectively (Magnolo, S.K. *et al.*, 1991, Bio/Technology 9:473-476; DeModena, J.A. *et al.*, 1993, Bio/Technology 11:926-929). At low dissolved oxygen levels (DO below 5% of air saturation), *S. coelicolor* transformed with the VHb gene produced ten fold more actinorhodin than non-transformed *S. coelicolor*. Magnolo *et al., supra.* However, when oxygen was not limiting (DO greater than 40% air saturation), both transformed and non-transformed strains produced similar amounts of antibiotic. *Id.* Production of cephalosporin C by the filamentous fungi *A. chrysogenum* is also severely reduced under low oxygen conditions. DeModena, J.A. *et al., supra.* Cultures of transformants expressing high levels of VHb yielded higher amounts of cephalosporin C, especially under oxygen limited conditions.

In contrast to these and many other antibiotics, erythromycin production does not appear sensitive to the levels of dissolved oxygen during culture. Heydarian *et al.*, 1996, Biotechnol. Letts. 18:1181-1186; Clark *et al.*, 1995, Microbiol. 141:663-669. Heydarian *et al.* reported that although growth of *S. erythraea* cultures is inhibited at a low constant dissolved oxygen tension (DOT) of 10% air saturation, the specific erythromycin production is virtually identical to that of a culture where the DOT did not fall below 65%. Similarly, Clark *et al.* found that erythromycin was produced in both oxygen limited and oxygen sufficient cultures. At the same time, however, Clark *et al.* discovered that a different actinomycete, *Amycolatopsis orientalis*, produced the antibiotic vancomycin only in oxygen-sufficient cultures. The results for both species were comparable in both shake flasks and bioreactors. Accordingly, availability of oxygen was not considered a critical limitation to erythromycin production, as long

as the dissolved oxygen concentration in culture are above minimal levels required for growth.

Summary of the Invention

The present invention is directed to methods and compositions for increased production of erythromycin. It has been discovered that the production of erythromycin by an erythromycin-producing organism is significantly increased when such organism also expresses a heterologous oxygen-binding protein.

In one aspect, the present invention provides an erythromycin-producing organism, wherein the organism also expresses a heterologous oxygen-binding protein.

Preferred oxygen-binding proteins for expression in erythromycin-producing organisms are those that bind oxygen reversibly such as globin proteins, and particularly *Vitreoscilla* hemoglobin and functional equivalents thereof.

Also encompassed by the instant invention are methods of increasing erythromycin production. In one aspect, the methods entail producing an erythromycin-producing organism that expresses a heterologous oxygen-binding protein. Methods of achieving the expression of oxygen-binding proteins in erythromycin-producing organisms include, but are not limited to, integrating a gene encoding the oxygen binding protein into the chromosome of the erythromycin-producing organism or transforming the organism to carry the gene extrachromasomally. In another aspect, the methods entail culturing an erythromycin-producing organism that expresses a heterologous oxygen-binding protein under conditions appropriate for production of erythromycin. The methods of the invention also encompass the collection of erythromycin produced from the cultured erythromycin-producing organism.

Finally, yet another aspect of the methods of the invention are novel methods of stably transforming *S. erythraea*.

Brief Description of the Figures

Figure 1. is a diagram of plasmid pETR419, which was used for intergeneric conjugation between *E. coli* and *S. erythraea*.

Figure 2. is a diagram of plasmid pETR432 used for chromosomal integration of an expression construct comprising VHb under the control of the *PmerR* promoter.

Figure 3. illustrates the cloning steps performed to construct pETR432.

Figure 4. summarizes the results of the shake-flask cultivations. Data points for the non-transformed *S. erythraea spp.* are circles. *S. erythraea::vhb* data points are represented by squares.

Figure 5. compares fermentation profiles from *S. erythraea ssp.* (Figure 5A) and *S. erythraea::vhb* (Figure 5B). The bars between Figures 5A and 5B indicate the duration of the respective feeds.

Figure 6. summarizes the results of 5 bioreactor cultivations, three fermentations with *S. erythraea ssp.* (circles) and two cultivations with the recombinant *S. erythraea::vhb* (squares).

Detailed Description of the Invention

A basis for the present invention is the unexpected discovery that erythromycin-producing organisms exhibit increased production of erythromycin when engineered to also express a heterologous oxygen-binding protein. In particular, a strain of *S. erythraea* stably transformed to express *Vitreoscilla* hemoglobin (VHb) produced up to 70% more erythromycin in bioreactor culture, as compared to the non-transformed parental strain.

As discussed more fully below in the Examples section, these surprising results appeared to be due to a direct effect of the heterologous VHb oxygen-binding protein on the erythromycin biosynthetic machinery. Therefore, the present invention is useful in any system for producing erythromycin that is known or can be used in the future.

Without intending to be limited to a particular mechanism, it is believed that the surprising results reported herein for production of erythromycin are due to either an increased level of intracellular oxygen, or an increased efficiency in intracellular oxygen utilization. Accordingly, the invention encompasses increasing

the levels of oxygen-binding proteins within the cell of an erythromycin-producing organism, so as to increase the production of erythromycin.

Erythromycins and Erythromycin Production

An erythromycin-producing organism is any organism, known or to be discovered, that produces erythromycin, either naturally or through genetic engineering. Particularly preferred erythromycin-producing organisms are microorganisms such as bacteria and fungi. While any microorganism can be used as an erythromycin-producing organism, gram positive bacteria are particularly preferred, and especially actinomycetes. For most applications, it is important that the erythromycin-producing organism also express an erythromycin resistance gene.

For purposes of the instant invention, erythromycin includes the naturally occurring erythromycin A, erythromycin B, erythromycin C and erythromycin D. However, the preferred naturally occurring erythromycin is the active antibiotic erythromycin A. Also encompassed by the term erythromycin are active derivatives of erythromycin, which can be produced *in vitro* or through genetic engineering, such as erythromycin E, erythromycin F, 6-deoxyerythromycin B and 6-deoxyerythromycin A (*see* Weber *et al.*, 1991, Science 252:114).

As noted above, erythromycin is naturally produced by *Saccharopolyspora erythraea*. These strains are commercially available from several different depositories (such as ATCC and NRRL), and can be selected through standard techniques well known in the field to obtain strains optimized for maximum production. For example, Paulus *et al.*, 1990, J. Bacteriol. 172:2541, describes a TLC assay to assess erythromycin production. Naturally occurring variants that produce greater amounts of erythromycin can be subject to a high through-put mechanized screen as is commonly used in the pharmaceutical industry. *See* for example, Horan *et al.*, 1993, Abstract, Joint Mtg of Soc. for Industr. Microbiol. and Canad. Soc. of Microbiol., July 31-August 6, 1996, Toronto, Canada, and Zak *et al.*, 1990, Eur. J. Clin. Mircorbiol. Infect. Dis. 9:462-465.

The entire erythromycin biosynthetic gene cluster has been cloned and sequenced. *See* Donadio *et al.*, 1991, Science 262:675-679; Bevitt *et al.*, 1992, Eur. J. Biochem. 204:39-49; and Cortes *et al.*, 1991, Nature 346:176-178. The

role of each domain in the erythromycin synthetic cluster has been proposed or deduced. Donadio *et al.*, 1992, Gene 111:51-60. Erythromycin is comprised of a polyketide-derived 14 membered macrolactone ring, 6dEB, to which are attached two deoxysugars, cladinose and desoamine. Synthesis of 6dEB requires three adjacent *ery*A genes encoding large multifunctional polypeptides. Together, the three adjacent *ery*A genes comprise six modules of repeated motifs, each encoding a different synthetic unit specific for one of the elongation steps. Donadio *et al.*, 1993, *supra*.

Efficient transformation systems and cloning vectors developed for manipulating *S. erythraea* are known and described in, for example, Donadio *et al.*, 1990, J. Bacteriol. 171:350-360, Kieser and Hopwood, 1991, Meth. Enzymol. 204:430-458, and Donadio and Hutchinson, 1991, Gene 100:231-235. Accordingly, other engineered derivatives of *S. erythraea* useful for the production of erythromycin are those in which additional copies of all or part of the erythromycin gene cluster have been introduced into the cell, or those in which particular genes have been targeted and disrupted. Further, novel erythromycins can be produced through domain exchanges of the synthetic units of the erythromycin gene cluster, as described by Pieper *et al.*, 1997, Biochem. 36:1846-1851; Luo *et al.*, 1996, Bioorg. Med. Chem. 4:995-999; Bedford *et al.*, 1996, Chem. Biol. 3:827-831; and Oliynyk *et al.*, 1996, Chem. Biol. 3:833-839. *See* for review Khosla and Zawada, 1996, Trends Biotechnol. 14:335-41; and McDaniel *et al.*, 1995, Nature 375:533.

For example, Hanel *et al., supra* was able to increase erythromycin production by inserting into the chromosome an extra copy of the *ery*C1 gene. Weber *et al., supra* used targeted gene disruption of the *ery*F gene to obtain an *S. erythraea* strain that produced 6-deoxyerythromycin A, an acid derivative of erythromycin A. Similarly, Donadio *et al.*, 1993, PNAS:USA 90, 7119 produced an erythromycin analog by reprogramming erythromycin synthesis.

Additionally, with the cloning and characterization of the erythromycin production gene cluster, other organisms may be engineered to produce erythromycin. Indeed, methods have been developed to clone the erythromycin biosynthetic genes into *E. coli* using an actinomycete/*E. coli* cosmid. Tuan *et al.*, 1990, Gene 111:21. Further, Kao *et al.*, 1994, Science 265:509-512, reported

expression of the entire 6-deoxyerythronolide B synthase complex in *Streptomyces coelicolor*. Any engineered erythromycin-producing organisms, including but not limited to these examples, are within the scope of the invention.

Erythromycin may be collected from the cultured erythromycin-producing organism using methods well known to those of skill in the fermentation and pharmaceutical fields.

Expression Of Heterologous Oxygen-Binding Proteins In Erythromycin-Producing Organisms

Heterologous expression of oxygen-binding proteins in erythromycin-producing organisms may be achieved by a variety of techniques known in the art. For purposes of the invention, the term "heterologous" is defined as expression of a protein that does naturally exist in that organism, or expression of a protein in a cellular location where it does not naturally occur, or an engineered increase in endogenous expression of a protein.

Oxygen-binding proteins expression in an erythromycin-producing organism may advantageously be achieved by recombinant DNA technology using techniques well known in the art. Such methods can be used to construct expression vectors containing a chosen oxygen-binding protein nucleotide sequence and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, infra, and Ausubel et al., 1989, infra. Alternatively, RNA capable of encoding oxygen-binding protein nucleotide sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

The particular expression vector systems utilized to express nucleotide sequences encoding oxygen-binding proteins will depend upon the erythromycin-producing organism. Generally, the expression vector will comprise a promoter operably linked to nucleotide sequences encoding an oxygen binding protein and containing a transcriptional initiation sequence, and a transcriptional terminator. The promoter should be one that is transcriptionally active in the erythromycin-

producing organism, and may be inducible or constitutive. The oxygen-binding protein encoded by the expression vector may be one that naturally occurs in the erythromycin-producing host, or may be from a different organism.

The expression construct should also encode appropriate recognition sequences for translational initiation in the erythromycin-producing organism. Optionally, the expression construct may contain signals for intracellular targeting of the oxygen-binding protein. For example, an oxygen-binding protein normally localized to the mitochondria, or the chloroplast, may instead be expressed in the cytoplasm by modifying the targeting signals on the translational product that are known to those of skill in the art. Finally, the vector may be designed for extrachromosomal maintenance, or for recombination into the chromosome.

Appropriate promoter sequences, transcriptional and translational initiation sequences, and terminators for a given host chosen as an erythromycin-producing organism are well known. Expression vectors for various hosts, such as plants, mammalian cells, insect cells, *E. coli, Bacillus,* and fungi (including *Saccharomyces, Aspergillus,* and *Penicillium*) are well known to those of ordinary skill in the art (*see* for a general review regarding expression in antibiotic-producing organisms, Lal *et al., supra*). Further, illustrated below by way of working examples are additional expression vectors appropriate for expression in actinomycetes, particularly *Saccharopolyspora* and *Streptomycetes*.

Alternatively, heterologous expression of an oxygen-binding protein may be achieved by altering expression of an oxygen-binding protein endogenous to the cell. As an example, homologous recombination may be used to replace an endogenous promoter with another more transcriptionally active promoter. Transcription may also be transactivated by inserting a transcriptional enhancer adjacent the endogenous gene encoding an oxygen-binding protein, or by increasing the activity of transcriptional enhancers.

Oxygen-Binding Proteins For Use In The Invention

Oxygen-binding proteins useful for expression in erythromycin-producing organisms include any proteins which bind oxygen, particularly those which bind oxygen reversibly such as the globins. Preferred oxygen-binding proteins are those which are capable of increasing erythromycin production in erythromycin-producing organisms.

Oxygen-binding proteins that may be used in the invention include, but are not limited to, *Vitreoscilla* hemoglobin (VHb), *Alcaligenes eutrophus* flavohemoprotein, horse heart myoglobin, *E. coli* hemoprotein, *B. subtilis* hemoprotein, yeast flavohemoglobin, soybean leghemoglobin, lupin leghemoglobin, and sperm whale myoglobin. As noted above, the oxygen-binding protein may also be one that is endogenous to the erythromycin-producing organism.

The genes encoding a large number of oxygen-binding proteins have been cloned and their sequence determined. For example, known polynucleotide sequences of globin proteins useful in the instant invention include but are not limited to those encoding a cyanobacterium myoglobin (Potts *et al.*, 1992, Science 256:1690-1692), *Scapharca inaequivalvis* hemoglobin (Gambacurta *et al.*, 1993, FEBS Lett. 330:90-94), *Aplysia limacina* myoglobin (Cutruzzola *et al.*, 1996, Biochem. J. 314:83-90), *Ascaris* hemoglobin (Sherman *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:11696-11700), *Pseudoterranova decipiens* nemotode hemoglobin (Dixon *et al.*, 1991, Natl. Acad. Sci. USA 88:5655-5659, and Dixon *et al.*, 1992, J. Mol. Evol. 35:131-136), *Paramecium caudatum* hemoglobin (Yamauchi *et al.*, 1992, Biochem. Biophys. Res. Commun. 182:195-200), *Rhizobium meliloti* haemoprotein (David *et al.*, 1988, Cell 54:671-683), and *Saccharomyces cerevisiae* (Shimada *et al.*, 1989, J. Biochem. 105:417-422).

Particularly suitable for use in the present invention are those oxygen-binding proteins which have relatively high k_{off} rates such as VHb (k_{off} 5600 s^{-1}; Orii and Webster, 1986, J. Biol. Chem. 261:3544-3547) or relatively low oxygen affinity such as horse heart myoglobin (K_D 0.79 μM; Wittenberg *et al.*, 1985, in Nitrogen fixation research progress, H.J. Evand et al. Eds. Martinus Nijhoff Publishers, Dordrecht, p. 354). Therefore, particularly preferred oxygen binding proteins can be those proteins with a k_{off} rate for oxygen of greater than 10 s^{-1}, more preferred greater than 100 s^{-1}, or a K_D for oxygen of more than 0.5 μM,

although it will be understood that oxygen-binding proteins with rate constants outside of these parameters will also be useful. Other examples of preferred oxygen-binding proteins are globins such as hemoglobin, myoglobin, and leghemoglobins. The properties of many oxygen-binding proteins, including globins, are disclosed in the literature. Additionally, techniques for determining the oxygen-binding properties of a protein such as a globin are well known to one of skill in the art and can be performed without undue experimentation.

An especially advantageous oxygen-binding protein for use in the instant invention, as described herein by way of working example, is *Vitreoscilla* hemoglobin ("VHb"). The complete sequence of the VHb gene is described in U.S. Patent No. 5,049,493, *supra*. Mutants of VHb which bind oxygen are also within the scope of the present invention.

Another particularly advantageous oxgygen-binding protein for use in the invention is *Alcaligenes eutrophus* flavohemoprotein (AeFH). This hemoglobin molecule shares 51% homology with VHb, and is very useful for biotechnological applications such as described herein. The complete primary sequence of AeFH and the nucleotide sequence encoding the protein are described in Cramm *et al.*, 1994, J. Biol. Chem. 269:7349-7354.

Also encompassed within the scope of the invention is any nucleotide sequence that (a) hybridizes to the complement of the nucleotide sequence of the VHb or AeFH genes under highly stringent conditions, i.e., hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. *et al.*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product that binds oxygen; and (b) any nucleotide sequence that hybridizes to the complement of the nucleotide sequence of the VHb or AeFH genes under less stringent conditions, such as moderately stringent conditions, i.e., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel *et al.*, 1989, supra), yet still encodes a functionally equivalent gene product that bind oxygen. Functional equivalents of VHb and AeFH include naturally occurring hemoglobin genes present in other species, and mutant VHb or AeFH whether naturally occurring or engineered that retain at least some reversible oxygen

binding function. Species from which genes encoding functional equivalents of VHb and AeFH include but are not limited to bacterial species, and, in particular, those bacterial species found in soil, dung, and other oxygen-accessible, but poorly aerated, environments.

The invention also encompasses degenerate variants of nucleotide sequences that encode the amino acid sequence of the VHb protein, VHb mutants, AeFH protein, AeFH mutants, and functional equivalents of VHb or AeFH encoded by nucleotide sequences which hybridize to the complement of the nucleotide sequence of the VHb gene or the AeFH gene, respectively. For example, the nucleotide sequence can be altered so as to optimize amino acid codon usage for expression in the chosen erythromycin-producing organism.

The following examples are provided to further illustrate, but not limit, the invention described above.

EXAMPLES

Example 1: Insertion of *VHb* expression vectors into *S. erythraea*

We used two different approaches to obtain expression of VHb protein in *S. erythraea*. In the first approach, an intergeneric conjugation plasmid was used for transfer of an expression plasmid from *E. coli* to *S. erythraea*. The second approach, designed to increase stability of the expression construct, used a vector for chromosomal integration of a VHb expression construct.

Material and Methods

S. erythraea:spp is an industrial strain obtained from SOLIDAGO. *Streptomyces lividans* TK64 is described in Hopwood *et al.*, 1983, J. Gen. Microbiol. 129:2257-2269. *E. coli* strains were grown at 37°C in either dYT or LB liquid medium (Sambrook *et al.*, 1989, "Molecular Cloning: A Laboratory Manual" Second Ed., Cold Spring Harbor Lab. Press.) or on dYT plates containing 1.6 % agar. *S. erythraea* strains were maintained on R5 plates (Hopwood *et al.*, 1985, "Genetic Manipulation of *Streptomyces*: A Laboratory Manual", John Innes Foundation, Norwich, U.K.) or incubated in SM liquid medium (Birr *et al.*, 1989, Appl. Microbiol. Biotechnol. 30:358-363) at 34°C. *S. lividans* was maintained in the same media incubated at 30° C. Mycelial stocks of

S. erythraea were kept at -80°C in 30% glycerol, whereas *S. lividans* stocks were stored as spore solutions in 20% glycerol at -20°C. Media was supplemented with the appropriate antibiotics (100 μg/ml ampicillin, 12.5 μg/ml thiostrepton, 50 μg/ml kanamycin, 30 μg/ml chloramphenicol and 40 μg/ml nalidixic acid) when needed.

Mini-preparations of plasmid DNA were done by an alkaline lysis method described by Lee and Rasheed, 1990, Biotechniques 9:976-971. Genomic DNA from *S. erythraea* and *S. lividans* was isolated according to protocols by Hopwood *et al.*, 1985, *supra*. Restriction enzymes, T4 DNA ligase, alkaline phosphatase, Klenow polymerase and polymerase were obtained from commercial sources and used as recommended by the manufacturers. Standard DNA techniques and Southern blot analysis were done as described by Sambrook *et al., supra.*

Polymerase chain reaction (PCR) for amplification of *PmerR* and *VHb* were performed with a GeneAmp 9600 PCR system (Perkin Elmer) using template specific conditions. All PCR fragments used for subsequent expression of *VHb* were confirmed by DNA sequencing with the dideoxy nucleotide chain termination method.

Competent *E. coli* XL1 Blue (Stratagene) and ET12567 (MacNeil et al., 1992) were prepared and transformed by the method of McKenney et al., 1981.

Preparation of *S. erythraea* protoplasts and PEG mediated transformation was performed according to a protocol of Hopwood *et al., supra* for *Streptomyces lividans*, with slight modifications. The cells were grown 4-5 days in TSB (Oxoid) containing 0.25% glycine. For protoplast formation, the final concentration of lysozyme was 8 mg/ml (instead of 4 mg/ml for *S. lividans*). Additionally, PEG3350 (Sigma) was used instead of PEG 1000 in the transformation reaction. For transformation of *S. erythraea*, non-methylated DNA isolated from *E. coli* ET12567 was used. Since regeneration of *S. erythraea* protoplasts seemed to be much slower compared to that of *S. lividans* protoplasts, the antibiotic overlay was done 48 hours after transformation.

Conjugational transfer of plasmids from *E. coli* to *S. erythraea* was performed on plates as described by Bierman *et al.*, 1992, Gene 116:43-49.

To detect VHb protein, strains of *S. erythraea* were grown in 200 ml of SM medium for 4-5 days at 34°C. Cells were harvested, washed twice in buffer (100

mM Tris-HCl pH7.5, 50 mM NaCl, 1 mM EDTA), resuspended in 20 ml of buffer and disrupted by passing 3x through a French press (Aminco SLM Instruments Inc.) operated at 1000-1500 psi. The soluble cellular fraction was used for Western blots (Winston et al., 1987) and for determination of the CO difference spectrum (Webster & Liu, 1974). Rabbit-anti-VHb serum was obtained from Cocalico Biologicals (Reanstown, PA). Protein concentration was determined by the method of Bradford (1976) using BIORAD dye reagent and bovine serum albumin as a standard.

Cultivation of *S. erythraea* was begun by inoculating 1.5 ml of a glycerol stock into a seed culture of 30 ml vegetative medium I (per liter: 16 g Argo corn starch, 10 g dextrin, 15 g soybean flour, 2.5 g NaCl, 5 ml corn steep liquor, 1 g $(NH_4)_2SO_4$, 6 ml soybean oil and 4 g $CaCO_3$, pH adjusted to 6.5) in a 250 ml baffled shake flasks. The seed culture was incubated for 40 h at 34°C with 250 rpm agitation in a humidified rotary shake incubator (Infors). Three mls of this seed culture were then used to inoculate 27 ml of half strength fermentation medium I (per liter: 35 g corn starch, 32 g dextrin, 33 g soybean flour, 7 g NaCl, 20 ml corn steep liquor, 2 g $(NH_4)_2SO_4$, 6 ml soybean oil and 8 g $CaCO_3$ with the pH adjusted to 6.5). Cultivations for erythromycin production were run for 9 days with the following feeding procedure: soybean oil 0.2 ml/day from day 0-6; and n-propanol 0.1 ml/day from day 0-5 and 0.15 ml/day from day 6-9. Shake flasks were weighted daily and sterile water was added as necessary to compensate for evaporation.

A standard bioassay was applied to determine the erythromycin titers. Briefly, square 12 by 12 cm petri dishes were filled with 35 ml test medium (27.5 g/l TSB, 2 g/l glucose, 2% agar). After the medium had solidified, a lawn of *M. luteus* was prepared by pouring 35 ml of test media containing 35 μl of an *M. luteus* overnight culture in TSB onto the plates. After cooling, up to 6 sterile antibiotic test disks (Difco) were placed onto the plates. Ten μl samples were pipetted on the disks and plates were incubated for 2 days at 34°C to allow a lawn of *M. luteus* to develop before the zone of growth inhibition was measured. Erythromycin titers were determined with erythromycin (Fluka) standards measured under the same conditions.

Results

Construction of a conjugable VHb expression plasmid

Intergeneric conjugation of plasmids from *E. coli* to *S. erythraea* has been described by Mazodier and coworkers (1989, J. Bacteriol. 171:3583-3585) and seemed in some cases even to be more efficient than transformation (Bierman *et al.*, 1992, *supra.*). Thus, a *VHb* expression vector was constructed for conjugation into *S. erythraea*. An expression cassette consisting of the *PermE** promoter (Bibb *et al.*, 1985, Gene 38:E375-E368, and as cited in Motamedi *et al.*, 1995, Gene 160:25-31), *VHb* (Khosla and Bailey, 1988, Mol. Gen. Genet. 214:158-161, and U.S. Patent No. 5,049,493, issued September 17, 1991, both of which are incorporated by reference herein) and the origin of transfer (*oriT*) from pPM927 (Smokvina *et al.*, 1990, Gene 94:53-59) was constructed as shown in Figure 1. This expression cassette was cloned into the *Streptomyces/E. coli* shuttle vector pJOE875 (Altenbuchner *et al.*, 1992, Meth. Enzymol. 216:457-466) containing the *Streptomyces* origin of replication from plasmid pIJ350 (Hopwood *et al., supra*) and the pUC origin for replication in *E.coli*. The resulting plasmid was designated pETR419. After conjugation from *E. coli* S17.1 (genotype *recA thi pro hsdR*$^-$M$^+$ RP4:2-Tc:Mu:KmTn7 Tp^R Sm^R; Simon *et al.*, 1983, Biotechnol. 1:784-791) into *S. erythraea*, thiostrepton resistant exconjugants were selected. Counter-selection against *E. coli* was done with 40 μg/ml of nalidixic acid. The isolated *S. erythraea* exconjugants showed some VHb activity as judged by CO-binding assays. As confirmed by plasmid isolations from exconjugants, the expression plasmids tended to undergo recombination in *S. erythraea*.

Chromosomal integration of a VHb expression cassette in S. erythraea spp.

Plasmid instability prompted us to construct a vector for chromosomal integration of a VHb expression cassette in *S. erythraea*. To further avoid possible recombination in *S. erythraea* the *PermE** promoter was replaced by another constitutive *Streptomyces* promoter. Previously it was shown that the two promoters of the mercury resistance determinant of *S. lividans* 1326 are constitutive in the absence of their negative regulator (MerR). Brünker *et al.*, 1996, Mol. Gen. Genet. 251:307-315. Since *S. erythraea* was not expected to contain this mercury regulated repressor, the *PmerR* promoter was used for VHb

expression. The complete sequence of the *PmerR* promoter has been reported in Sedlmeier and Altenbuchner, 1992, Mol. Gen. Genet. 236:76-85; Brunker *et al.*, 1996, Mol. Gen. Genet. 251:307-315; and Klein *et al.*, 1997, Exs. (Switzerland) 80:133-151.

PmerR and the VHb gene were amplified by PCR during which convenient restriction sites (*Sal*I and *Eco*RI for *PmerR*; *Eco*RI and *Bam*HI for VHb) were introduced at the end of the fragments and cloned into pIC19H (which only replicates in *E. coli*). The following primers were used to amplify the *PmerR* promoter:

forward: 5' TTGTCGACCCGCGGCGAATGCGCCGG (SEQ ID NO:1)

reverse: 5' TTGAATTCCCTTTCCACCAGCAGCTA (SEQ ID NO:2)

VHb sequence was amplified with appropriate restriction sites using the following primers:

forward: 5' TTGAATTCATGTTAGACCAGCAAACC (SEQ ID NO:3)

reverse: 5' GGATCCTTATTCAACCGCTTGAGC (SEQ ID NO:4)

In addition the thiostrepton resistance gene (*tsr*) (Hopwood *et al.*, 1985, *supra*) for antibiotic selection in *S. erythraea* was inserted into this plasmid. The *Streptomyces* phage ϕC31 attachment site (*att*) was included in the expression vector to facilitate homologous recombination with the *S. erythraea* chromosome. The ϕC31 att sequence is described in Kuhstoss *et al.*, 1991, J. Mol. Biol. 222:897-908 and Rausch and Lehmann, 1991, Nucl. Acids Res. 19:5187-5189, and can be found at GenBank accession: X60954. This construction was done under the assumption that *S. erythraea* also carries the ϕC31 *att* site. The resulting plasmid, pETR432 containing *tsr*, *PmerR-vhb* and *att* ϕC31 is shown in Figure 2, and a diagram of its construction illustrated in Figure 3. This plasmid was transformed using a modified transformation procedure into *S. lividans* TK64 and *S. erythraea* , as described above, resulting in strains *S. lividans::vhb* and *S. erythraea::vhb*.

Expression and activity of *VHb* in transformants of both strains was confirmed by Southern and Western blot analysis and PCR (data not shown). In addition, a DNA fragment with *VHb* specific primers was amplified from chromosomal DNA of *S. erythraea::vhb* that was transformed with pETR432. The

amplified fragment was cloned into pIC19H and sequenced to confirm the correct DNA sequence of *VHb*.

Biological activity of synthesized VHb was demonstrated by carbon monoxide (CO)-binding assays. A specific CO difference spectrum was observed in crude extracts of *S. lividans::vhb* and *S. erythraea::vhb* after treatment with CO while no peak at about 418 nm could be detected with the control strains that did not express VHb.

Stability of the recombinant S. erythraea strain

The genetic stability of the chromosomally integrated *VHb* expression cassette was determined by assaying retention of thiostrepton resistant in long term production cultures grown in the absence of thiostrepton. Cells of each sample taken for the erythromycin assays were plated onto R5 agar medium and replica plated on thiostrepton-containing agar plates. After 9 days of cultivation in the production medium without thiostrepton selection, the ratio of thiostrepton resistant to thiostrepton sensitive colonies was greater 97% (data not shown) demonstrating that the integrated *VHb* expression cassette in the chromosome of *S. erythraea* was stably maintained.

Discussion

Intergeneric conjugation of plasmids from *E. coli* into several *Streptomyces* strains is fairly efficient. In addition to *S. lividans* and *S. coelicolor*, *S. pristinaespiralis* and *S. viridochromogenes* can be used as recipients in such conjugation experiments (Mazodier *et al., supra*). Furthermore, it has been reported that plasmids could be conjugated from *E. coli* into *S. fradiae*, *S. ambofaciens* and even into *Sac. spinosa*. These latter strains are relatively resistant to transformation by PEG mediated protoplast transformation (Bierman *et al., supra*). All these conjugation systems require the origin of transfer (*oriT*) from RK2 in *cis* (Guiney & Yakobson, 1983, PNAS:USA 80:3595-3598), and transfer functions supplied in *trans* from the donor strain *E. coli* S17.1 (Mazodier *et al., supra*). Therefore a conjugable *VHb* expression plasmid, pETR419, was constructed as described above.

Plasmid pETR419 was transformed into *E. coli* S17.1 and then conjugated with *S. erythraea* to yield thiostrepton resistant exconjugants. Although the

selected clones seemed to synthesize small amounts of active VHb, the expression plasmids were unstable in *S. erythraea*. This instability could result from homologous recombination between the *PermE** fragment and the chromosomal *ermE* region within the erythromycin biosynthesis cluster of this strain.

Thus, it was decided to integrate a *VHb* expression cassette into the chromosome of *S. erythraea*. spp. and to replace *PermE** by another constitutive promoter (*PmerR*) from *S. lividans* 1326. This heterologous promoter should reduce the chances for homologous recombination with the erythromycin biosynthetic genes. As the target for site specific integration, the *Streptomyces* phage ϕC31 attachment site was chosen which has previously been used for successful integration of plasmids into the chromosomes of *S. lividans, S.fradiae* and *S. ambofaciens* (Bierman *et al., supra*).

The resulting *VHb* expressing construct, pETR432, containing the *PmerR-vhb* expression cassette, the thiostrepton resistance gene (*tsr*) and a fragment carrying the ϕC31 attachment site was successfully transformed to *S. erythraea*. The presence of chromosomal integration of *VHb* was demonstrated by Southern blot analysis and amplification of *VHb* by PCR from chromosomal DNA of *S. erythraea::vhb*. All tested transformants showed the same restriction pattern in Southern blots which indicates that the plasmid integration occurred at a specific site, probably the ϕC31 attachment site of the *S. erythraea* chromosome. Integration at this site did not have any negative effect on the metabolism or viability of the recombinant strain. Furthermore, the integration was shown to be stable for at least 9 days in the absence of thiostrepton. Under these conditions, over 97% of the cells still retained the thiostrepton resistance phenotype.

CO-binding assays confirmed synthesis of active VHb. A typical VHb CO difference spectrum with an absorption maximum at 420 nm was observed in *S. lividans*. With crude extracts of *S. erythraea::vhb*, two absorption maxima were detected, one at 450 nm and one at 424 nm, respectively. While the peak at 424 nm is related to *VHb* expression the peak at 450 nm probably refers to a cytochrome P-450 monooxygenase (EryF) from *S. erythraea* (Katz and Donadio, 1995) as it is also found in *S. erythraea* without *VHb*. The absorption maximum at 424 nm shows synthesis of active VHb, although a slight shift in maximal absorption from 420 nm to 424 nm is observed.

From CO-binding assays it can be concluded that VHb activity in *S. lividans::vhb* is about 5-fold as high as in *S. erythraea::vhb* under the same conditions indicating that *PmerR* is more active in the strain from that it originally was isolated than in *S. erythraea* spp.

Example 2: Increased Production of Erythromycin

In this experiment, the effect of co-expression of an oxygen-binding protein, VHb, on erythromycin production in batch and bioreactor culture was investigated.

Materials and Methods

The bacterial strains used in this study were *S. erythraea NRRL2338*, and the erythromycin production strain *S. erythraea ssp.*, and transformant *S. erythraea::vhb* described above in Example 1.

S. erythraea NRRL2338 was grown on R5 (Hopwood *et al.*, 1985, *supra*) plates to obtain spores and to prepare spore suspensions for storage at -20°C. The production strain and its recombinant variant which do not sporulate were maintained either on R5 plates at 4°C for up to 2 months or as mycelial cultures grown on V1 medium (see below), centrifuged and resuspended in 30% glycerol for storage at -70°C in 1.5 ml aliquots. These stocks were used directly as inoculum for the different cultivations.

Micrococcus luteus (ATCC 9341) was used as challenge strain in the erythromycin bioassay.

Vegetative 1 (V1), Vegetative 2 (V2) and Fermentation (F1) medium were used for liquid cultures. Medium V1 contains per liter of reverse osmosis (RO) water 16 g Argo corn starch (CPC International Inc, Englewood Cliffs, N.J., USA), 10 g dextrin (D-2256, Sigma), 15 g soybean flour (32HO41, Sigma), 2.5 g NaCl (Merck), 5 ml corn steep liquor (C-Plus Cerestar F15855, about 50 % solids, Cerestar France SA, Haubourdin), 1 g (NH_4) SO_4 (Roth), 6 m/l pure soybean oil (Nef Lebensmittel AG, Zurich, CH, catalog #01190) and 4 g $CaCO_3$ (Sigma). The pH was adjusted to 6.5. After autoclaving 20 minutes at 121°C the medium should have a pH of about 7. One liter of V2 medium used for the second stage inoculum was made of 18 g corn starch, 12 g dextrin, 5 g soybean flour, 3 g NaCl, 6 ml corn steep liquor, 1.2 g $(NH_4)SO_4$, 6 ml soybean oil, and 5 g $CACO_3$, resuspended in RO water. After adjustment of the pH to 6.8 the medium was

autoclaved 20 min at 121°C. The erythromycin production medium F1 contained, per liter of RO water, 35 g corn starch, 32 g dextrin, 33 g soybean flour, 7 g NaCl, 20 ml corn steep liquor, 2 g $(NH_4)SO_4$, 3 ml soybean oil, and 8 g $CACO_3$. After adjustment of the pH to 6.5 the medium was autoclaved 20 minutes at 121°C.

Sterile antifoam agent, Madzu DF 204 (PPG Ouvrie, Lesquin, France) was added prior to sterilization (about 0.5ml/l) and during cultivations as needed. All media containing soybean flour started boiling at around 70°C causing foaming problems when sterilized in bioreactors. Therefore, V2 medium was sterilized in the bioreactor without soybean flour which was autoclaved separately and added aseptically to the bioreactor. The original F1 medium was used at half strength only. All salts and dextrin were autoclaved separately and were added aseptically to the sterilized bioreactor containing all the other compounds. Due to the high portion of undissolved material, sterilization times for bioreactors were set to 50 min at 121°C. Test medium containing tryptic soy broth (TSB, Oxoid) supplemented with 2 g/l glucose and 1.5% agar was used for *M. luteus* based erythromycin bioassay.

Cultivations were performed in both shake flasks and bioreactors according to the following outline. For shake flask fermentations, 250 ml baffled Erlenmeyer flasks containing 30 ml culture were used. Flasks were incubated at 34°C in an Infors RFI-150 incubator (Infors AG, Bottmingen, CH) with shaking at 250 rpm and a 2" stroke. Medium V1 was inoculated with 1.5 ml of a frozen stock culture and incubated with shaking. After 38 hrs, 3 mls of the seed culture was withdrawn to inoculate 27 mls of half strength F1 production medium. During the 9 day fermentation, the following compounds were fed once a day: soybean oil, 0.2 ml/day, was added from day 0 to day 6; and n-propanol was added at 0.1 ml/day from day 0 to day 5 and 0.15 ml/day from day 6 until the end of the fermentation. In addition, the flasks were weighed daily and sterile water was added to compensate for evaporation.

Bioreactor cultivations were performed in 3 stages. The first stage seed culture was grown in 35 ml V1 as described above for shake flask incubations. After 48 hours this culture was used to inoculate 3.5 liters of V2 medium. This second stage seed cultivation was done in an LH 210 5 liter bioreactor (Inceltech

LH SGI S.A., France) equipped with a pitched blade turbine. The agitation speed was set to 800 rpm, air flow rate to 0.6 vvm, temperature was controlled at 34°C and dissolved oxygen tension (DOT), pH and redox potential profiles were monitored. CO_2 and O_2 in the exhaust gas was monitored on-line with a VG Prima 600 mass spectrometer (VG Gas Analysis Systems, Middlewich, UK). After 40 hours, 1.5 liters of culture were transferred into an Infors ISF200 bioreactor (Infors AG, Bottmingen, CH) equipped with 2 Rushton turbines containing 10 liters of half strength Fl production medium. Cultivation conditions were as follows: temperature was set to 34°C, pH was controlled with H_2SO_4 to not exceed 7.2, agitation speed was set to 700 rpm and controlled by DOT signal to increase to 900 rpm if DOT was below 45% saturation, air flow rate for the first 12 hours was set to 0.37 vvm then changed to 0.83 vvm and falling to 0.7 vvm as feeding adds to the reactor volume, pressure was set to 0.1 bar. Continuous feeding of n-propanol, 2.4 ml/l/day from 12 to 160 hours, soybean oil, 4.8 ml/l/day from 25 hours until the end of the cultivation, and 15% dextrin, 48 ml/l/day from 30 to 90 hours, was realized with a Reglo-Digital MS4/8-100 four channel pump (Ismatec SA. Zurich, CH). Proper selection of tubing diameters compensated for the different flow rates. Low flow rates were achieved by the pump's discontinuous mode activating the pump once a minute to add the respective amounts of feed compounds. Redox potential, and CO_2 and 0_2 concentrations in the exhaust gas, were monitored on-line as described above. In addition, free glucose was monitored hourly with a YSI 2700 Biochemistry Analyzer fitted with the 2730 Monitor and Control Module (YSI Inc., Yellow Springs, OH). Samples were drawn aseptically through a cross flow filter assembly (BioEngineering, Wald, CH) fitted with a 0.2 μm IRIS 6502 membrane (Phone-Poulenc Tech -Sep, Miribel, France). Daily samples of 50 or 100 ml were drawn for the determination of the erythromycin titer, CO binding studies and Western Blot analysis, and for microscopic inspection for mycelial morphology and possible contamination. Samples were stored at -20°C.

Erythromycin titers were determined as described above in Example 1.

CO-binding assay and Western blot analysis of the *S. erythraea* cultures were performed as described elsewhere (Kallio *et al.*, 1996, Biotechn. Prog. 12:31-39; Sambrook *et al., supra*) with the following modifications. Following

cell disruption using a French press (Aminco, SLM Instruments Inc., Urbana, IL) an ultracentrifugation step for 4 hours at 35,000 rpm in an SW41Ti rotor (Beckmann) was added in order to separate soy oil, soybean oil-bound small particles and cell debris from the cleared cell extract.

Results

Erythromycin production in fed-batch shake flask cultivations was compared between the original production strain *S erythaea::vhb* and *S erythraea* NRRL2338. Experiments were conducted in 30 ml medium using baffled 250 ml shake flasks as described above. Samples were taken during the cultivation in production medium and analyzed for erythromycin titers. Figure 4 summarizes the results of the shake-flask cultivations. The industrial strain produced 3.8 - 3.9 g/l under these conditions. This titer was confirmed in a second cultivation. The recombinant strain *S. erythraea::vhb* yielded between 5.6 and 6.3 *g/l* erythromycin. This represents a 45 to 63% increase in volumetric productivity. Furthermore, *S. erythaea::vhb* showed a more rapid onset of biosynthesis during the first three days and an additional jump in production between day 7 and 8 before reaching the final titer. These titers compared to 50 mg/l obtained with *S erythraea* NRRL2338 after 9 days and indicate a 100 fold increased erythromycin production in the highly developed industrial strains. Because of the genetic stability of *S. erythromycin::vhb* shake flask cultivations described above, thiostrepton was not used in any of the cultivations.

Bioreactor fed-batch cultivations were performed on a 10-15 liter scale. This scale is a useful representation of pilot production scale and permits withdrawal of the larger samples required to monitor VHb expression by Western blot analysis and VHb activity by CO-binding assays. As described above the second stage preculture was performed in a bioreactor. This cultivation method improved the reproducibility with which V2 cultivations could be performed. The pH and redox potential profiles for duplicate and triplicate cultivations were almost identical for the respective strains. During the first 16 hours of second stage cultivation, pH dropped to almost 6. After 16 hours, pH then increased sharply to above 8, reaching a maximum at around 24-26 hours after which pH fell again to values > 7 at 40 hours. During production in half strength Fl medium, pH was controlled at less than 7.2.

Fermentation profiles from *S erythraea ssp.* and *.S erythraea::vhb* are compared in Figure 5. The bars on top indicate the duration of the respective feeds. It is interesting to note that with 3 exceptions no free glucose could be detected. The first and highest peak of free glucose, about 1 g/l, was measured during the first 12 hours after inoculation, the second peak after 38 hrs (following the start of the dextrin feed), and the third peak occurred at the end of the dextrin feed. Differences between the recombinant *S. erythraea::vhb* and the original *S. erythraea ssp.* were observed. These include the higher concentration of 0.4 g/l free glucose after 38 hours in the *S. erythraea ssp.* cultivation as compared to 0.25 g/l with the recombinant strain and the appearance of the third peak only with the original strain. These differences point to a slower growth of the original strain and a reduction of the growth rate after about 72 hours. This assumed reduction in growth rate coincided with a decrease in erythromycin production rate.

Figure 6 summarizes the results of 5 such bioreactor cultivations, three fermentations with *S. erythraea ssp.* and two cultivations with the recombinant *S. erythraea::vhb*. Final erythromycin titers obtained were 7.25 g/l for the recombinant *S. erythraea* and 4.25 g/l for the original production strain. This corresponds to an increase in erythromycin production of about 70%. This increase in productivity was the result of a higher rate of erythromycin biosynthesis. The production rates were calculated for the time interval from 48 to 144 hours, and found to be 4.2 mg/h/l with the recombinant VHb expressing strain *S. erythraea::vhb* as compared to only 1.9 mg/h/l with the original production strain.

Microscopic analysis of the samples revealed that starting at about day 8 (192 hours) a progressive fragmentation of the mycelium could be observed. The disintegration of the mycelium was more pronounced in *S. erythraea ssp.* than in the recombinant *S. erythraea::vhb*.

Expression of the heterologous VHb protein in *S. erythraea::vhb* was analyzed to prove that this change in production is correlated with the expression of VHb in *S. erythraea::vhb*. Western blots were made with samples from cultivations with the recombinant and the original strain. A clear signal that corresponds to the positive control was detected in all samples from the *S. erythraea::vhb* throughout the cultivation. No *VHb* specific signal was detected

in samples from cultivations with *S. erythraea ssp.* This result is additional proof that the recombinant strain is genetically stable and that the chromosomally integrated gene is transcribed throughout the erythromycin production phase.

The activity of the expressed gene was examined by CO binding studies. VHb is characterized by a well defined peak at 420 nm in CO binding assays. This VHb specific peak was detected in *S. erythraea::vhb* throughout the cultivation. No peak at 420 could be identified in the original *S. erythraea ssp.* The peak at 450 nm belongs to the cytochrome P-450 monoxygenase, *eryF,* of the erythromycin gene cluster which converts 6-deoxyerythonolide B to erythronolide B.

Discussion

The goal of this study was the development of an improved strain that would be genetically stable throughout the production process. We integrated only a single copy of our vector confirming the *Vitreoscilla* hemoglobin gene, a thiostrepton marker used for the initial isolation of transformants, and the phage ϕc31*att* site into the chromosome (Example 1, *supra*). Integration is expected to take place at the putative *att* site of the chromosome, a region that has no function in secondary metabolism. This is in contrast to the approach generally used in which modifications are made in the genes encoding for the biosynthesis or the regulation of biosynthesis of the antibiotics (Hänel *et al., supra*; Lal *et al., supra*).

Both shake flask and bioreactor cultivations at a scale of at least 10 liter proved that this genetically engineered *S. erythraea::vhb* was superior to the original strain. Bioreactor cultivations done as described above closely resemble an industrial process. *S. erythraea ssp.* was reported to produce between 5 to 7 g/l erythromycin under these conditions. In our hands the strain reproducibly produced around 4.5 g/l which is a little lower than the expected productivity. This is most likely due to modifications made to the original media formulation to substitute for certain complex compounds that were not available in Europe. Since we were primarily interested in comparing the original strain with its recombinant variant, we did not attempt to improve on our media or culture conditions.

Comparing the volumetric yields of erythromycin for the two strains showed a 70% increased volumetric yield with the recombinant strain *S.*

erythraea::vhb relative to the original strain. This significant difference could be attributed to a change in erythromycin biosynthesis rate. While after about 90 hours of cultivation the biosynthesis rate in *S. erythraea ssp.* dropped from initial high rate to a reduced rate of 1.9 ml/l/h, erythromycin production rate with *S. erythraea:: vhb* remained at the high rate of 4.2 mg/h/l (Figure 3). It is not clear why this reduction in erythromycin production occurs with *S. erythraea ssp.* Interestingly, at about the same time, which is also the end of the dextrin feed, a transient accumulation of free glucose was observed in the cultivation with *S. erythraea ssp.* This points to a reduction in growth rate that could not be observed with the recombinant strain. The limitation that caused this apparent change in growth and erythromycin production rates remains unknown. All differences, however, should be the result of the genetic manipulation of the recombinant strain. Fortunately, *S. erythraea::vhb* was genetically stable and it was not required to supplement the medium for selection with thiostrepton, a substance that is known to cause pleiotropic effects. Thus, it can be argued that the increase in erythromycin productivity was due to the expression of the *vhb* gene. That the expressed VHb was functional throughout the cultivation was shown by Western blot analysis and CO-binding assays.

How VHb expression aids growth or antibiotic production is unknown. It is believed that VHb improves oxygen availability by increasing the intracellular oxygen concentration under microaerobic culture conditions (Kallio *et al.*, 1994, Eur. J. Biochem. 219:201-208). Despite a high >45% DOT throughout the cultivations, oxygen might become limited in the mycelium aggregated in a viscous medium. Interactions of VHb and cytochrome *o* and cytochrome *d* in *E. coli* have been reported (Wakabayashi *et al.*, 1986, Nature 322:481-483; Tsai *et al.*, 1996, Biotechnol. Bioeng. 49:151-160). The interaction with these terminal oxidases is argued to increase microbial respiration and growth. This apparent requirement for oxygen is in contrast to observations that specific erythromycin production remains unchanged at aerobic, 60% DOT, microanaerobic, 10% DOT, or even anaerobic culture conditions (Heydarian *et al., supra*; Clark *et al., supra*).

Erythromycin biosynthesis involves the action of a cytochrome P450 monoxygenase, *eryFl*, which performs the C6-hydroxylation converting 6-deoxyerythronolide B to erythronolide B. This reaction step, taken in conjunction

with the results presented herein, raises the possibility of a direct interaction between VHb and the monoxygenase of erythromycin biosynthesis. Regardless, the results demonstrate the usefulness of genetic engineering for the improvement of erythromycin production strains by providing a stable recombinant strain expressing the functional heterologous oxygen-binding *VHb* gene.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. An erythromycin-producing organism, wherein the organism further expresses a heterologous oxygen-binding protein.

2. The organism of claim 1 which is *Saccharopolyspora erythraea.*

3. The organism of claim 1, wherein the oxygen-binding protein is a globin protein.

4. The organism of claim 3, wherein the globin protein is selected from the group consisting of *Vitreoscilla* hemoglobin, *Alcaligenes eutrophus* flavohemoprotein, horse heart myoglobin, *E. coli* hemoprotein, *B. subtilis* hemoprotein, yeast flavohemoglobin, soybean leghemoglobin, lupin leghemoglobin, and sperm whale myoglobin, or their functional equivalents.

5. The organism of claim 4 wherein the globin protein is *Vitreoscilla* hemoglobin.

6. The organism of claim 2 wherein the gene is integrated into the *Saccharopolyspora erythraea* chromosome.

7. The organism of claim 1, wherein the erythromycin is selected from the group consisting of erythromycin A, erythromycin B, erythromycin C, erythromycin D, erythromycin E, erythromycin F, and 6-deoxyerythromycin A.

8. A method of increasing erythromycin production, the method comprising: culturing an erythromycin-producing organism that expresses a heterologous gene encoding an oxygen-binding protein under conditions appropriate for production of erythromycin.

9. The method of claim 8 wherein the organism is *Saccharopolyspora erythraea.*

10. The method of claim 8, wherein the oxygen-binding protein is a globin protein.

11. The method of claim 9, wherein the globin protein is selected from the group consisting of *Vitreoscilla* hemoglobin, *Alcaligenes eutrophus* flavohemoprotein, horse heart myoglobin, *E. coli* hemoprotein, *B. subtilis* hemoprotein, yeast flavohemoglobin, soybean leghemoglobin, lupin leghemoglobin, and sperm whale myoglobin, or their functional equivalents.

12. The method of claim 11 wherein the globin protein is *Vitreoscilla* hemoglobin.

13. The method of claim 9 wherein the heterologous gene is integrated into a chromosome of the cell.

14. The method of claim 8 wherein the erythromycin is selected from the group consisting of erythromycin A, erythromycin B, erythromycin C, erythromycin D, erythromycin E, erythromycin F, and 6-deoxyerythromycin A.

15. A method of producing erythromycin, the method comprising:
collecting erythromycin from a culture of an erythromycin-producing organism that expresses a heterologous gene encoding an oxygen-binding protein.

16. A method of making an improved erythromycin-producing organism, the method comprising producing an erythromycin-producing organism that expresses a heterologous oxygen-binding protein.

17. The method of claim 16, wherein the method comprises transforming the erythromycin-producing organism with an expression construct that directs expression of the oxygen-binding protein in the erythromycin-producing organism.

18. The method of claim 17, wherein the expression construct is integrated into the chromosome of the erythromycin-producing organism.

19. The method of claim 17, wherein the expression construct is maintained on a plasmid in the erythromycin-producing organism.

20. The method of claim 16, wherein the organism is *Saccharopolyspora erythraea*.

21. A method of stably transforming *Saccharopolyspora erythraea* with an exogenous DNA construct, the method comprising directing integration of the exogenous DNA construct at the ϕC31 attachment site of the *S. erythraea* chromosome.

FIG. 1

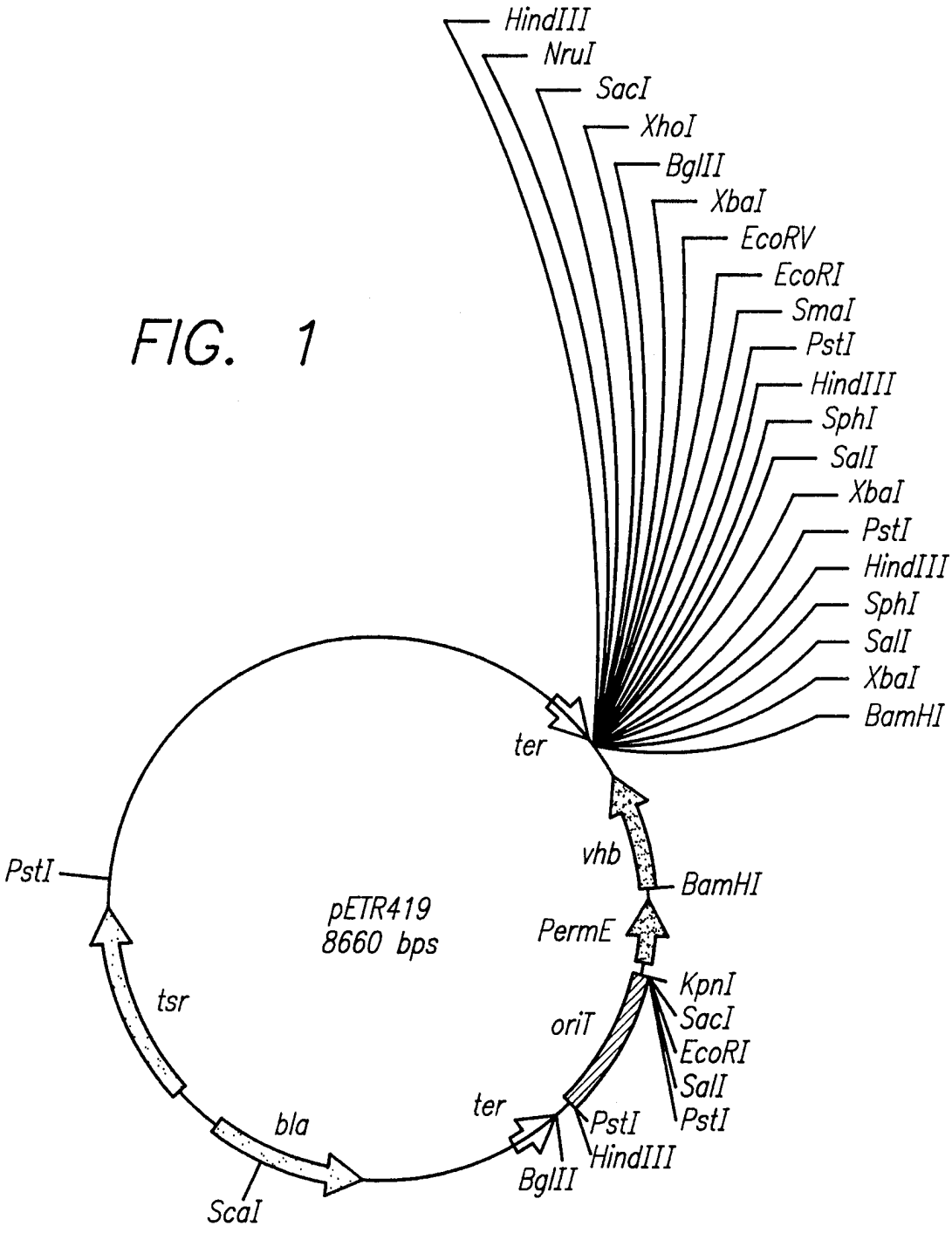

HindIII
NruI
SacI
XhoI
BglII
XbaI
EcoRV
EcoRI
SmaI
PstI
HindIII
SphI
SalI
XbaI
PstI
HindIII
SphI
SalI
XbaI
BamHI
ter
vhb
BamHI
PermE
KpnI
SacI
EcoRI
SalI
PstI
oriT
ter
PstI
HindIII
BglII
bla
ScaI
tsr
PstI
pETR419
8660 bps

FIG. 2

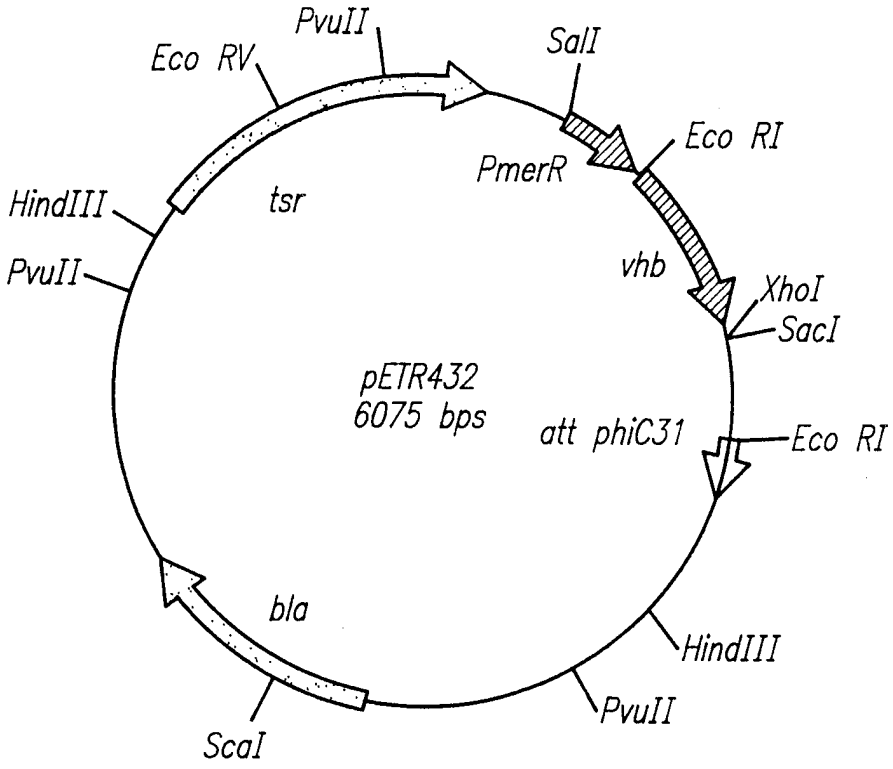
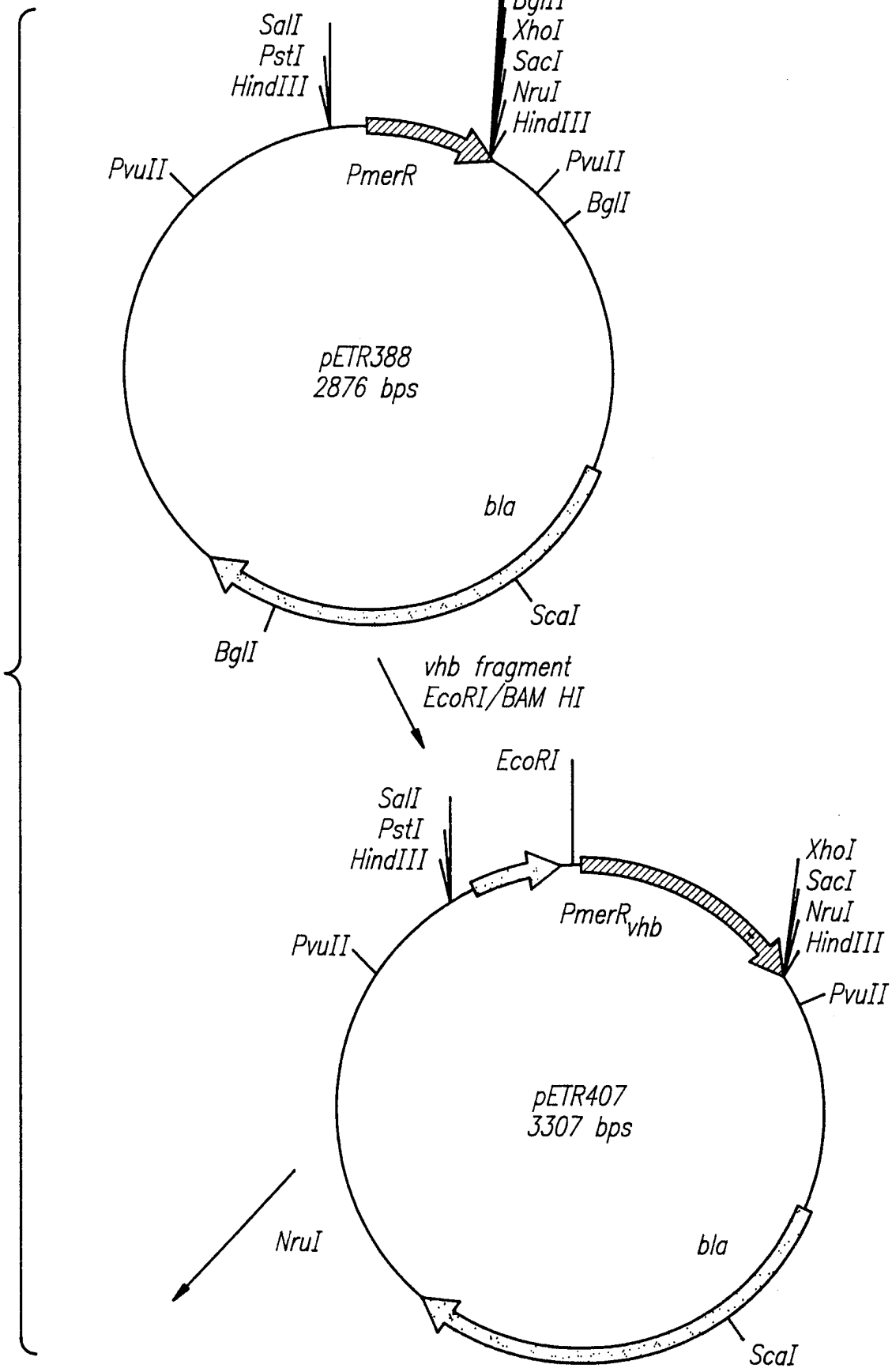

PvuII
SalI
Eco RV
Eco RI
PmerR
HindIII
tsr
vhb
PvuII
XhoI
SacI
pETR432
6075 bps
att phiC31
Eco RI
bla
HindIII
PvuII
ScaI

FIG. 3-1


EcoRI
EcoRV
XbaI
BglII
XhoI
SacI
NruI
HindIII
SalI
PstI
HindIII
PvuII
PmerR
PvuII
BglI
pETR388
2876 bps
bla
ScaI
BglI
vhb fragment
EcoRI/BAM HI
EcoRI
SalI
PstI
HindIII
XhoI
SacI
NruI
HindIII
PmerRvhb
PvuII
PvuII
pETR407
3307 bps
NruI
bla
ScaI

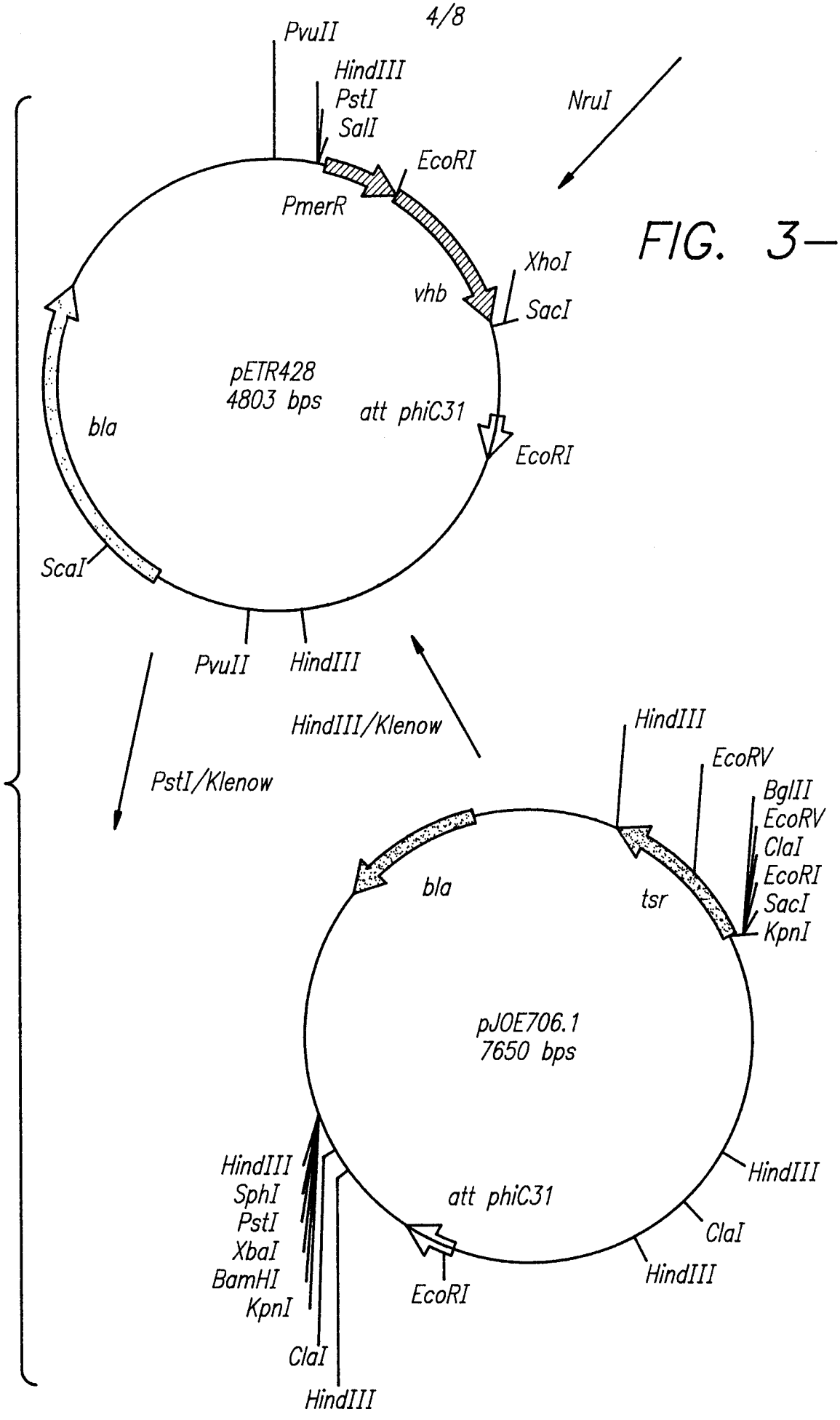

PvuII
HindIII
PstI
SalI
NruI
EcoRI
PmerR
XhoI
vhb
SacI
pETR428
4803 bps
att phiC31
bla
EcoRI
ScaI
PvuII
HindIII
HindIII/Klenow
PstI/Klenow
HindIII
EcoRV
BglII
EcoRV
ClaI
EcoRI
SacI
KpnI
bla
tsr
pJOE706.1
7650 bps
HindIII
SphI
PstI
XbaI
BamHI
KpnI
ClaI
HindIII
att phiC31
HindIII
ClaI
HindIII
EcoRI

FIG. 3-2

FIG. 3-3

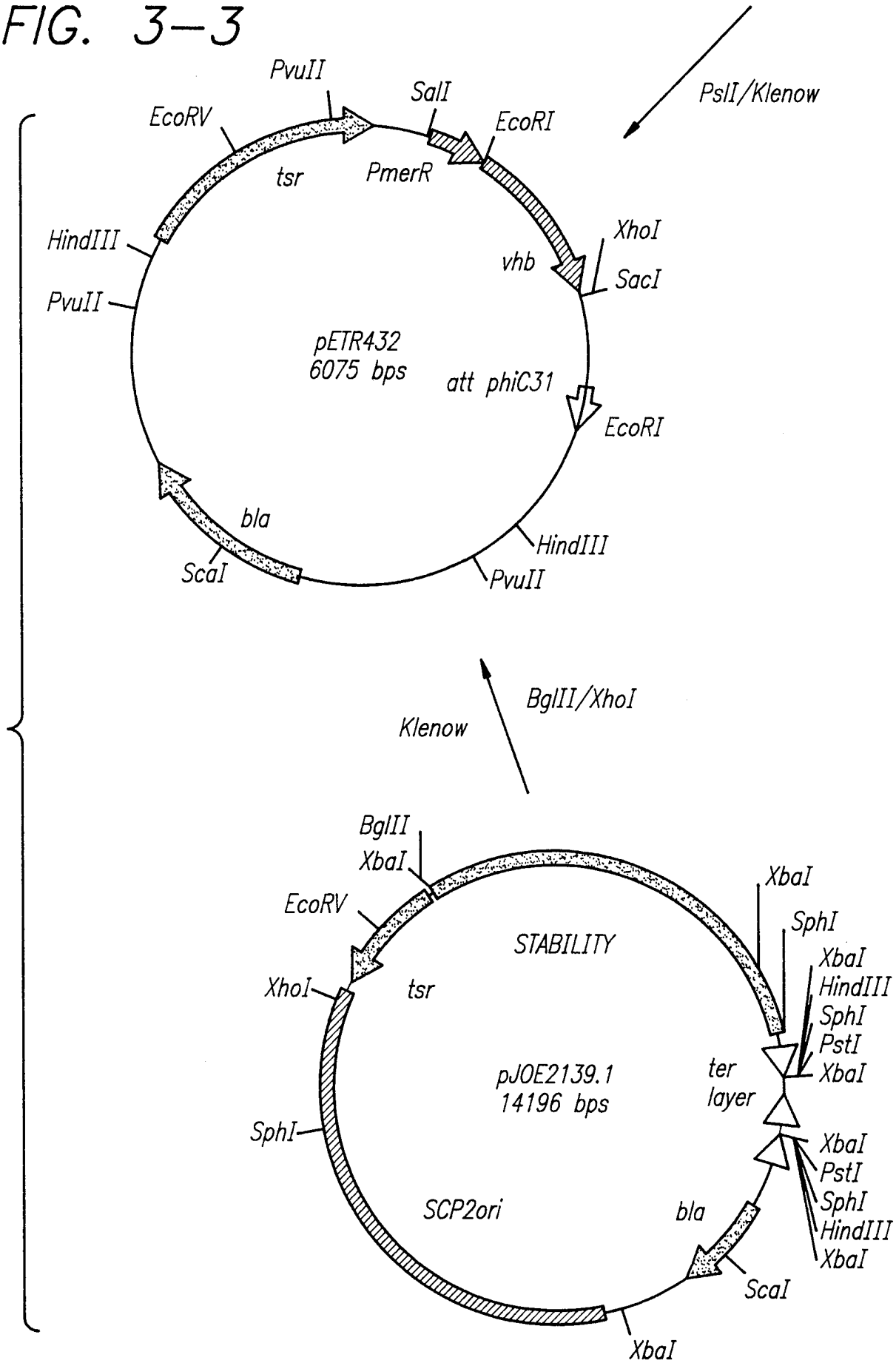

PvuII
SalI
EcoRV
EcoRI
PslI/Klenow
tsr
PmerR
HindIII
XhoI
vhb
SacI
PvuII
pETR432
6075 bps
att phiC31
EcoRI
bla
HindIII
ScaI
PvuII
Klenow
BglII/XhoI
BglII
XbaI
XbaI
EcoRV
SphI
STABILITY
XbaI
HindIII
XhoI
tsr
SphI
PstI
pJOE2139.1
14196 bps
ter
layer
XbaI
XbaI
PstI
SphI
SCP2ori
bla
HindIII
XbaI
ScaI
XbaI

FIG. 4

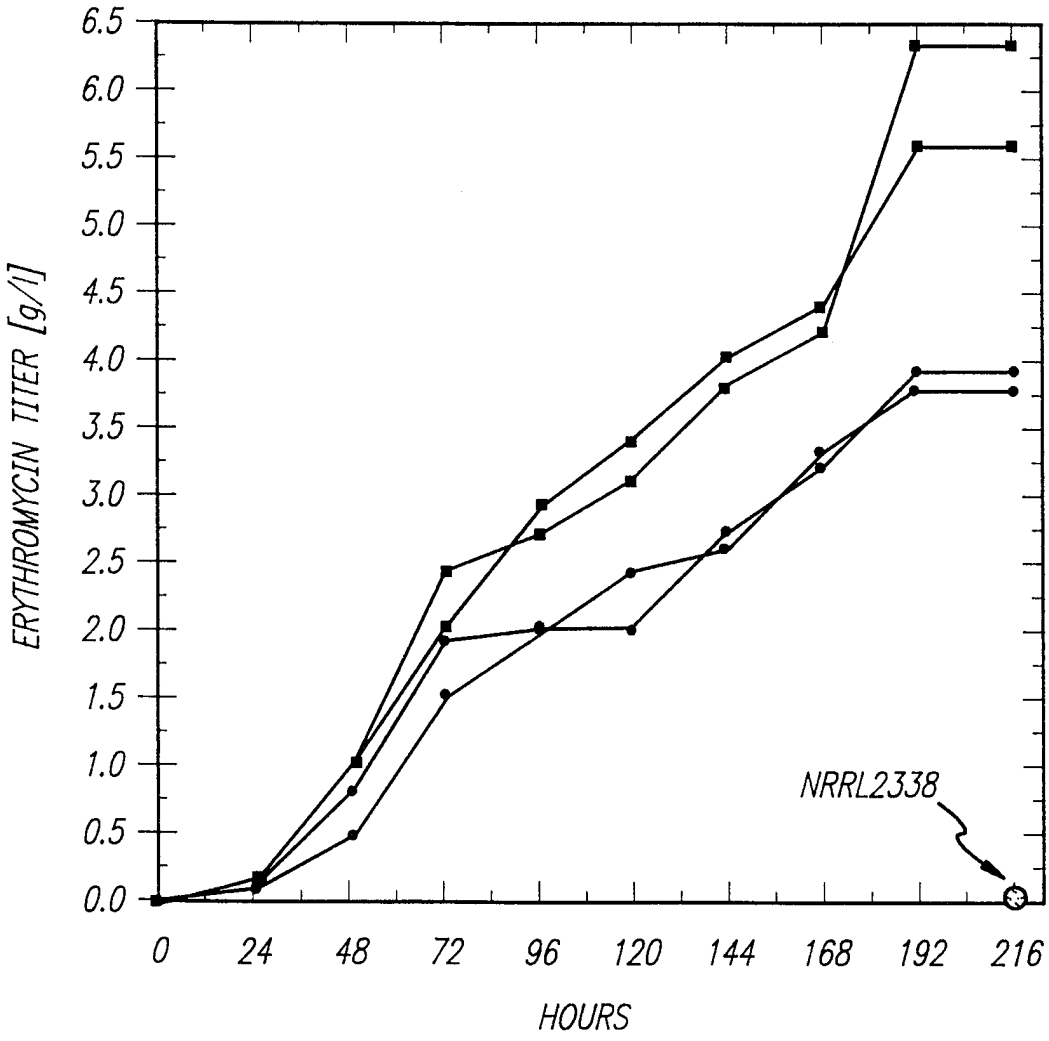

ERYTHROMYCIN TITER [g/l]
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
24
48
72
96
120
144
168
192
216
HOURS
NRRL2338

FIG. 5A

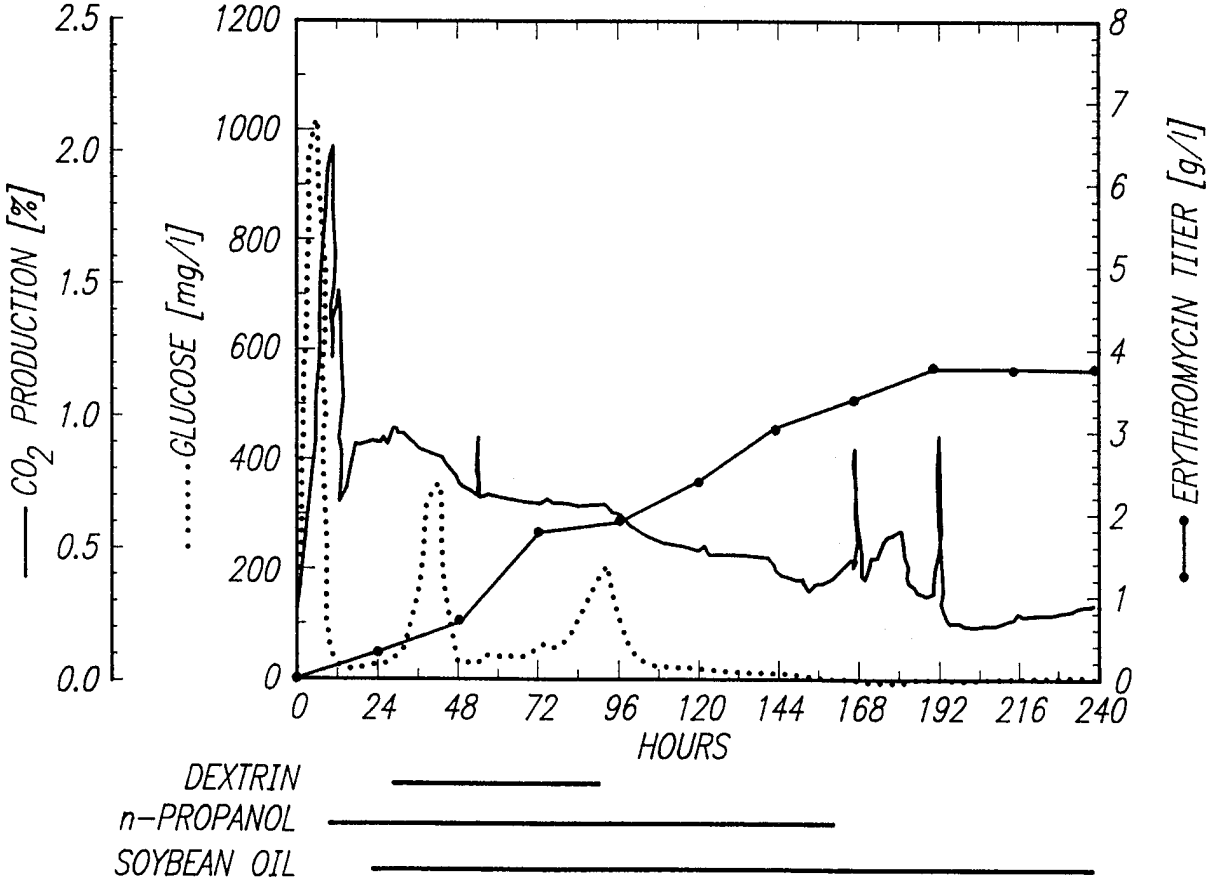

2.5
2.0
1.5
1.0
0.5
0.0
CO_2 PRODUCTION [%]
1200
1000
800
600
400
200
0
GLUCOSE [mg/l]
8
7
6
5
4
3
2
1
0
ERYTHROMYCIN TITER [g/l]
0 24 48 72 96 120 144 168 192 216 240
HOURS
DEXTRIN
n-PROPANOL
SOYBEAN OIL

FIG. 5B

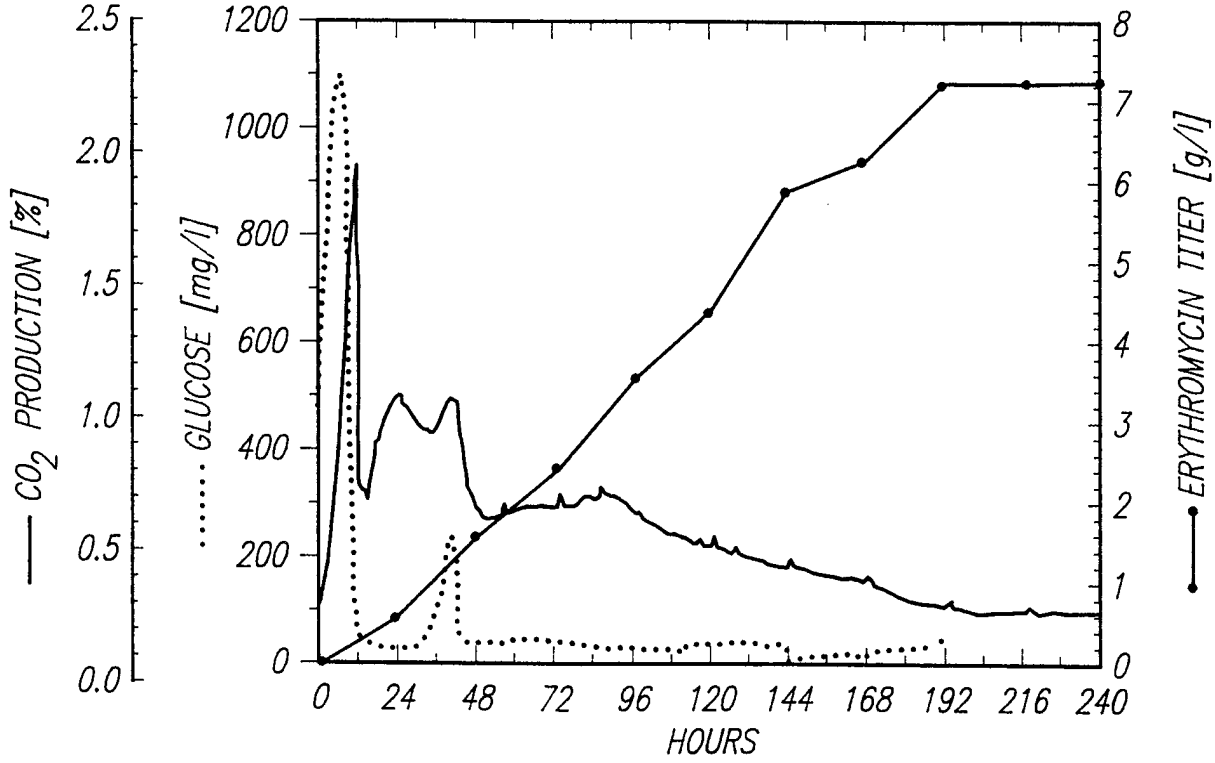
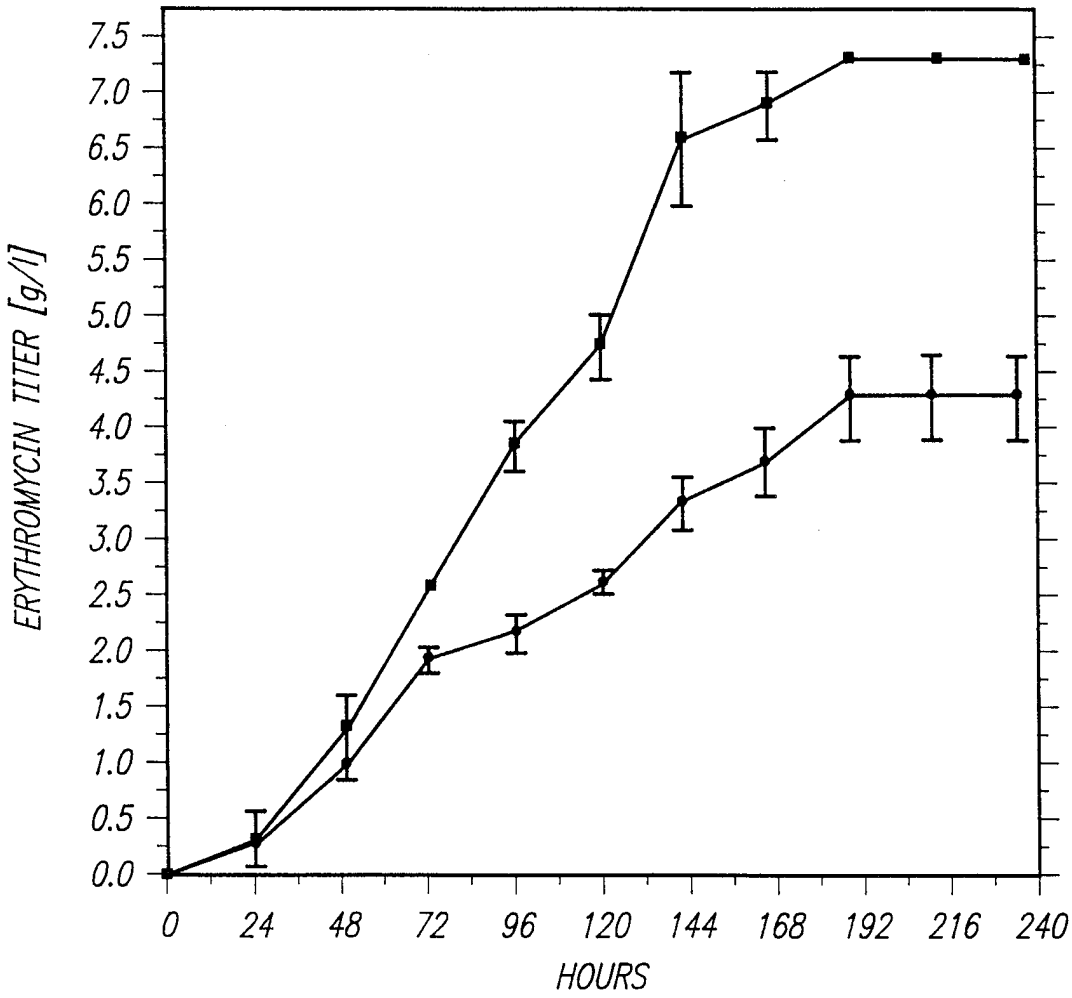

2.5
2.0
1.5
1.0
0.5
0.0
CO_2 PRODUCTION [%]
1200
1000
800
600
400
200
0
GLUCOSE [mg/l]
8
7
6
5
4
3
2
1
0
ERYTHROMYCIN TITER [g/l]
0 24 48 72 96 120 144 168 192 216 240
HOURS

FIG. 6


ERYTHROMYCIN TITER [g/l]
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
24
48
72
96
120
144
168
192
216
240
HOURS

INTERNATIONAL SEARCH REPORT

Internatio. Application No
PCT/IB 98/00790

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/76 C12N15/90 C12P19/62 C12N1/21 C07K14/805

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P C12N C07K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 25697 A (CALIFORNIA INST OF TECHN) 23 December 1993 see abstract see examples 17-20 ---	1-20
X	MAGNOLO S K ET AL: "Actinorhodin production by Streptomyces coelicolor and growth of Streptomyces lividans are improved by the expression of a bacterial hemoglobin." BIO/TECHNOLOGY, vol. 9, May 1991, pages 473-476, XP002076830 cited in the application see abstract see page 475, column 1, paragraph 3 --- -/--	1-20

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 September 1998	12/10/1998

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

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INTERNATIONAL SEARCH REPORT

Internatio. Application No
PCT/IB 98/00790

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 06628 A (EXOGENE) 16 May 1991 see abstract see example 4	1-20
A	WO 89 03883 A (CALIFORNIA INST OF TECHN) 5 May 1989 cited in the application see abstract	1-20
A	DEMODENA J A ET AL: "The production of cephalosporin C by Acremonium chrysogenum is improved by the intracellular expression of a bacterial hemoglobin." BIO/TECHNOLOGY, vol. 11, August 1993, pages 926-929, XP002076831 cited in the application see abstract	1-20
A	STASSI D ET AL: "IDENTIFICATION OF A SACCHAROPOLYSPORA ERYTHRAEA GENE REQUIRED FOR THE FINAL HYDROXYLATION STEP IN ERYTHROMYCIN BIOSYNTHESIS" JOURNAL OF BACTERIOLOGY, vol. 175, no. 1, January 1993, pages 182-189, XP000608396 see abstract	1,2
A	HEYDARIAN S M ET AL: "The effect of culture conditions on the production of erythromycin by Saccharopolyspora erythraea in batch culture." BIOTECHNOLOGY LETTERS, vol. 18, 1996, pages 1181-1186, XP002078396 cited in the application see abstract	1-20
X	EP 0 403 173 A (LILLY CO ELI) 19 December 1990 see page 39; claims 1,2	21
A	WO 97 06266 A (ABBOTT LAB) 20 February 1997 see claim 1	21
T	MINAS W ET AL: "Improved erythromycin production in a genetically engineered industrial strain of Saccharopolyspora erythraea." BIOTECHNOLOGY PROGRESS, vol. 14, 11 July 1998, pages 561-566, XP002078390 see the whole document	1-21

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 98/00790

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-20

   An erythromycin-producing organism, wherein the organism further expresses a heterologous oxygen-binding protein.

2. Claim: 21

   A method of stably transforming Saccharopolyspora erythraea with an exogenous DNA construct, the method comprising directing integration of the exogenous DNA construct at the phage C31 attachment site of the Saccharopolyspora erythraea chromosome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatio Application No
PCT/IB 98/00790

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9325697	A	23-12-1993	NONE		
WO 9106628	A	16-05-1991	AU	6891291 A	31-05-1991
			CA	2072115 A	01-05-1991
			EP	0497916 A	12-08-1992
WO 8903883	A	05-05-1989	AU	2721388 A	23-05-1989
			DK	310089 A	23-08-1989
			EP	0342221 A	23-11-1989
			EP	0683230 A	22-11-1995
			JP	7108226 B	22-11-1995
			JP	2500248 T	01-02-1990
			US	5049493 A	17-09-1991
EP 0403173	A	19-12-1990	US	5190871 A	02-03-1993
			AU	634243 B	18-02-1993
			AU	5701190 A	13-12-1990
			CA	2018164 A	12-12-1990
			JP	3019692 A	28-01-1991
WO 9706266	A	20-02-1997	CA	2201481 A	20-02-1997
			EP	0783584 A	16-07-1997
			JP	10507087 T	14-07-1998