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Genetic engineering of an industrial strain of *Saccharopolyspora erythraea* for stable expression of the *Vitreoscilla* haemoglobin gene (*vhb*)

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Several *Actinomycetes/Streptomycetes* expression vectors are described for expression of the *Vitreoscilla* haemoglobin gene (*vhb*) in an industrial erythromycin-producing strain of *Saccharopolyspora erythraea*. Cloning of *vhb* under the control of either the thiostrepton-inducible *PtipA* promoter or the constitutive *PermE promoter led to the production of chemically active haemoglobin (VHb) in *Streptomyces lividans* TK24 transformed with these constructs. However, the plasmids could not be transformed into *Sac. erythraea*. Transformants of *Sac. erythraea* and/or exconjugants were obtained using a novel *Escherichia coli/Streptomyces* shuttle vector comprised of *vhb* under the control of the *PermE** promoter, the *Streptomyces* plasmid pIJ350 origin of replication, the thiostrepton-resistance gene (*tsr*) for selection, and the *oriT* region which is necessary for conjugal transfer. Increased plasmid stability in *Sac. erythraea* was obtained by construction of a vector for chromosomal integration. This vector contained the *Streptomyces* phage ϕC31 attachment site for chromosomal integration and *vhb* expressed under the *PmerR* promoter and was stably maintained in the chromosome of *Sac. erythraea*. Shake-flask cultivations of the transformed *Sac. erythraea* strain with the chromosomally integrated *vhb* gene show that *vhb* is expressed in an active form. The corresponding amount of erythromycin produced in the *vhb*-expressing strain was approximately 60% higher relative to the original VHb-negative strain.**

Keywords: erythromycin, integration vector, phage ϕC31

INTRODUCTION

Erythromycin is a clinically important and potent macrolide antibiotic produced by the Gram-positive actinomycete *Saccharopolyspora erythraea* (Weber *et al.*, 1985). It is used to treat infections by several prokaryotic pathogens such as *Streptococcus*, *Staphylococcus*, *Mycoplasma*, *Ureaplasma*, *Chlamydia* and *Legionella* (Nakayama, 1984). The current annual production of erythromycin is more than 2 tonnes and the pharmaceutical demand for this antibiotic is increasing annually.

Despite intensive efforts using classical strain development techniques and bioprocess optimization methods, maximum attainable final concentrations of erythromycin using *Sac. erythraea* are low in comparison to other industrially produced antibiotics such as penicillin or cephalosporin C. Since traditional methods have not greatly increased erythromycin production, our approach was to modify *Sac. erythraea* by metabolic engineering (Bailey, 1991) to improve its overall productivity. Special emphasis was placed on improving oxygen metabolism, as this might be a limiting factor for erythromycin synthesis in this organism.

Previous experiments using other industrially important organisms have shown that expression of a bacterial haemoglobin gene (*vhb*) originally isolated from *Vitreoscilla* sp. (Khosla & Bailey, 1988a) can significantly improve cell growth and productivity (Khosla &

Abbreviation: VHb, *Vitreoscilla* haemoglobin.

Bailey, 1988b). It was demonstrated that expression of *vhb* in *Acremonium chrysogenum* led to a threefold increase in cephalosporin C production (DeModena *et al.*, 1993). Furthermore, the yield of human tissue plasminogen activator (tPA) from Chinese hamster ovary cells (Pendse & Bailey, 1994), L-lysine production of *Corynebacterium glutamicum* (Sander *et al.*, 1994), and total protein secretion, neutral protease activity and α-amylase activity of *Bacillus subtilis* (Kallio & Bailey, 1996) were all increased in the presence of active *Vitreoscilla* haemoglobin (VHb) in these organisms. Even in transgenic tobacco plants expressing *vhb*, a positive effect on an oxygen-dependent step in nicotine synthesis was observed which led to a 34% increase in nicotine content (Holmberg *et al.*, 1997). The recombinant tobacco plants also showed earlier germination and flowering and were able to produce plant material (dry weight) faster relative to the controls.

These examples of the beneficial effects of VHb in a variety of classes of organisms prompted us to clone and express the haemoglobin gene from *Vitreoscilla* sp. into *Sac. erythraea*.

In this work we describe for the first time the stable chromosomal integration of a *vhb* expression cassette into an industrial erythromycin-producing strain of *Sac. erythraea* and show that erythromycin production in the recombinant *vhb*-expressing strain was significantly enhanced compared to the original strain.

METHODS

Bacterial strains, plasmids and culture conditions. All bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown at 37 °C in either 2 × YT or LB liquid medium (Sambrook *et al.*, 1989) or on 2 × YT plates containing 1·6% (w/v) agar. *Streptomyces lividans* strains were maintained on R5 plates (Hopwood *et al.*, 1985) or incubated in SM liquid medium (Birr *et al.*, 1989) at 30 °C. *Sac. erythraea* was grown in the same media at 34 °C. *S. lividans* stocks were stored as spore solutions in 20% (v/v) glycerol at −20 °C, whereas mycelial stocks of *Sac. erythraea* were kept at −80 °C in 30% (v/v) glycerol. Media were supplemented with the appropriate antibiotics (100 μg ampicillin ml^{-1}, 12·5 μg thiostrepton ml^{-1}, 50 μg kanamycin ml^{-1}, 30 μg chloramphenicol ml^{-1}, or 40 μg nalidixic acid ml^{-1}) as needed.

Isolation and manipulation of DNA. Minipreparations of plasmid DNA were done by an alkaline lysis method as described by Lee & Rasheed (1990). Genomic DNA from *S. lividans* and *Sac. erythraea* was isolated according to protocols by Hopwood *et al.* (1985). Restriction enzymes, T4 DNA ligase, alkaline phosphatase, Klenow polymerase and Pwo polymerase were obtained from commercial sources and used as recommended by the manufacturers. Standard DNA techniques and Southern blot analyses were performed as described by Sambrook *et al.* (1989). PCR for amplification of *PmerR* and *vhb* was 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 using the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977).

Transformation of bacterial cells. Competent *E. coli* XL-1 Blue (Bullock *et al.*, 1987) and ET12567 (MacNeil *et al.*, 1992) were prepared and transformed by the method of McKenney *et al.* (1981). Preparation of *S. lividans* protoplasts and PEG-mediated transformation were performed according to the protocol of Hopwood *et al.* (1985). For *Sac. erythraea*, this protocol was slightly modified. The cells were grown for 4–5 d in TSB (Oxoid) containing 0·25% (w/v) glycine. For protoplast formation, the final concentration of lysozyme was 8 mg ml^{-1} (instead of 4 mg ml^{-1} for *S. lividans*) and PEG-3350 (Sigma) was used instead of PEG-1000 in the transformation reaction. Furthermore, for transformation of *Sac. erythraea*, non-methylated DNA isolated from *E. coli* ET12567 was used. Since regeneration of *Sac. erythraea* protoplasts seemed to be much slower than that of *S. lividans* protoplasts, the antibiotic overlay was performed 48 h after transformation. Conjugational transfer of plasmids from *E. coli* to *Sac. erythraea* was performed on plates as described by Bierman *et al.* (1992).

Detection of VHb and activity assays. *vhb*-expressing strains of *S. lividans* and *Sac. erythraea* were grown in 200 ml SM medium for 4–5 d at 30 °C and 34 °C, respectively, in a rotary shaker incubator at 250–300 r.p.m. Cells were harvested, washed twice in buffer (100 mM Tris/HCl pH 7·5, 50 mM NaCl, 1 mM EDTA), resuspended in 20 ml buffer and disrupted by passing three times through a French press (Aminco SLM Instruments) operated at 1000–1500 p.s.i. (6·9–10·3 MPa). The soluble cellular fraction was used for Western blots (Winston *et al.*, 1987) after separating the proteins by 15% SDS-PAGE and for determination of the CO-difference spectrum (Webster & Liu, 1974). The rabbit anti-VHb serum was obtained from Cocalico Biologicals. The protein concentration in the samples was determined by the method of Bradford (1976) using Bio-Rad dye reagent and bovine serum albumin as the standard. Total protein concentration of samples from shake-flask cultivations was determined as described by Gerhardt *et al.* (1994).

Shake-flask cultivations of *Sac. erythraea*. A seed culture of 30 ml vegetative medium I [per litre: 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 250 ml baffled shake flasks was inoculated with 1·5 ml glycerol stock and incubated for 40 h at 34 °C with 250 r.p.m. agitation [2 inch (5 cm) stroke] in a humidized rotary shaker incubator (Infors). Seed culture (3 ml) was inoculated into 27 ml half-strength fermentation medium I (F1) [per litre: 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 $CaCO_3$, pH adjusted to 6·5]. Cultivations for erythromycin production were run for 9 d with the following daily addition of soybean oil (0·2 ml, days 0–6) and *n*-propanol (0·1 ml, days 0–5 and 0·15 ml, days 6–9) starting at the day of inoculation of F1. The shake flasks were weighed daily and sterile water was added to compensate for evaporation if necessary.

Erythromycin bioassay. The titres of erythromycin produced by the industrial *Sac. erythraea* strain and its genetically engineered derivative were determined using a conventional bioassay with commercially available erythromycin (Fluka) as a standard. Portions (35 ml) of test medium [27·5 g TSB l^{-1}, 2 g glucose l^{-1}, 2% (w/v) agar] were poured into Petri dishes (12 × 12 cm). Once the medium was solidified, a second layer consisting of 35 ml test medium containing 35 μl of a *Micrococcus luteus* overnight culture (grown 18 h at 30 °C in LB), was added. The erythromycin titre was determined by pipetting 10 μl culture supernatant (or appropriate dilutions in

Table 1. Bacterial strains and plasmids

Strain/plasmid	Description	Reference/source
Strains		
E. coli XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^{R})]	Bullock *et al.* (1987)
E. coli ET12567	*supE44 hsdS20* ($r^{-}_{B}m^{-}_{B}$) *ara-14 proA2 lacY galK2 rpsL20 xyl-5 mtl-1 dam*$^{-}$ *dcm*$^{-}$ *hsdM*$^{-}$ Cm^{R}	MacNeil *et al.* (1992)
E. coli S17.1	*recA thi pro hsdR*$^{-}$ M^{+} RP4:2-Tc:Mu:KmTn7 Tp^{R} Sm^{R}	Simon *et al.* (1983)
S. lividans TK24	SLP2^{-} SLP3^{-} *str-6*	Hopwood *et al.* (1983)
S. lividans TK64	SLP2^{-} SLP3^{-} *pro-2 str-6*	Hopwood *et al.* (1985)
Sac. erythraea	Industrial erythromycin-producing strain	Solidago AG
M. luteus ATCC 9341	Test organism for erythromycin bioassay	ATCC
Plasmids		
pIC19H	*lacZ*α	Marsh *et al.* (1984)
pIJ6021	*PtipA*	Takano *et al.* (1995)
pIJ4090	*PermE**	Bibb & Janssen (1986)
pETR355	*vhb* under the control of *PermE**	This work
pETR364	*vhb* under the control of *PtipA*	This work
pETR388	*PmerR* in pIC19H	This work
pETR407	*vhb* under the control of *PmerR* in pETR388	This work
pETR419	*PermE*-vhb* and *oriT* in pJOE875	This work
pETR428	ϕC31 *att* site (from pJOE706) in pETR407	This work
pETR432	pETR428 containing *tsr*	This work
pETR451	As pETR432 without *vhb* expression cassette	This work
pJOE875	*E. coli/Streptomyces* shuttle vector	Altenbuchner *et al.* (1992)

methanol) on antibiotic test disks (6·6 mm diameter; Difco) placed onto the solidified test plates. After 48 h incubation at 30 °C, the growth-inhibition zones of *M. luteus* were measured and the erythromycin titre (g l^{-1}) was calculated using a standard curve.

RESULTS

Expression of *vhb* in *S. lividans*

As no suitable expression vectors were available for *Sac. erythraea* in the public domain, the *vhb* gene from *Vitreoscilla* sp. was first subcloned into *S. lividans* expression plasmids. After the expression of active *vhb* in *S. lividans* TK24 was confirmed, *Sac. erythraea* could be transformed with these constructs.

The *vhb* gene was amplified by PCR using pRED2 (Khosla & Bailey, 1988a) as a template. The primers for this PCR reaction were designed to generate *Bam*HI sites at both the 5′ and 3′ ends of the gene. An additional *Nde*I site at the 5′ end of the *vhb* gene was also introduced. The PCR fragment was ligated into the *Bam*HI-digested vector pIC19H (Marsh *et al.*, 1984), resulting in the plasmid pETR352. After the nucleotide sequence of *vhb* was confirmed by DNA sequencing, the gene was cloned as a *Nde*I/*Bam*HI or *Bam*HI fragment into the *Streptomyces* expression vectors, pIJ6021 and pIJ4090, respectively (Bibb & Janssen, 1986; Takano *et al.*, 1995), resulting in the plasmids pETR364 and pETR355 (Fig. 1a). In pETR364, *vhb* is under the control of the thiostrepton-inducible *PtipA* promoter, whereas in pETR355, *vhb* expression occurs from the constitutive *PermE** promoter.

S. lividans TK24 cells containing either pETR364 or pETR355 were grown in 10 ml SM medium for 4 d to study *vhb* expression. In the case of *S. lividans* TK24 harbouring pETR364, thiostrepton (2–20 μg ml^{-1}) was added after 3 d to induce the *PtipA* promoter. VHb synthesis was observed in both expression systems, as shown by Western blot analysis (Fig. 1b). Whilst the expression of *vhb* under the control of the *PermE** promoter was constitutive, as expected, the *PtipA* promoter required induction with thiostrepton: 5 μg ml^{-1} thiostrepton was sufficient for full induction of the *PtipA* promoter under these conditions. Further increases in the thiostrepton concentration did not result in higher VHb production. Without addition of thiostrepton, almost no VHb-specific band was detected in Western blots. This indicates that the *PtipA* promoter is relatively tightly regulated in *S. lividans* TK24. Furthermore, the results from the Western blots indicate that the *PtipA* promoter is approximately five times more active in the induced state than is the *PermE** promoter.

To verify that the synthesized VHb is biologically active, CO-difference spectrum assays were performed with crude extracts of *S. lividans* TK24 carrying pETR364 or pETR355. CO-difference spectra typical of active VHb were observed for both constructs: these showed a single specific peak at about 418–420 nm after CO

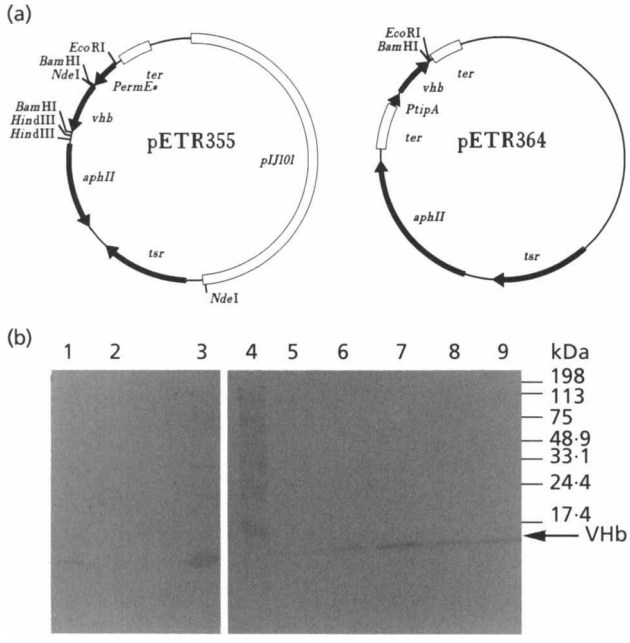

Fig. 1. (a) Restriction map of the *vhb* expression plasmids pETR355 and pETR364. The *vhb* gene was amplified by PCR using pRED2 as the template with the primers 5′-GGATCCCATATGAACTTAAGGAAGACC-3′ and 5′-GGATCCTTATTCAACCGCTTGAGC-3′, and cloned as described. (b) Western blot analysis of crude extracts from *S. lividans* TK24 containing pETR355 (a pIJ101 derivative; Hopwood *et al.*, 1985) or pETR364. 10 μg soluble protein was loaded in each lane. Lanes: 1, TK24/pETR355; 2, TK24; 3, positive control (crude extract from *E. coli* DH5α/pRED2); 5, TK24/pETR364 uninduced; 6–9, TK 24/pETR364 induced with different thiostrepton concentrations (lane 6, 2 μg ml^{-1}; lane 7, 5 μg ml^{-1}; lane 8, 10 μg ml^{-1}; lane 9, 20 μg ml^{-1}) for 18 h; 4, protein marker broad range (Bio-Rad).

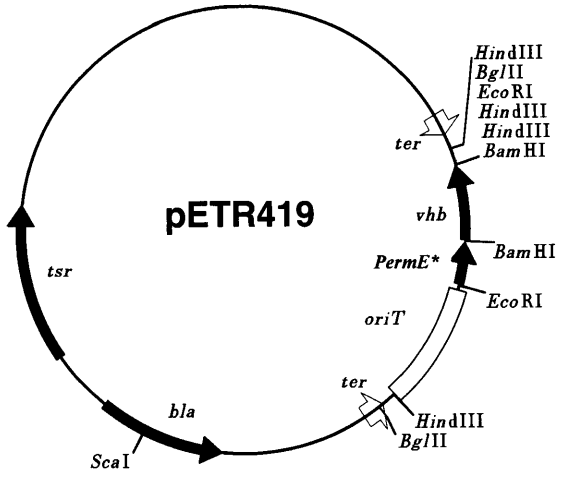

Fig. 2. Restriction map of the plasmid pETR419 containing the *PermE*-vhb* expression cassette and the origin of transfer (*oriT*) for intergeneric conjugation flanked by transcriptional terminators (*ter*). The plasmid is a shuttle vector which replicates in *E. coli* and *Actinomycetes*.

treatment of the soluble fraction, following reduction with sodium hydrosulphite (data not shown).

Although the *vhb* expression vectors described above worked well in *S. lividans* TK24, we were unable to transform *Sac. erythraea* with these plasmids. Therefore, a set of alternative expression plasmids for VHb production was constructed based on different promoters and transformation procedures.

Construction of a conjugable *vhb* expression plasmid

Intergeneric conjugation of plasmids from *E. coli* to *Sac. erythraea* has been described by Mazodier *et al.* (1989) and seems in some cases to be even more efficient than transformation (Bierman *et al.*, 1992). Thus, a *vhb* expression vector was constructed for conjugation into *Sac. erythraea*. An expression cassette consisting of the *PermE** promoter, *vhb* and the origin of transfer (*oriT*) from pPM927 (Smokvina *et al.*, 1990) was cloned into the *Streptomyces/E. coli* shuttle vector pJOE875 (Altenbuchner *et al.*, 1992), which contains the *Streptomyces* origin of replication from plasmid pIJ350 (Hopwood *et al.*, 1983) and the pUC origin for replication in *E. coli*. The resulting plasmid was designated pETR419 (Fig. 2). After conjugation from *E. coli* S17.1 into *Sac. erythraea*, thiostrepton-resistant exconjugants were selected. Counterselection against *E. coli* was done with nalidixic acid (40 μg ml^{-1}). The isolated *Sac. erythraea* exconjugants showed only very weak and not reproducible VHb activity, as judged by CO-difference spectrum assays. As confirmed by plasmid isolations from exconjugants, the expression plasmids were not stably maintained and underwent recombination in *Sac. erythraea* (data not shown).

Chromosomal integration of a *vhb* expression cassette in *Sac. erythraea*

Plasmid instability prompted us to construct a vector for chromosomal integration of a *vhb* expression cassette in *Sac. erythraea*. To avoid possible recombination in *Sac. erythraea*, the *PermE** promoter was replaced by another constitutive *Streptomyces* promoter. It has previously been 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). Since *Sac. erythraea* was not expected to contain this mercury-regulated repressor, the *PmerR* promoter was used for *vhb* expression. *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 to the end of the fragments and cloned into pIC19H, which only replicates in *E. coli*. In addition, the thiostrepton resistance gene (*tsr*) was inserted into this plasmid for antibiotic selection in *Sac. erythraea*. The *Streptomyces* phage φC31 attachment site (*att*) (Chater, 1986) was included in the expression vector to facilitate homologous recombination with the *Sac. erythraea* chromosome. This was done on the assumption that *Sac. erythraea* also carries the φC31 *att* site. The resulting

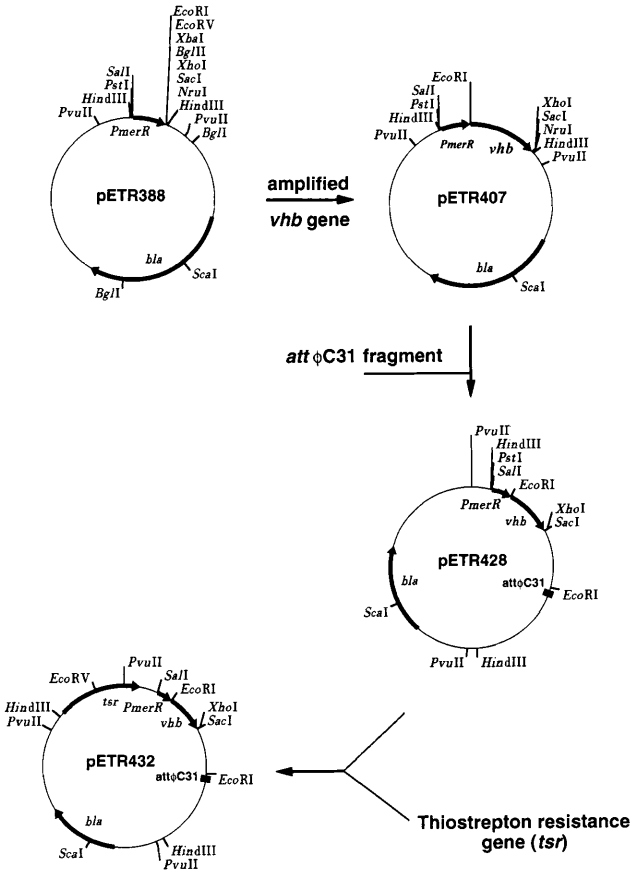

Fig. 3. Construction of pETR432. The *PmerR* promoter and *vhb* were amplified by PCR and cloned into the vector pIC19H to give the plasmid pETR407. Primers used in the PCR reactions were 5′-TTGTCGACCCGCGGCGAATGCGCCGG-3′ and 5′-TTGAATTCCCTTTCCACCAGCAGCTA-3′ for *PmerR*, and 5′-TTGAATTCATGTTAGACCAGCAAACC-3′ and 5′-GGATCCTTATTCAACCGCTTGAGC-3′ for *vhb*, respectively. The *φ*C31 *att* site was isolated as a *Hin*dIII fragment (carrying the *att* site and the 5′ end of the integrase gene) from pJOE706 (not shown) and after filling in the ends with Klenow polymerase was inserted into the *Nru*I-digested plasmid pETR407 to give pETR428. For selection the thiostrepton-resistance gene (*tsr*) from pJOE2139.1 (not shown) was cloned (*Bgl*II/*Xho*I, Klenow) into pETR428 (*Pst*I, Klenow), resulting in pETR432.

plasmid, pETR432, contains *tsr*, *PmerR-vhb* and *att φ*C31, as shown in Fig. 3. This construct was transformed using a modified transformation procedure into *S. lividans* TK64 and *Sac. erythraea* as described above.

The chromosomal integration and expression of *vhb* in transformants of both strains was demonstrated by Southern and Western blot analysis (Fig. 4a) and by PCR (data not shown). In addition, a DNA fragment was amplified from chromosomal DNA of *Sac. erythraea* that had been transformed with pETR432 using *vhb*-specific primers. The amplified fragment was cloned into pIC19H and sequenced to confirm that it had the correct DNA sequence of *vhb*.

Biological activity of synthesized VHb was demonstrated by CO-difference spectrum assays (Fig. 4b). A VHb-specific CO-difference spectrum was observed in crude extracts of *S. lividans* TK64::pETR432 and *Sac. erythraea*::pETR432 after treatment with CO, but no such peak could be detected with the untransformed control strains.

Erythromycin accumulation in fed-batch shake-flask cultivations

Erythromycin productivities of the recombinant *vhb*-expressing strain, *Sac. erythraea*::pETR432, the original *Sac. erythraea* strain and *Sac. erythraea*::pETR451 (lacking the *vhb* expression cassette) were evaluated in shake-flask cultivations. The strains were grown in 30 ml half-strength F1 medium in 250 ml baffled shake flasks as described above. Samples (200 µl) were taken every 24 h during cultivation and the erythromycin titres were determined by a bioassay. Results of these assays are shown in Fig. 5.

Sac. erythraea produced 3·8–3·9 g erythromycin l^{-1}. A similar result (3·6 g l^{-1}) was obtained with the recombinant strain *Sac. erythraea*::pETR451. Under identical conditions, *Sac. erythraea*::pETR432 produced up to 6·3 g erythromycin l^{-1}, a value which was also obtained for other transformants (Fig. 5). This represents an increase in the product titre of approximately 60%. With all strains, the maximum titre was reached at day 8 of the cultivation and then remained constant until the end of the fermentation, at day 9. In contrast to the original strain, erythromycin accumulation in *Sac. erythraea*::pETR432 seemed to be faster during the first 3 d of cultivation. After that the erythromycin accumulation rates remained the same in both strains until, at around days 7 and 8, a drastic increase in erythromycin accumulation was observed for the genetically engineered strain, compared to the original *Sac. erythraea* strain. As calculated from the total protein concentrations, the original *Sac. erythraea* and the recombinant strain *Sac. erythraea*::pETR432 produced similar biomasses during the cultivation (1·6 g l^{-1} and 1·4 g l^{-1} at day 9, respectively).

Stability of the recombinant strain of *Sac. erythraea*

To determine the genetic stability of the chromosomally integrated *vhb* expression cassette, the proportion of cells showing thiostrepton resistance was determined in cultures grown in the absence of thiostrepton. Cells from each sample taken for the erythromycin assays were plated onto R5 agar. Single colonies that emerged were replica-plated onto agar plates containing thiostrepton. The fraction of the colonies that remained thiostrepton resistant after 9 d cultivation in production medium without thiostrepton was greater than 97% (data not shown), demonstrating that the *vhb* expression cassette was stably integrated into the chromosome of *Sac. erythraea*.

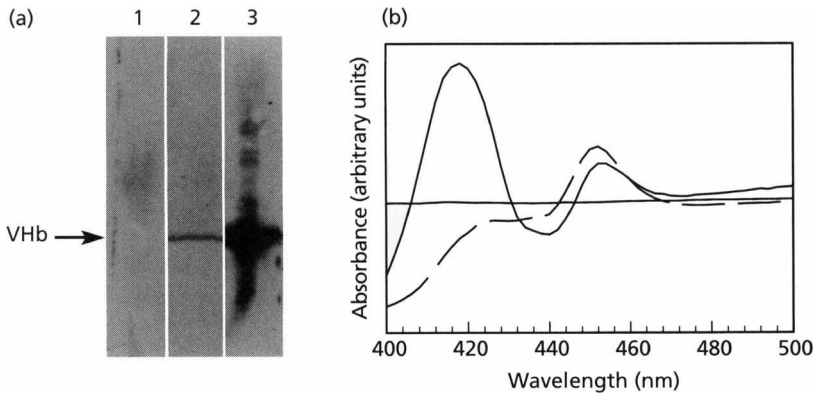

Fig. 4. Western blot analysis (a) and CO-difference spectra (b) of cleared cell extracts (10 μg protein loaded) from *Sac. erythraea* (broken line) and *Sac. erythraea*::pETR432 (full line) after cultivation for 5 d in shake flasks. The difference in absorbance of CO-treated and untreated samples is plotted. Lanes: 1, *Sac. erythraea*; lane 2, *Sac. erythraea*::pETR432; lane 3, *E. coli*/pRED2 (positive control).

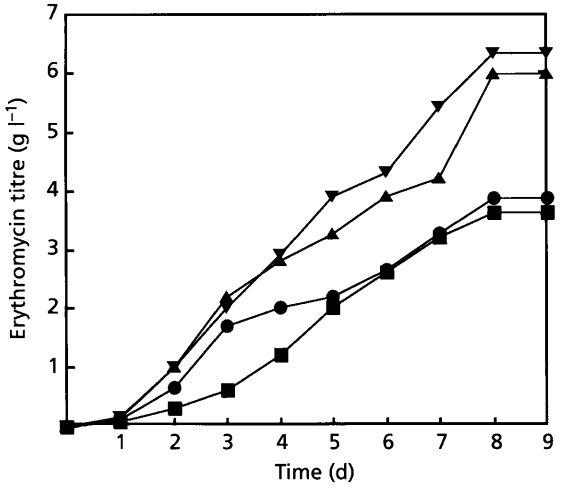

Fig. 5. Erythromycin accumulation with the strains *Sac. erythraea* (●) and *Sac. erythraea*::pETR432 no. 1 (▲) during a 9 d shake-flask cultivation. The mean product titres from two independent cultivations for each strain are shown. In addition, the results obtained with a second transformant, *Sac. erythraea*::pETR432.6 (▼), and the control strain, *Sac. erythraea*::pETR451 (■), are shown.

DISCUSSION

Several cloning strategies for the expression of the *Vitreoscilla* sp. *vhb* gene in *Streptomycetes* have been tested. Initially, the *vhb* gene was cloned and actively expressed in *S. lividans* TK24 using two different *Streptomyces* expression vectors (pIJ6021 and pIJ4090). Both plasmids have high copy numbers and can only replicate in *Streptomyces* spp. However, none of these expression plasmids could be transformed into *Sac. erythraea* by conventional transformation procedures. Even electroporation of mycelia or protoplasts did not yield any transformants. This inefficient transformation could be due in part to the small quantity and poor quality of the DNA isolated from *S. lividans*. However, transformation of poorly characterized, highly developed, and randomly mutagenized industrial production strains is often difficult, if not impossible, to achieve.

Reports on efficient intergeneric conjugation of plasmids from *E. coli* into several *Streptomyces* strains pointed to a reasonable alternative. It has been shown that, besides *S. lividans* and *Streptomyces coelicolor*, *Streptomyces pristinaespiralis* and *Streptomyces viridochromogenes* could be used as recipients in conjugation experiments (Mazodier *et al.*, 1989). Furthermore, it was reported that plasmids could be conjugated into *Streptomyces fradiae*, *Streptomyces ambofaciens* and even into *Saccharopolyspora spinosa*, strains that are barely transformable by PEG-mediated protoplast transformation (Bierman *et al.*, 1992). All of these conjugation systems require the origin of transfer (*oriT*) from RK2 *in cis* (Guiney & Yakobson, 1983) and transfer functions supplied *in trans* from the donor strain *E. coli* S17.1. Therefore, a conjugable *vhb* expression plasmid was constructed. The resulting plasmid, pETR419, was transformed into *E. coli* S 17.1 and then conjugated with *Sac. erythraea* to yield thiostrepton-resistant exconjugants. Although the selected clones seemed to synthesize small amounts of active VHb, it turned out that the expression plasmids were unstable in *Sac. erythraea*. This instability could be in part a result of homologous recombination between the *PermE** fragment of the expression plasmid 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 *Sac. erythraea* and to replace *PermE** with another constitutive promoter (*PmerR*) from *S. lividans* 1326. This construction was expected to reduce the likelihood of homologous recombination with the erythromycin biosynthetic genes. As the target for site-specific integration, the *Streptomyces* phage φC31 attachment site was chosen. This had previously been used for the successful integration of plasmids into the chromosomes of *S. lividans*, *S. fradiae* and *S. ambofaciens* (Bierman *et al.*, 1992).

The resulting *vhb*-expressing construct, pETR432, which contained the *PmerR-vhb* expression cassette, the thiostrepton resistance gene (*tsr*) and a fragment carrying the φC31 attachment site, was successfully transformed into *Sac. erythraea*. The chromosomal

integration of *vhb* was demonstrated by Southern blot analysis and PCR amplification of *vhb* from chromosomal DNA extracted from *Sac. erythraea*::pETR432. All tested transformants showed the same restriction pattern in Southern blots. This indicates that integration of the plasmid had occurred at a specific site, which was probably the ϕC31 or similar phage attachment site of the *Sac. erythraea* chromosome. Integration at this site did not have negative effects on the erythromycin production or growth of the recombinant strain. Furthermore, the integration was shown to be stable for at least the duration of a single erythromycin production batch process (9 d) in the absence of selection pressure with thiostrepton.

CO-difference spectrum assays confirmed the 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 *Sac. erythraea*::pETR432, two absorption maxima were detected: one at 450 nm and one at 418 nm. Whilst the peak at 418 nm was clearly related to *vhb* expression, the peak at 450 nm was probably generated by cytochrome P-450 monooxygenases in *Sac. erythraea* (Katz & Donadio, 1995). The same 450 nm peak was also observed in CO-difference spectra of *Sac. erythraea* not expressing *vhb*. The absorption maximum at 418 nm demonstrated that active VHb was synthesized.

The most important outcome of this study was that erythromycin production in the genetically modified industrial production strain was not adversely affected. By contrast, shake-flask cultivations with *Sac. erythraea*::pETR432 reproducibly showed a 60% higher erythromycin titre compared to the original, VHb-negative strain. The increase was mostly due to the higher erythromycin production rate during the first 3 d of cultivation and an additional strong increase after day 7. The increase in erythromycin accumulation in the recombinant strain is not due to a higher biomass production since the biomass yields of both strains were similar throughout the cultivation. Furthermore, no significant difference in mycelial fragmentation was observed between the two strains (decreased mycelial fragmentation may lead to increased productivity: Bushell *et al.*, 1997). Therefore we assume that the improved erythromycin production in the *vhb*-expressing strain is a consequence of an increased erythromycin biosynthetic flux. This might be the result of an increased activity of an oxygen-dependent step in erythromycin synthesis, most likely the C-6 hydroxylation of 6-deoxyerythronolide B by EryF (Katz & Donadio, 1995) or/and the final hydroxylation step by EryK (Stassi *et al.*, 1993).

We have described here for the first time successful genetic manipulation of an industrial erythromycin-producing strain of *Sac. erythraea*. The preliminary erythromycin production titres from shake flasks probably do not reflect the production potential of the new *vhb*-expressing strain since the culture conditions have not been optimized for this strain.

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