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Stable long-term indigo production by overexpression of dioxygenase genes using a chromosomal integrated cascade expression circuit

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Abstract

In our laboratory we have analyzed different factors to maximize the yield in heterologous protein expression for long-term cultivation, by combination of an efficient cascade expression system and stable integration in the bacterial chromosome. In this work, we have explored this system for the production of indigo dye as a model for biotechnological production, by expressing in *Escherichia coli* the *thnA1A2A3A4* genes from *Sphingomonas macrogolitabida* strain TFA, which encode the components of a tetralin dioxygenase activity. We compared P_{tac}, and the *Pm*-based cascade expression circuit in a multicopy plasmid and stably integrated into the bacterial chromosome. Plasmid-based expression systems resulted in instability of indigo production when serially diluted batch experiments were performed without a selective pressure. This problem was solved by integrating the expression module in the chromosome. Despite the gene dosage reduction, the synergic effect of the cascade expression system produced comparable expression to the dioxygenase activity in the plasmid configuration but could be stably maintained for at least 5 days. Here, we show that the cascade amplification circuit integrated in the chromosome could be an excellent system for tight control and stable production of recombinant products.

Keywords: Dioxygenase; *nahR*; P_{sal}; *xylS2*; *Pm*; Indigo

1. Introduction

Expression vectors appear to be an indispensable tool for both biological studies and biotechnological applications. Controlling gene overexpression becomes an important issue when protein production has

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to be scaled up. In addition to several aspects regarding toxic subproducts or plasmid stability, tight control of gene expression is an important factor among biotechnologists. Expression systems available reach such a recruitment of the cellular transcription and translation resources that protein yields can be up to 20–30% of the total soluble protein (Cebolla et al., 2002; Chao et al., 2002). Several studies have highlighted that high levels of continuous overexpression from plasmid vectors rapidly led to loss of the overproducing phenotype due to plasmid loss or when antibiotics were used to select plasmid maintenance, due to domination of the culture by a population of faster growing mutants, which do not overproduce the desired product (Baheri et al., 2001; Bhattacharya and Dubey, 1995; Camajova et al., 2002; Cebolla et al., 2002). This might be due to the toxicity resulting from the expressed peptide (and/or its metabolic products), and therefore, the cultures are dominated by bacteria carrying knock out mutations in the cloned gene (Corchero and Villaverde, 1998), or to a more general problem relating very high rates of gene expression and plasmid replication. A high transcription rate may interfere with plasmid replication, and consequently, "expression-down" mutants arising from the bacterial population have selective advantage and rapidly dominate the growing culture (Cebolla et al., 2002).

To overcome these problems, a suitable strategy is to integrate the expression module into the bacterial chromosome, which allows maintenance of a stable phenotype even in the absence of a selective pressure, therefore, use of antibiotic in the culture is unnecessary. Nevertheless, the reduction of the gene dose usually results in a dramatic reduction of the protein yield. Recently, a heterologous *nahR*/P_{sal}-*xylS2*/*Pm* cascade expression system, which provides very high protein production even when inserted as a single copy in the bacterial chromosome, has been described as a suitable option for long-term overproduction of β-galactosidase (Fig. 1) (Cebolla et al., 2002). However, a validation of the cascade expression system with a more complex production process of biotechnological relevance has not been tested.

Indigo is one of the largest selling textile dyes, used on cotton and wool fabrics. The textile industry is currently using chemically synthesized indigo. However, indigo can be microbiologically produced from indole by enzymatic formation of a dihydrodiol in a reaction

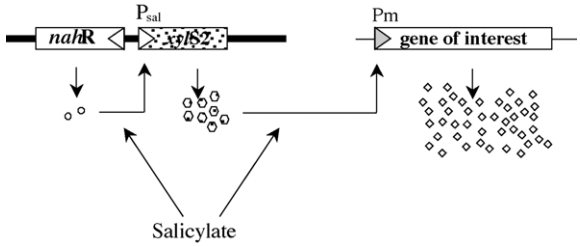

Fig. 1. Scheme of the cascade expression system showing the regulatory and the expression module.

catalyzed by enzymatic complexes with dioxygenase or even monooxygenase activities, which are involved in degradation of aromatic compounds (Ensley et al., 1983; O'Connor et al., 1997). The resulting dihydrodiol is chemically dehydrated to form indoxyl, which in turn dimerizes to indigo (Fig. 2). When the genes coding for both subunits of the dioxygenase and those for the specific electron transport system are cloned and expressed in *Escherichia coli*, the engineered bacteria is able to transform indole to indigo. When growing in rich medium, *E. coli* strains bearing some dioxygenase genes are able to produce indigo from the tryptophan present in the medium by combining the tryptophanase activity of *E. coli* and the dioxygenase activity (Ensley et al., 1983). A number of scientists are seeking for a greener and cost-effective alternative method of indigo production (Murdock et al., 1993; Bialy, 1997). Most modifications involved metabolic engineering of *E. coli* to increase endogenous production of indole (Berry et al., 2002; Berry, 1996), but the specific activity of the cloned dioxygenase, and a high and stable expression of them are also important issues to design engineered *E. coli* strains for indigo production.

In this paper, we analyze the performance of the cascade amplification circuit to co-express four genes necessary for the dioxygenase activity, which was required for the production of the metabolite indigo. The dioxygenase genes utilized in this work are those involved in degradation of the organic solvent tetralin by *Sphingomonas macrogolitabida*, which have recently been characterized in our laboratory (Moreno-Ruiz et al., 2003). The requirement in *E. coli* of the ferredoxin reductase component of the dioxygenase enzymatic complex has been tested. Additionally, we have explored the effect of heterologous co-expression of the gene coding for the tryptophanase activity on indigo production.

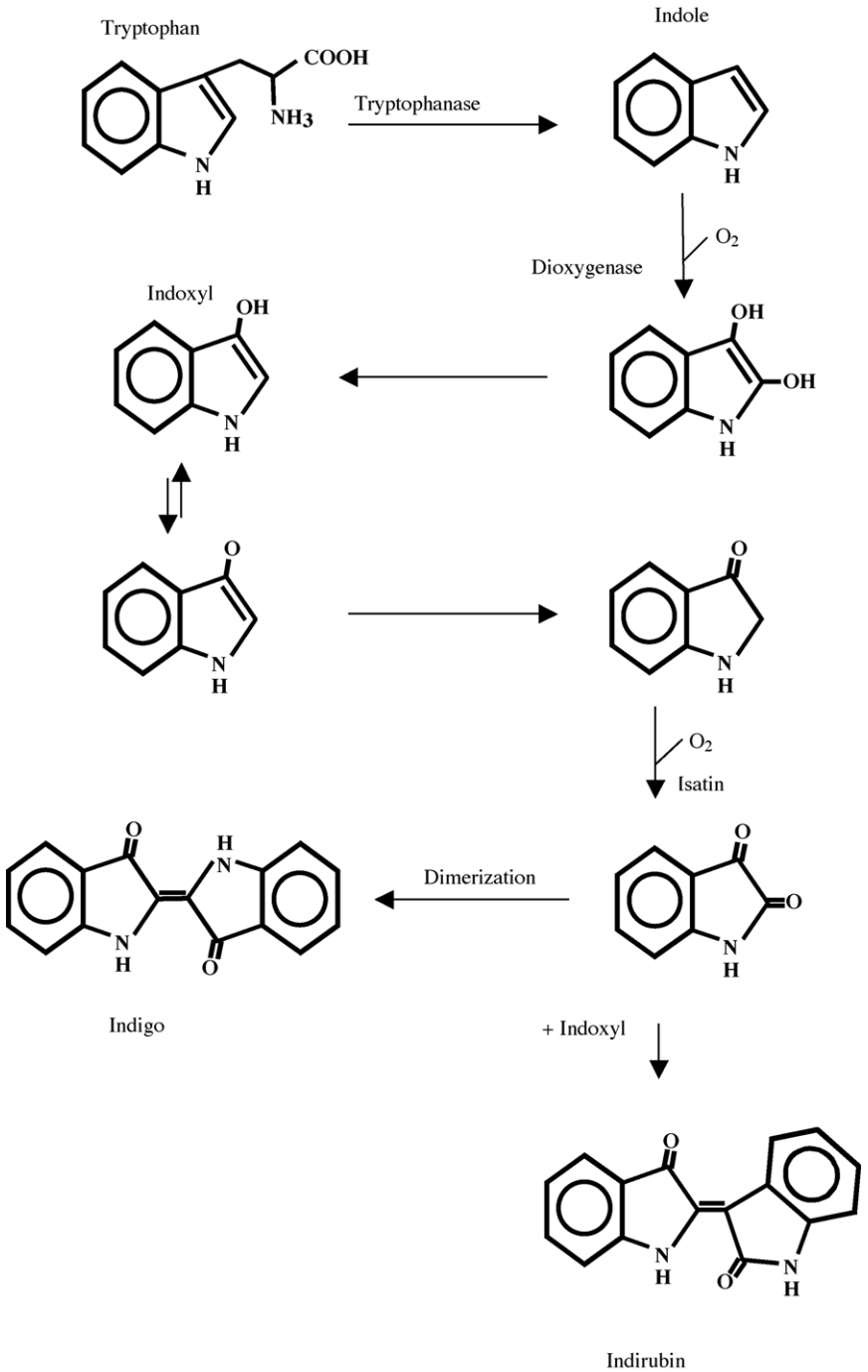

Fig. 2. Scheme of the modification reactions of tryptophan, which lead to formation of indigo and its related dyes.

Table 1
Strains and plasmids used in the present work

	Description	Reference
Strains		
E. coli DH5α	deoR, endA1, gyrA96, recA1, supE44	Laboratory collection
E. coli S171-λpir	F^- recA, hsdR, RP4-2 (Tc::Mu) (Km::Tn7) lysogenized with λpir phage	de Lorenzo et al. (1993)
E. coli MPO12	*E. coli* K12 spontaneous Rif^R with a Km^R-linked miniTn5 bearing a *nahR*/P_{sal}-*ylS2* module	This study
E. coli MPO13	MPO12 with a Tc^R-linked miniTn5 with a rrnBT1-*Pm-thnA1A2A3A4*	This study
E. coli NCM631	BL21 (DE3) derived strain with a Tn10 linked to Δlac	Govantes et al. (1996)
Plasmids		
pBluescript	Ap^R, cloning vector with a *T7* promoter	Stratagene
pCAS	Ap^R, expression vector with rrnBT1-*Pm*-polilinker	Active Motif
pCNB4-S2	Ap^R, Km^R, miniTn5 vector with the *nahR*/P_{sal}-*xylS2* fusion cloned between the I and O sites	Cebolla et al. (2001)
pIZ227	Cm^R, expression vector expressing both $lacI^q$ and the lysozyme	Govantes et al. (1996)
pIZ633	Ap^R, Bluescript derived plasmid expressing *thnA3* from the *T7* promoter	This study
pIZ651	Ap^R, Bluescript derived plasmid expressing *thnA1A2* from the *T7* promoter	This study
pIZ652	Ap^R, Bluescript derived plasmid expressing *thaA1A2A3A4* from the *T7* promoter	This study
pIZ682	Ap^R, pIZ651 derived plasmid with *thnA1A2A3* expressed from the *T7* promoter	This study
pIZ693	Gm^R, pIZ1016 with *thnA1A2A3A4* expressed from a *tac* promoter	Moreno-Ruiz et al. (2003)
pIZ1016	Gm^R, Expression vector derived from pBBR bearing $lacI^q$ and *tac* promoter	Moreno-Ruiz et al. (2003)
pMPO3	Ap^R, pCAS with rrnBT1-*Pm-thnA1A2A3A4*	This study
pMPO5	Ap^R, pCAS with rrnBT1-*Pm-tnaA*	This study
pMPO17	Ap^R, Tc^R. MiniTn5 vector with the rrnBT1-*Pm-thnA1A2A3A4* cloned between the I and O sites	This study

2. Materials and methods

2.1. Plasmids, strains and culture media

Plasmids and strains are described in Table 1. Different combinations of genes encoding dioxygenase components, which are organized in two divergent operons in *S. macrogolitavida* (Moreno-Ruiz et al., 2003), were cloned together as follows: the *thnA1* and *thnA2* genes were cloned as a *Sal*I–*Nae*I (filled in) fragment between the *Sal*I and *Eco*RV sites of pBluescript SKII(+), yielding pIZ651. Plasmid pIZ652 was constructed by cloning a *Kpn*I (filled in)–*Xba*I fragment containing *thnA3* and *thnA4* between the *Sma*I and *Xba*I sites of pIZ651. An *Eco*RI fragment from pIZ652 harbouring *thnA3* was cloned in the *Eco*RI site of pBluescript SKII(+) to obtain pIZ633. The *thnA3* gene was then subcloned from this plasmid as an *Eco*RV–*Xba*I fragment and ligated between the *Sma*I–*Xba*I sites of pIZ651, thus obtaining pIZ682. A *Sal*I–*Xba*I fragment of pIZ652, containing *thnA1A2A3A4* was cloned in pIZ1016 (Moreno-Ruiz et al., 2003), thus placing the operon downstream of the *tac* promoter generating pIZ693.

To express dioxygenase genes from the *Pm* promoter, the gene cluster coding for both the dioxygenase subunits (*thnA1* and *thnA2*) and the dioxygenase reductase (*thnA3* and *thnA4*) genes from pIZ652 as a *Sal*I(filled in)–*Xba*I fragment were cloned between the *Nco*I (filled in) and *NheI* sites of pCAS-B (Active Motif, USA), thus resulting in pMPO3. A *Not*I fragment from pMPO3 containing *Pm-thnA1A2A32A4* was cloned into the unique *Not*I site of pUT-Tc generating pMPO17.

The *tnaA* gene coding for the tryptophanase in *E. coli* was amplified by polymerase chain reaction (PCR) using the following primers: TNA-1: 5′-ATCTAGAGAGGTAAAATAAATGAAGGAT-3′ and TNA-2: 5′-ATCCTTATAGGAGCTCTGTAGTA-3′. Underlined nucleotides correspond to *Xba*I and *Sac*I restriction sites, respectively. PCR was performed at a final volume of 25 μL containing 25 ng of *E. coli* chromosome as template, 10 pg of each primer and 2.5 mM MgCl. Initial 5 min of denaturation at 95 °C

were followed by 30 cycles of amplification (95 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min), and a final extension of 5 min at 72 °C. The amplicon was purified, digested with *Xba*I (filled in) and *Sac*I, and cloned into a pCAS vector linearized with *Nco*I (filled in) and *Sac*I to yield pMPO5.

The insertion of the regulator module *nahR*/P_{sal}-*xylS2* into the chromosome was constructed as follows: a spontaneous rifampicin-resistant mutant of *E. coli* K12 was used as a recipient in matings with *E. coli* S17-1λpir carrying pCNB4-S2 (Cebolla et al., 2002). Conjugation was performed in 1% sodium citrate at 30 °C for 3 h. Recipients with transposition events were selected at 37 °C on Luria–Bertani (LB) agar plates with rifampicin 20 mg L^{-1} and kanamycin 50 mg L^{-1}. Colonies were also checked for ampicillin sensitivity (100 mg L^{-1}), thus ruling out unspecific plasmid integration. A prototrophic candidate of the resultant strain was chosen and named MPO12. This strain was subsequently transformed chemically with pMPO3 or pIZ693 and the corresponding empty vectors. A second transposition event was carried out, using S17-1λpir (pMPO17) as donor and MPO12 as recipient. Selection of a kanamycin-resistant, tetracycline-resistant and ampicillin-sensitive transconjugant resulted in the strain MPO13, which carried both the *nahR*/P_{sal}-*xylS2* and the *Pm-thnA1A2A3A4* modules.

E. coli strains were grown in minimal M9 medium containing glucose, 1 g L^{-1}; NH_4, 1 g L^{-1}; Na_2PO_4, 6 g L^{-1}; KH_2PO_4, 3 g L^{-1}; $MgSO_4$, 0.26 g L^{-1} and Cl_2Ca, 11.1 mg L^{-1} or in LB medium containing tryptone, 10 g L^{-1}; NaCl, 5 g L^{-1} and yeast extract, 5 g L^{-1}.

2.2. Induction of batch cultures

Cells were grown in LB at 37 °C and shook until OD_{660} reached 0.3. Then, they were induced with either 2 mM sodium salicylate (Sigma) or 1 mM isopropyl β-D-galactopiranoside (IPTG) (Sigma), and incubated at 30 °C and 150 rpm. After 5, 7 and 24 h, OD_{660} was measured and samples were collected for indigo extraction, as described (O'Connor et al., 1997). Briefly, 1 mL of each bacterial culture was centrifuged at maximum speed for 5 min. Supernatants were discarded and blue pellets were resuspended in 1 mL of DMF (Sigma). Samples were then centrifuged again and the blue supernatant was stored in darkness and −20 °C until absorbance (λ = 610 nm) was determined. The extinction coefficient used for calculations was $\varepsilon = 16{,}230\ M^{-1}\ cm^{-1}$ according to standard indigo curves and data previously reported (Moreno-Ruiz et al., 2003).

2.3. Serial batch cultures

Serially diluted batch cultures were performed as follows: 20 mL LB cultures were induced in early-exponential phase and incubated at 30 °C and 150 rpm. Cultures were diluted 100-fold every 12 h in new medium containing 2 mM salicylate. When plasmid maintenance was selected, cultures also contained 100 mg L^{-1} ampicillin. Samples were collected, and OD_{660} and indigo production were monitored during the experiments for 108 h (4.5 days).

2.4. Tetralin dioxygenase activity in E. coli

E. coli cultures induced with IPTG for 7 h were harvested, concentrated to an $OD_{600} = 4$ in 10 mL of a mineral M9 medium supplemented with 300 μM tetralin, and incubated in a shaker at 30 °C. Samples of 1 mL were taken at different times, harvested and the supernatant pipetted to a new vial and acidified to transform the *cis*-dihydrodiol into the mixture of monohydroxylated aromatic derivatives (Boyd et al., 1994; Sikkema and de Bont, 1993). The concentrations of 1-hydroxytetralin (1-HT) and 2-hydroxytetralin (2-HT) in the acidified supernatants were determined by HPLC, as previously described (Moreno-Ruiz et al., 2003).

2.5. Tryptophanase activity assay

In vitro tryptophanase activity was indirectly assayed by using resting cells expressing dioxygenase genes. Crude extracts were obtained from overnight-induced cultures of MPO12 (pMPO5) grown in M9 medium. Cells were concentrated up to $OD_{600} = 50$ in phosphate buffer 100 mM pH 7.8, broken by sonication (five 30 s pulses on ice), centrifuged at 13,000 rpm for 5 min and the supernatant was taken. Resting cells with dioxygenase activity were prepared from a culture of MPO12 (pIZ693) growing in M9 medium which had been induced at $OD_{600} = 0.2$–0.3 by addition of 1 mM IPTG. Cells were collected 7 h after induction, and con-

centrated up to OD_{600} = 50 in phosphate buffer 100 mM pH 7.8. Rate of indigo production from 1 mM tryptophan by the resting cells is measured with or without the addition of equal volume of crude extract of MPO12 (pMPO5) (final OD_{600} = 25) enriched in tryptophanase activity. Rate of indigo production from 1 mM indole by the resting cells was also measured as a positive control. All assays were carried out in the presence of 200 μg mL^{-1} rifampicin to avoid de novo expression of the chromosomal *tnaA* gene in the resting cells.

2.6. Thin layer chromatography and indigo analysis

The pigment mixture obtained, as described above, was subjected to analytical thin layer chromatography at room temperature (silica gel 60 F_{250} plates 20 cm, Merck, Darmstadt, Germany) along with standard indigo. The running solvent was 20% diethyl ether in chloroform. Samples were also subjected to a spectrophotometric scan ranging from 250 to 700 nm.

3. Results

3.1. Indigo production by tetralin dioxygenase activity

Genes responsible for the tetralin dioxygenase activity of *S. macrogolitabida* strain TFA have been recently identified (Moreno-Ruiz et al., 2003). Functional analysis indicated that *thnA4*, encoding a ferredoxin reductase, was involved in the dioxygenase activity. However, an insertion mutant in *thnA4* still maintained some dioxygenase activity, suggesting that other reductases of strain TFA could provide electrons to the enzyme complex (Moreno-Ruiz et al., 2003). To directly confirm in *E. coli* the requirement of each *thnA* genes products in the dioxygenation of tetralin, the *thnA1A2* and *thnA3A4* genes, which are located separately in two divergent operons (Moreno-Ruiz et al., 2003), were cloned together in expression vectors. Different combinations of *thnA* genes were assembled in pBluescript SK II (+), which in the appropriate strain drives transcription of downstream genes from the *T7* promoter, in response to IPTG.

The overproducing *E. coli* strain NCM631 (pIZ227) (Govantes et al., 1996) was transformed with each pBluescript derivative, and overproduction experiments were performed. Induced *E. coli* cells were tested for tetralin ring-hydroxylating dioxygenase activity in a resting cell assay. As shown in Fig. 3, cells bearing pIZ651, which just have the α (ThnA1) and β (ThnA2) subunits of the dioxygenase enzyme, did not produce 2-HT from tetralin. However, *E. coli* cells bearing pIZ682, which have the ferredoxin ThnA3, or those bearing pIZ652 with both ThnA3 and ThnA4 also present, produced significant amounts of 2-HT over time. Interestingly, cells having just ThnA1A2A3 produced 2-HT at a rate even higher than cells also having ThnA4. Taken together, these data clearly indicate that ThnA1, ThnA2 and ThnA3 are essential, but the ferredoxin reductase ThnA4 is fully dispensable for tetralin dioxygenase activity in *E. coli*.

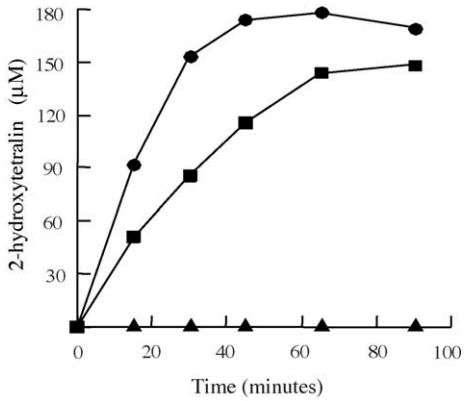

Fig. 3. Production of 2-hydroxytetralin from tetralin by strains bearing different combinations of *thnA* genes. Induced *E. coli* NCM631 (pIZ227) bearing the plasmids pIZ561 (*thnA1A2*, ▲), pIZ682 (*thnA1A2A3*, ●), or pIZ652 (*thnA1A2A3A4*, ■).

Accumulation of a blue pigment by strains overproducing different ThnA products during growth in LB medium fully correlated to their tetralin dioxygenase activity. The blue dye accumulated in the induced cultures was extracted with DMF, as described in Section 2, and its absorption spectrum characterized (Fig. 4a). Maximum absorbance peaks corresponding to 286 and 610 nm were detected, with the same profile as the commercial indigo available resuspended in the same solvent (Gillam et al., 1999). Thin layer chromatography did not show any indirubin contamination at the concentration tested (Fig. 4b). The retention factor (R_f) calculated was 0.85 for both the commercial indigo and our samples. No indigo formation was detected if

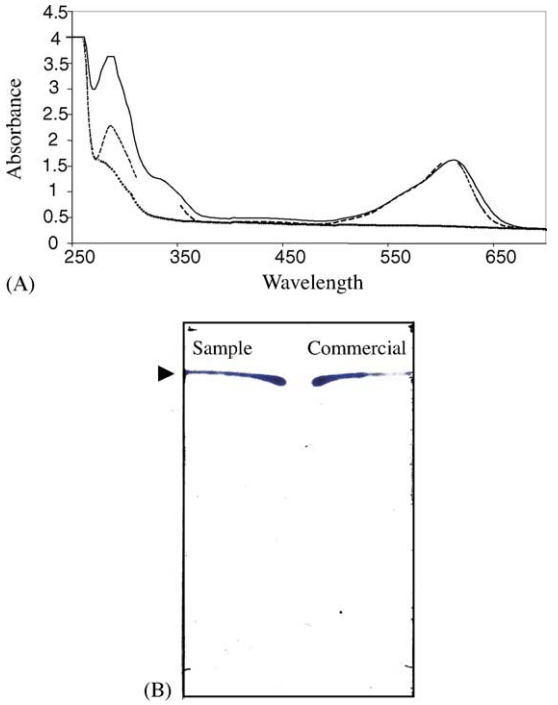

Fig. 4. (A) Absorption spectra of commercial indigo (- - -) and extractions from cultures overexpressing (continuous) or not overexpressing dioxygenase genes (· · ·). (B) Thin layer chromatography of both commercial and biologically produced indigo samples.

cultures were uninduced, or when induced but bearing empty vectors (data not shown). Taken together, these data clearly demonstrate that the blue dye produced by cloning tetralin dioxygenase genes in *E. coli* is indigo, and that the dye may be an excellent reporter for monitoring the heterologous gene co-expression.

3.2. Cascade amplification circuit and gene dosage

The cascade expression system (Fig. 1) consists of a regulation module, already inserted into the chromosome of the strain MPO12, and the expression module where the genes of interest have to be cloned. The *thnA1A2A3A4* genes, already cloned together in plasmid pIZ652, were subcloned downstream of the *Pm* promoter of the expression module present in plasmid pCAS, and subsequently placed in a miniTn5 delivery vector to insert it into the chromosome. The same gene arrangement, transcribed from the *tac* promoter,

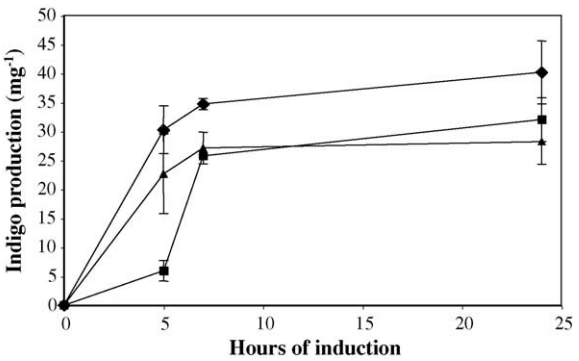

Fig. 5. Accumulation of indigo after induction by the *E. coli* MPO12 strain bearing the *thnA1A2A3A4* genes expressed from the P_{tac} promoter (pIZ693) (▲), from the cascade expression module in a plasmid (pMPO3) (♦), or form the expression module integrated in the chromosome (MPO13) (■). Data represent the average of four independent induction experiments; error bars represent standard deviations.

was already cloned in the broad host range expression vector pIZ1016 (Moreno-Ruiz et al., 2003). In order to evaluate the expression capacity of the cascade amplification circuit, depending on the gene dosage of the *Pm-thnA1A2A3A4* operon, we compared productions between MPO13 and MPO12 (pMPO3) (monocopy versus multicopy). Moreover, induction levels were compared also to those obtained from either P_{T7} (pIZ652) or P_{tac} (pIZ693). Cultures were grown aerobically in LB at 37 °C until early exponential phase when cultures were induced by addition of 1 mM IPTG or 2 mM salicylate, as stated in Section 2, and indigo concentration in the culture estimated at different time intervals. As can be observed in Fig. 5, indigo production from the strains bearing the expression systems in plasmids rapidly increased after induction and reached at least 75% of the maximum production within the first 5 h. Indigo accumulation was slightly lower (70%) in the strain expressing the dioxygenase genes from the *tac* promoter in the plasmid. On the other hand, the strain expressing dioxygenase genes from the chromosome produced four- to six-fold less indigo during the first 5 h after induction. However, after 7 h the gene circuit in the chromosome allowed enough dioxygenase activity to obtain levels of indigo similar to those obtained from the *tac* promoter expression system and just slightly lower (70–75%) than those obtained from the expression module of the cascade in a plasmid. Results of the expression system based on the *T7* promoter were

very similar to those of the system based on the *tac* promoter; not shown.

3.3. Stability of the indigo-producing phenotype

One important aspect of biotechnological interest is to know whether the high performance achieved by the plasmid configurations could be maintained in a long-term continuous production. In order to test this, batches were set with the strains bearing the chromosomal configuration of the cascade amplification circuit (MPO13), or the plasmid configuration (MPO12 bearing pMPO3). Cultures with the plasmid configuration were incubated with or without selective pressure for plasmid maintenance, whilst the chromosomal configuration was not selected at all. Serial dilutions of 100-fold after reaching stationary phase were done up to eight times, and indigo production and growth rate was monitored for several days. Results from the serial batch experiments are summarized in Fig. 6. Indigo production by the strain with the expression module in the plasmid started to decrease after 12 h (one dilution). Loss of the indigo-producing phenotype was very fast in the culture without selection for plasmid maintenance, whose production capacity was reduced almost 10-fold after 48 h (three serial dilutions) and fully eliminated after 60 h (four serial dilutions). Selective pressure reduced the rate of loss of the indigo-producing phenotype very slightly. Thus, selection could not stabilize the production, which was also undetectable after 60 h (four dilutions). The maximal levels of indigo production by the strain with the expression module in the chromosome were 65% of the maximal levels obtained by the strain with the plasmid configuration. However, the indigo-producing phenotype was maintained for at least 108 h (eight serial dilutions). These data clearly indicate that for an indigo-producing process that involved production and growth for longer than 24 h, inserting the expression module bearing the dioxygenase genes into the chromosome is a preferred option in order to prevent loss of indigo yield.

Interestingly, when indigo accumulation was tested in intermediate samples (30, 42, 54 h, etc.), with cultures in the middle or late exponential phase, no significant accumulation of indigo was detected (data not shown). This effect was invariably observed in the four experiment repetitions, regardless of the stability of the indigo-producing phenotype. Thus, it seems that indigo is only produced after the onset of the stationary phase. Since the cascade expression system could be induced to maximal levels during exponential phase (Cebolla et al., 2002), it is unlikely that lack of indigo production during exponential phase was due to low expression of dioxygenase genes. However, since indigo comes from indole, which is in turn produced from the tryptophan in the growth medium, one possibility is that tryptophanase, the enzyme required to produce indole from tryptophan was limiting during the exponential phase of growth. With this idea in mind, we decided to clone and heterologously co-express the tryptophanase gene (*tnaA*) in order to obtain a continuous high rate of hydrolysis of tryptophan.

3.4. Effect of tryptophanase co-expression on indigo production

The *tnaA* gene of *E. coli* was cloned downstream of the *Pm* promoter in the expression module present in pCAS to test if its increased activity could favor the generation of indole, thus increasing the indigo production. For this purpose, we measured the indigo production in a strain expressing tryptophanase from *Pm* promoter and dioxygenase from the *tac* promoter. Bioconversion from indole provided in the growth medium to indigo has been previously reported as a common way of measuring dioxygenase activity. However, our purpose was to achieve endogenous indole production from tryptophan by means of the tryptophanase reaction. As shown in Table 2, induction by salicylate for the overexpression of *tnaA* from the plasmid pMPO5 did not affect the indigo yield at all. To ensure that salicylate induction actually resulted in an increase of the tryptophanase activity, we developed an indirect assay for testing this reaction. Resting cells overproducing the dioxygenase activity were challenged with tryptophan in the presence of a crude extract of a tryptophanase-producing culture (induced MPO12 bearing pMPO5), or crude extracts of cultures not overproducing the tryptophanase, and indigo production was monitored over time. As can be observed in Fig. 7, resting cells of MPO12 (pIZ693) were not capable of producing indigo from tryptophan, though they readily produced it from indole. However, when a crude extract of the strain overexpressing *tnaA* was added, the tryptophanase activity in the extract readily hydrolyzed tryptophan to indole, which was transformed to indigo by the dioxy-

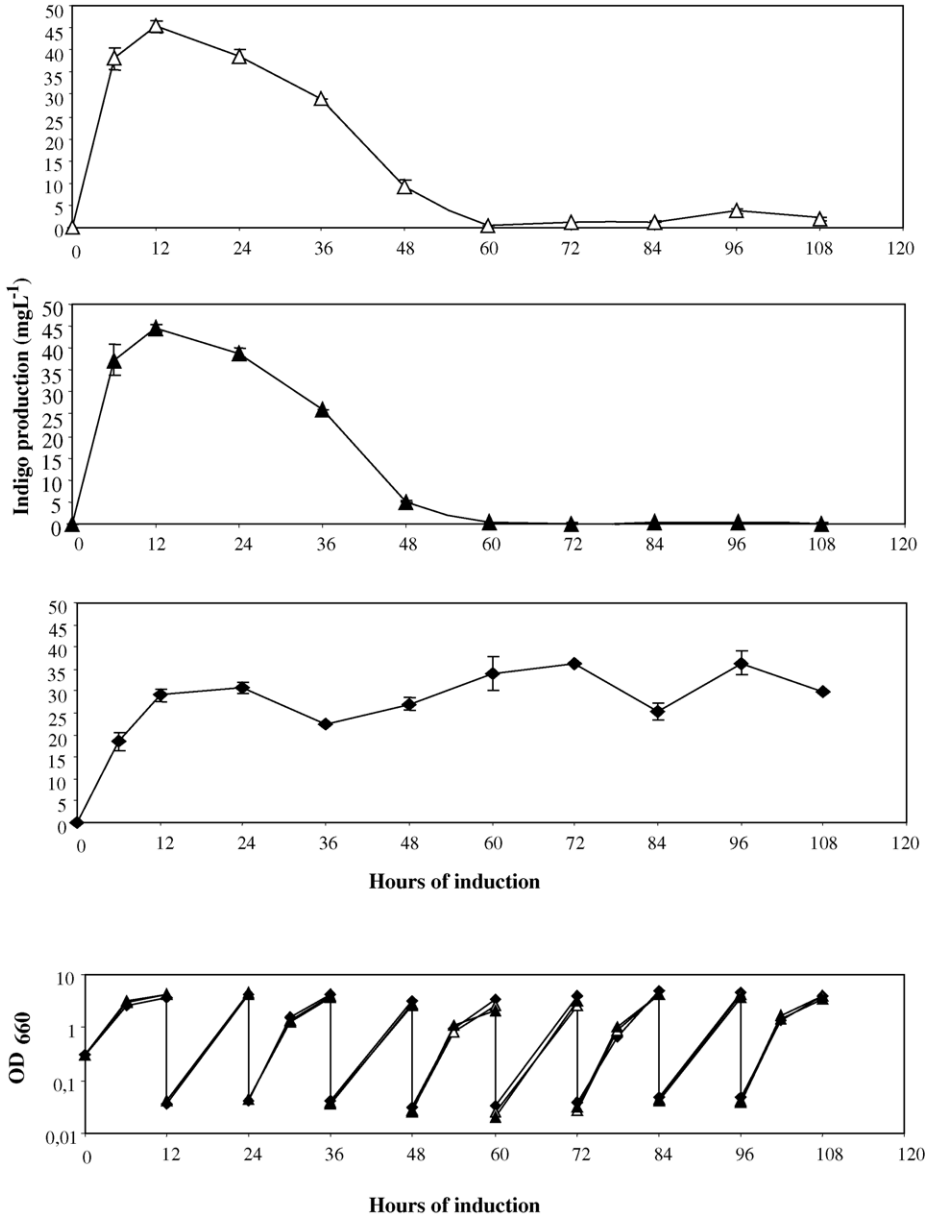

Fig. 6. Stability of the indigo-producing phenotype of the strains expressing dioxygenase genes from a plasmid, or from the chromosome. (A) Indigo accumulation of induced cultures of MPO13 strain (♦) and MPO12 (pMPO3) after growth in LB (▲), or LB plus 100 mg L^{-1} ampicillin (△) for 12 h after each dilution. (B) Optical densities of the growing cultures after each dilution. Data are the average of four repetitions carried out in parallel.

genase activity of the resting cells. Similar experiments using crude extracts from the same strain, but not overproducing the tryptophanase since it was grown in the absence of salicylate, did not result in indigo production from tryptophan. Thus, co-expressing the tryptophanase in cells overexpressing dioxygenase genes did not improve the rate of indigo production from tryptophan in cultures growing in rich medium, probably

Table 2
Effect of tryptophanase co-expression together with the dioxygenase activity on indigo production (mg L^{-1})

Time after induction (h)	pMPO5	pMPO5 + pIZ1016	pMPO5 + pIZ693			pIZ693 + pCAS	pIZ693
	+/−[a]	+/+[a]	+/+[a]	+/−[a]	−/+[a]	+/+[a]	−/+[a]
5	ND	ND	10.1 ± 6.9	0.2 ± 0.1	16.1 ± 6.0	10.1 ± 6.5	15.9 ± 9.2
7	ND	ND	23.8 ± 6.2	0.3 ± 0.2	23.2 ± 3.5	21.5 ± 5.7	22.8 ± 3.6
24	ND	ND	27.3 ± 10.7	0.3 ± 0.1	25.4 ± 4.9	31.9 ± 13.1	23.0 ± 4.1

tnaA (in pMPO5) and *thnA1A2A3A4* genes (in pIZ693) were expressed from the *Pm* and P_{tac} promoters, respectively. Three independent experiments were performed and average ± standard errors are represented. ND: not detected.
[a] Salicylate IPTG.

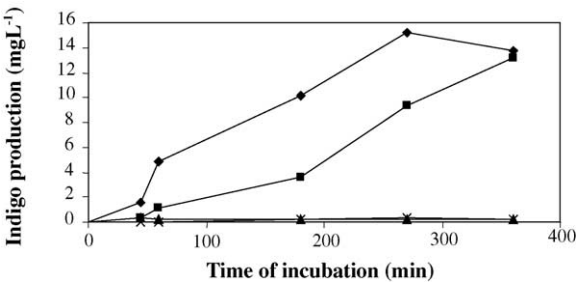

Fig. 7. Tryptophanase activity assayed using resting cells expressing dioxygenase genes. Indigo production by induced MPO12 (pMPO3) resting cells after addition of 1 mM indole (♦), 1 mM tryptophan (×), 1 mM tryptophan plus equal volume of crude extract from the strain overproducing tryptophanase MPO12 (pMPO5), grown in the presence of salicylate (induced, ■), or in its absence (uninduced, ▲). Tryptophan was added together with rifampicin to avoid endogenous *tnaA* induction in the resting cells.

because tryptophanase production is not limiting under these conditions.

4. Discussion

Versatile expression vectors have been developed in *E. coli* for different purposes. Most of them are single expression systems engineered to carry strong promoters, such as *tac*, *trc* or *T7* promoters. However, these expression systems are unstable in plasmids, and very little is known about the performance of these expression systems once placed in a single copy in the chromosome. Some examples have indicated that expression levels from the chromosome were largely reduced (de Lorenzo et al., 1993; Suarez et al., 1997). Moreover, potential toxicity and high cost of IPTG have restricted industrial application of many of these expression systems, and its use for producing human therapeutic proteins (Chao et al., 2002; Figge et al., 1988).

In this paper, we have tested the feasibility of using a cascade expression system integrated into the chromosome for the stable production of dye indigo in long-term fermentations. Production of indigo from indole involves a dioxygenase activity acting on aromatic compounds. The most commonly used dioxygenase genes encode a naphthalene dioxygenase activity (Berry et al., 2002; Bhushan et al., 2000; Bialy, 1997). However, genes encoding the components of a tetralin dioxygenase and its electron transfer system from *S. macrogolitabida* strain TFA have been used in this work as an alternative source of indigo-producing enzymatic activity. Testing the requirement of each gene for dioxygenase activity in *E. coli* has shown that *thnA4*, which encodes the ferredoxin reductase component of the electron transfer system to the dioxygenase, is not required at all (Fig. 3). It was previously shown in *Sphingomonas* that the function of the ferredoxin reductase could be partially provided by other ferredoxin reductases present in the cell. Apparently, the ferredoxin ThnA3 can accept electrons from undefined ferredoxin reductases of *E. coli* so efficiently that the function of ThnA4 is dispensable, at least under the conditions tested. This indicates that cloning the fourth gene of the enzymatic complex is not necessary if tetralin dioxygenase genes are used to engineer *E. coli* strains for indigo production.

When the expression system bearing the dioxygenase genes was integrated into the chromosome, significantly lower amount of indigo was accumulated during the first 5 h after induction (Fig. 5). Initially, this could be explained by a delay in the induction of dioxygenase genes from the chromosome, in comparison to the system in the plasmid. However, no delay at all was ob-

served from the system integrated in the chromosome when overexpressing *lacZ* (Cebolla et al., 2002). The delay in indigo accumulation may rather indicate a low specific activity of the *thn* dioxygenase using indole as substrate. This suggests the need of maintaining high levels of dioxygenase production in long-term fermentations in order to keep a high rate of bioconversion of indole to indigo.

Despite the initial slower accumulation of indigo using the system integrated in the chromosome, maximal accumulation of indigo was comparable to those obtained using the same expression system in a multicopy plasmid, or a different plasmid system based on the *tac* promoter. This indicates that dioxygenase genes are expressed from the system in the chromosome to sufficiently high levels to obtain a high rate of indole bioconversion, which represents 65–70% of the rate obtained with the expression module in a plasmid (Fig. 6). Most importantly, when overexpression was from the chromosome, the indigo-producing phenotype was maintained for at least 108 h without selective pressure, while strains overexpressing from systems in plasmids lost the indigo-producing phenotype very rapidly. These results clearly indicate that in order to develop an indigo-producing bioprocess, integration of dioxygenase genes in the chromosome of the engineered strain is strongly recommended. This recommendation may be extensible to other bioengineered processes, which involve either continuous or fed batch cultures of overexpressing strains.

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