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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Study of the role played by NfsA, NfsB nitroreductase and NemA flavin reductase from *Escherichia coli* in the conversion of ethyl 2-(2′-nitrophenoxy)acetate to 4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one (D-DIBOA), a benzohydroxamic acid with interesting biological properties

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Abstract Benzohydroxamic acids, such as 4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one (D-DIBOA), exhibit interesting herbicidal, fungicidal and bactericidal properties. Recently, the chemical synthesis of D-DIBOA has been simplified to only two steps. In a previous paper, we demonstrated that the second step could be replaced by a biotransformation using *Escherichia coli* to reduce the nitro group of the precursor, ethyl 2-(2′-nitrophenoxy)acetate and obtain D-DIBOA. The NfsA and NfsB nitroreductases and the NemA xenobiotic reductase of *E. coli* have the capacity to reduce one or two nitro groups from a wide variety of nitroaromatic compounds, which are similar to the precursor. By this reason, we hypothesised that these three enzymes could be involved in this biotransformation. We have analysed the biotransformation yield (BY) of mutant strains in which one, two or three of these genes were knocked out, showing that only in the double *nfsA/nfsB* and in the triple *nfsA/nfsB/nemA* mutants, the BY was 0%. These results suggested that NfsA and NfsB are responsible for the biotransformation in the tested conditions. To confirm this, the *nfsA* and *nfsB* open reading frames were cloned into the pBAD expression vector and transformed into the *nfsA* and *nfsB* single mutants, respectively. In both cases, the biotransformation capacity of the strains was recovered (6.09±0.06% as in the wild-type strain) and incremented considerably when NfsA and NfsB were overexpressed (40.33%±9.42% and 59.68%±2.0% respectively).

Keywords Biotransformation · *Escherichia coli* · Benzohydroxamic acids · Nitroreductases NfsA and NfsB · Flavin reductase NemA · D-DIBOA

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Introduction

Some benzohydroxamic acids isolated from plants such as 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one, known as DIBOA, and 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxacin-3(4H)-one, known as DIMBOA (Honkanen and Virtanen 1960; Hamilton et al. 1962), exhibit a variety of interesting biological activities as phytotoxic properties, antimicrobial activity as well as antifeedant, antifungal and insecticidal properties (Duke 1986; Macías et al. 2006a, 2008, 2009).

Recently, the chemical synthesis of an analogue of these compounds known as D-DIBOA [4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one] was simplified to only two steps

(Macías et al. 2001, 2006b). The product of the first step is the ethyl 2-(2′-nitrophenoxy)acetate (Macías et al. 2006b) (Fig. 1a), which is henceforth referred to as the precursor (Valle et al. 2011). The second step involves the reduction of the nitro group followed by a cyclisation (Fig 1b). This second step, which occurs in complex conditions, requires the usage of an expensive catalyst and renders undesired products (Macías et al. 2006b). In a previous work, we demonstrated that this second step could be carried out by *Escherichia coli* JM109 (Fig. 1b) (Valle et al. 2011), and by this reason, we have approached microbial biotransformation as a new strategy of synthesis.

Aromatic nitroreductases have been widely studied in Gram-negative bacteria, mainly in the family Enterobacteriaceae. Phylogenetic analyses suggest that type I nitroreductases can be classified into two main groups, which are represented by the *E. coli* nitroreductases; NfsA group A (Blattner et al. 1997) and NfsB group B (Michael et al. 1994) encoded by the *nfsA* and *nfsB* genes, respectively. Group A nitroreductases are usually NADPH-dependent (Bryant et al. 1981; Zenno et al. 1996a), while group B nitroreductases may use both NADH or NADPH as electron donors (Bryant et al. 1981; Whiteway et al. 1998; Zenno et al. 1996b, 1998). In addition to the nitroreductases, bacteria also possess flavoreductases of the so-called old yellow enzyme (OYE) family that are capable of denitrating some explosives such as the nitroesters pentaerythritol tetranitrate (PETN), glycerol trinitrate (Roldán et al. 2008) and trinitrotoluene (TNT) (González-Pérez et al. 2007) using NADPH as the electron donor. The best known flavoreductases have been characterised in the species that belong to the family Enterobacteriaceae: the PETN reductase of *Enterobacter cloacae* (French et al. 1998), and the N-ethylmaleimide reductase xenobiotic (NemA) of *E. coli* encoded by the *nemA* gene (Williams et al. 2004). The physiological role of these enzymes has not been elucidated yet. Despite these flavoreductases have the same substrates range as the nitroreductases, they present significant differences in their amino acid sequences (Williams and Bruce 2002; Williams et al. 2004).

These three enzymes, NfsA, NfsB and NemA, which contain the flavin mononucleotide as prostetic group, can reduce up to two nitro groups ($-NO_2^-$) from different nitroaromatics compounds as TNT (Esteve-Nuñez et al. 2001; Yin et al. 2005) to the corresponding amines ($-NH_2$) via hydroxylamino (–NHOH) intermediates. The reduction is catalysed through the addition of one or two electrons from the cofactors NADH and/or NADPH (Roldán et al. 2008). This biotransformation is similar to the second step of the chemical synthesis of D-DIBOA from the precursor (Valle et al. 2011).

These antecedents motivated us to analyse whether the *nfsA*, *nfsB* and *nemA* gene products were involved in the conversion of the ethyl 2-(2′-nitrophenoxy)acetate to D-DIBOA.

Materials and methods

Bacterial strains, media and plasmids

E. coli strain W3110 [$F^-\lambda^-$ *rph1* INVrrnD-rrnE] was purchased from the American Type Culture Collection (ATCC 27325) and used as the wild-type strain in biotransformation and genes inactivation experiments. Strain AB502NemA is a derivative of *E. coli* AB1157 in which the three genes *nfsA*, *nfsB* and *nemA* have been inactivated and was kindly donated by Dr. J.L. Ramos (Estación Experimental del Zaidín-CSIC, Granada, Spain; González-Pérez et al. 2007). *E. coli* DH5α (*supE44* Δ*lacU169* ϕ80lacZΔM15 *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used as the host for cloning the *nfsA* and *nfsB* genes in the expression vector pBAD/His A (Invitrogen, Carlsbad, CA, USA). *E. coli* strains were cultured at 37°C in Luria–Bertani broth (LB) for molecular biology experiments and at 30°C for biotransformation experiments. When required, LB was supplemented with different antibiotics at the following final concentrations: ampicillin (Amp, 100 μg/mL), kanamycin (Kan, 50 μg/mL) or chloramphenicol (Cm, 25 μg/mL). The pKD4, pKD46 and pCP20 plasmids were used for the inactivation of genes with PCR products as described by Datsenko and Wanner (2000). pKD46 is an easily curable, low copy number plasmid containing the lambda phage Red recombinase under the control of an arabinose inducible promoter containing the Cre recombinase (Datsenko and Wanner 2000). pKD4 is a template plasmid carrying a Kan resistance marker flanked by FLP recombinase recognition target sites. pCP20 is a plasmid conferring resistance to Amp and Cm that shows

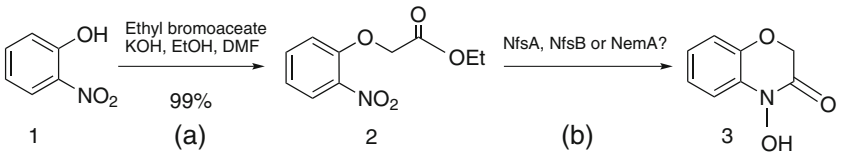

Fig. 1 Benzohydroxamic acid synthesis: **a** first step: synthesis of ethyl 2-(2′-nitrophenoxy)acetate (precursor) (2) using 2-nitrophenol (1) as starting material described by Macías et al. (2006b); **b** second step: hypothesis tested in this work of the precursor reduction by biotransformation with the NfsA, NfsB and/or NemA from *E. coli*

temperature-sensitive replication and thermal induction of FLP recombinase synthesis. The pBAD/His A vector containing the *araC* promoter, which is inducible by arabinose was used in expression experiments.

Construction of the single mutants, *nfsA*, *nfsB* and *nemA*, and double mutants, *nfsA*/*nemA*, *nfsB*/*nemA* and *nfsA*/*nfsB*

The single and double mutants with *nfsA*, *nfsB* and *nemA* genes inactivated were obtained from PCR products of these genes disrupted with a Kan resistance marker following the method of Datsenko and Wanner (2000). For this purpose, pairs of primers (Table 1) were designed in order to have short 5′ (H1) or 3′ (H2) homology sequences of the target genes (in capital letters) that were flanking P1 or P2 priming sequences of the pKD4 vector (in small letters). Using these primers and the pKD4 vector as the template, PCR products containing the Kan^R gene, flanked by FLP recognition targets (FRTs) were obtained. The PCR-generated products were introduced by electroporation into *E. coli* W3110 previously transformed with the pKD46 plasmid. The transformants were selected in LB agar plates supplemented with Kan and Amp. Gene disruptions were confirmed by PCR amplification using the following primers described in Table 1. The Kan resistance markers were removed after transformation with the pCP20 plasmid. Clones were selected by replica plating in LB agar plates supplemented with Cm or Kan. For plasmid curing, several clones were randomly selected, and replica was plated in LB agar plates with no antibiotic and incubated at 42°C. The double mutants were generated from the single mutants using the same procedure described above.

Cloning of the *nfsA* and *nfsB* genes

The *nfsA* open reading frame (ORF) was PCR-amplified from *E. coli* W3110 chromosomal DNA using the primers in Table 1 containing *Nco*I or *Hin*dIII sites. PCR was performed using the i-pfU™ DNA polymerase (Intron, Gyeonggi-do, Korea) following the manufacturer's instructions. The PCR products were then digested with *Nco*I and *Hin*dIII (Takara, Shiga, Japan) and ligated into the pBAD/His A vector digested with the same restriction enzymes. The resulting plasmids were named pBAD-NfsA and pBAD-NfsB. Approximately 25 ng of each plasmid were transformed into the *nfsA* and *nfsB* single mutants of *E. coli* W3110, respectively, to complement the inactivated genes. The resulting strains were named *nfsA*pBAD-NfsA and *nfsB*pBAD-NfsB. In both cases, the cloned DNAs were sequenced with 3730XL DNA sequencer (Macrogen Inc, Seoul, Korea) and compared with the *nfsA* and *nfsB* sequences using the ClustalW software. The single mutants transformed

Table 1 Names and sequences of the primers designed for this study

Primers	Sequences
H1P1-*nfsA*	CGC TGA CCG GCG GGC AAA AAC ACG TAG CGC AAG CGG gtg tag gct gga gct gct tc
H2P2-*nfsA*	AGA TCC GGA TTA TCC GCA GGC CAG CCA AGG CAC AGC cat atg aat atc ctc ctt a
H1P1-*nfsB*	ATG GAT ATC ATT TCT GTC GCC TTA AAG CGT CAT TCC gtg tag gct gga gct gct tc
H2P2-*nfsB*	CCA TCC ACT CTG CAT CAT CAT GCA GAT CTT TAC GGT GCc ata tga ata tcc tcc tta
H1P1-*nemA*	TAT TAG TGA AGC CAC GCA AAT TTC TGC CCA GGC AAA gtg tag gct gga gct gct tc
H2P2-*nemA*	CAG CGT TTC AGC TTT TTC TAC TGT GTA TGC ACC TGC cat atg aat atc ctc ctt a
nfsA-F	GCG CGA TCG GTA AAT TGC CTA AAG
nfsA-R	CAC GCA GCC GCT TAC ACG AAT AG
nfsB-F	TCA GTG TAT CCC GGC GAA GAA ATC
nfsB-R	CAC GTT TTT CGA AGG TCC GCA G
nemA-F	GCT GGA AAA TGG CCG TGA GAA CC
nemA-R	CCA CGC CCC AGT TGT AGG TCA GTT C
*Nco*I-*nfsA*-F	GGG CC**A TG**G GGA CGC CAA CCA TTG AAC TTA TTT GTG GCC
*Hin*dIII-*nfsA*-R	GGG AAG C**TT A**GC GCG TCG CCC AAC CCT G
*Nco*I-*nfsB*-F	GGG CC**A TG**G ATA TCA TTT CTG TCG CCT TAA AGC G
*Hin*dIII-*nfsB*-R	GGG AAG C**TT A**CA CTT CGG TTA AGG TGA TG

The primers from 1 to 6 were used to obtain the single and double mutants of the *nfsA*, *nfsB* and *nemA* genes. Capital letters indicate the homologous sequences of the target genes, and the lower case letters indicate the priming sequences of the pKD4 vector. In the names of the primers, H1P1 indicates the forward primers and H2P2 the reverse primers. Primers from 7 to 12 were used for PCR verification of the deletion of the targeted genes. The primers from 13 to 16 were designed for the cloning of the *nfsA* and *nfsB* ORFs into the pBAD vector. The start and stop codons are shown in *bold letters*. These primers incorporate restriction enzyme targets at 5′ end (*underlined* in the sequences). The restriction enzymes used are indicated in the name of each primer. Forward (-F) and reverse (-R) primers are also indicated

with pBAD/His A empty vector were used as control and named *nfsA*pBAD and *nfsB*pBAD.

The vector pBAD-NfsA construct was introduced into the *nfsB* single mutant and into the wild-type strain (named *nfsB*pBAD-NfsA and wtpBAD-NfsA respectively). The *nfsA* single mutant and the wild-type strain were also transformed with the pBAD-NfsB plasmid (named *nfsA*pBAD-NfsB and wtpBAD-NfsB.

Biotransformation experiments

The inocula were prepared in 10 mL of LB medium and grown overnight. The cultures were then inoculated into 90 mL of LB medium in 250-mL Erlenmeyer flasks and supplemented with 1 mL of stock solution of precursor diluted in methanol (MeOH) to achieve a final concentration of 2.22 mM (0.5 mg/mL) and 1% (*v/v*) of MeOH, respectively. Cells were grown at 30°C and 150 rpm for up to 26 h. For biotransformation experiments in which the expression of NfsA or NfsB were controlled using the pBAD A vector, the LB medium was supplemented with Amp, and the cells were grown to an optical density (OD_{600nm}) of 0.6, and then the expression proteins were induced by addition of L-arabinose to a final concentration of 0.02, 0.002 or 0.0002% (*w/v*).

Analytical techniques

For quantitative analysis of the precursor and D-DIBOA, 1 mL samples were withdrawn from the cultures, centrifuged at 10,000×*g* for 10 min and filtered through 0.22-μm nylon filters (VWR International Eurolab S.L., Barcelona, Spain) before analysis by reverse-phase high performance liquid chromatography (HPLC) on a Merck HITACHI HPLC system using a Phenomenex® Gemini C18 4.6×250 mm column as previously described by Valle et al. (2011). The biotransformation yield (BY) was calculated as follows:

$$\mathrm{BY} = \frac{\mathrm{molD-DIBOA}}{\mathrm{molprecursor}} \times 100$$

The cell dry weight (CDW) was quantified using standard procedures (Greenberg et al. 1992) in order to calculate the specific productivity (SP):

$$\mathrm{SP} = \frac{\mathrm{mg(D-DIBOA)}}{\mathrm{mg(CDW)} \times \mathrm{h}} \left(\mathrm{h}^{-1}\right)$$

The mutants and the wild-type BY and SP average data and standard deviation (SD) were calculated at least from three replicates. StatGraphics centurion for Windows version 16.0 was used for statistical analysis. The results were considered significantly different at α two-tailed level of 0.05 using the Student's *t* statistic function. Previously, the data were performed with Kolmogorov–Smirnov test for normality.

The GenBank/EMBL accession numbers for *nfsA* and *nfsB* ORF are EG11261 and EG20151, respectively.

Results

D-DIBOA in vivo synthesis by *E. coli* W3110

The biotransformation ability of the *E. coli* wild-type strain W3110 was evaluated in shake flasks cultures in which the LB medium was supplemented with the precursor by monitoring the bacterial growth, D-DIBOA and precursor concentrations at several times (Fig. 2). The analysis of the data obtained in these experiments showed that the precursor completely disappeared from the medium after 15 h of culture, but the BY remain virtually unchanged from 15 to 26 h (around 5.5%). Therefore, the production of D-DIBOA could be evaluated at any moment during this interval of time. The disappearance of the precursor was due in part to the biotransformation to D-DIBOA; however, there was also a spontaneous degradation. This effect was previously observed in precursor's stability experiments in different aqueous solution (data not shown). This phenomenon can be observed in Fig. 2, in which the BY of D-DIBOA is 5.5%; however, the precursor that remains after 15 h is not around 95% as can be expected, but the precursor is transformed completely in the medium. Despite the yield of the biological transformation is significantly lower than the chemical synthesis (5.5% versus 77% respectively), these experiments are necessary to establish the wild-type biotransformation yield as a reference and therefore to be able to elucidate the role of *nfsA*, *nfsB* and *nemA* genes in the biotransformation of the precursor to D-DIBOA by knocking out these genes.

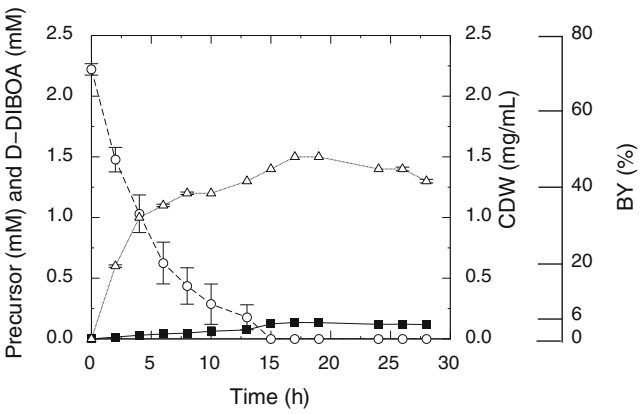

Fig. 2 Wild-type growth in the presence of precursor (—○—) and production of D-DIBOA (—■—). Concentrations are expressed in millimolars; CDW (·····△·····) is expressed in milligram per milliliter. The mean and SD values were calculated at least in three replicates

Effect of the inactivation of the *nfsA*, *nfsB* and *nemA* genes on the synthesis of D-DIBOA

To study the possible role of the *E. coli* nitroreductases NfsA and NfsB nitroreductases and NemA flavin reductase in the synthesis of D-DIBOA, we constructed *nfsA*, *nfsB* and *nemA* single mutants and *nfsA*/*nfsB*, *nfsA*/*nemA* and *nfsB*/*nemA* double mutants with these genes inactivated in *E. coli* W3110. The mutants and the wild-type strains were then grown for 26 h to ensure that the precursor had completely disappeared from the culture medium.

The analysis of these experiments showed that *nfsA* and *nfsB* but not *nemA* single mutants had significant lower BY than the wild-type strain (Fig. 3). In this sense, the BY for *nfsA* single mutant was 2.2±0.13% and 3.49±0.42% for *nfsB* single mutant, which represent a decrease of biotransformation capacity of 59.7±2.38% and 35.92±7.64%, respectively, compared to that of the wild-type strain (5.45±0.56%). These results suggested that these two nitroreductases are involved in the synthesis of D-DIBOA. According to the BY presented in Fig. 3, NfsA has a more active role than NfsB in the in vivo synthesis of D-DIBOA. On the other hand, the *nemA* single mutant BY (5.27±0.57%) was no significantly different from the wild-type, retaining a 96.70±10.42% of the biotransformation ability of the wild-type strain, indicating that the flavin reductase NemA is not involved in the synthesis of D-DIBOA.

We also carried out the same analysis for the double mutants mentioned above and we found, as expected, that the BY of the double mutants *nfsA*/*nemA* and *nfsB*/*nemA* were lower than that of the wild-type strain, with values of 2.75±0.18% and 3.81±0.63%, respectively, representing a 49.49±3.30% and 29.99±11.50% decrease compared to the wild-type (Fig. 3). On the other hand, the BY of the *nfsB*/*nemA* double mutant did not present significant differences compared to the *nfsB* single mutant (3.49%±0.42%). However, unexpectedly, the BY of the double mutant *nfsA*/*nemA* was slight but significantly higher than for the *nfsA* single mutant (2.20%±0.13%).

The *nfsA*/*nfsB* double mutant and the *nfsA*/*nfsB*/*nemA* triple mutant (strain AB502NemA kindly provided by Dr. J.L. Ramos), were unable to synthesise D-DIBOA from the precursor and the BY and the SP were therefore 0% (Fig. 3). These results indicated that the NfsA and NfsB nitroreductases are the sole enzymes involved in the in vivo synthesis of D-DIBOA in our experimental conditions.

The SP observed in all mutants and wild-type, presented the same pattern as the BY, indicating that the bacterial growth did not affect D-DIBOA synthesis (Fig. 3).

Recovery of the biotransformation capacity of *nfsA* and *nfsB* single mutants

To further confirm the role of NfsA and NfsB in the synthesis of D-DIBOA, the *nfsA* and *nfsB* ORFs were cloned into the pBAD/His A expression vector and reintroduced into the single mutants in order to evaluate the complementation of the lost activities. In both cases, the BY at 16 h were calculated as the average of three different experiments using three clones, named c1, c2, and c3, selected from the same transformation (Fig. 4).

The BY obtained indicated that *nfsA*pBAD-NfsA (Fig. 4a) and *nfsB*pBAD-NfsB (Fig. 4b) recovered their capacity to synthesise D-DIBOA from the precursor when the expression of both proteins were induced with 0.0002% (*w*/*v*) of L-arabinose (BY of 5.69%±0.60% and 5.44%±1.14%, respectively, versus 6.23%±1.74% for the wild-type strain). Furthermore, the biotransformation capability of both strains was considerably increased when protein expression was induced at higher L-arabinose concentrations, reaching a maximum BY value of 40.33%±9.42% in the case of NfsA (Fig. 4a) and 59.68%±2.0% in the NfsB (Fig. 4b) with 0.02% (*w*/*v*) of L-arabinose. These yields represented 6.5- and 9.6-fold increments in the BY for *nfsA*pBAD-NfsA and for *nfsB*pBAD-NfsB, respectively, compared to the wild-type, reaching yields of a similar range to the chemical synthesis (77%). These increments were not

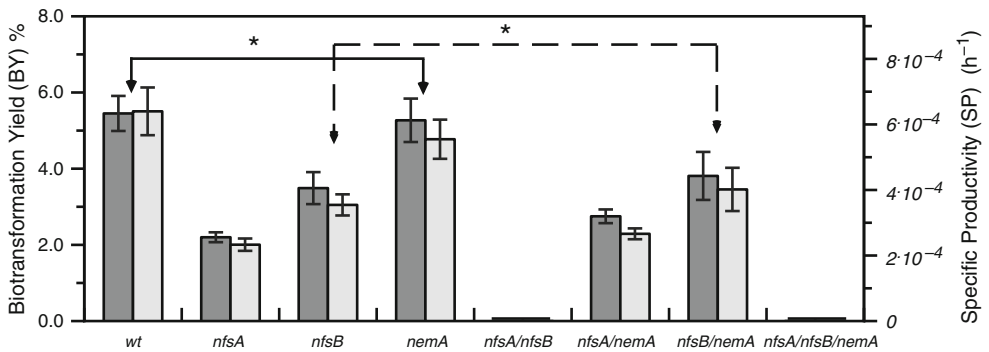

Fig. 3 Histograms of biotransformation yield (BY) coloured in dark grey and the specific productivity (SP) coloured in clear grey of singles and doubles mutants and the triple mutant at 26 h post inoculum. The *nfsA/nfsB* double mutant and the AB502NemA triple mutant were 0%, for both parameters. *Asterisks* show the pair of strains that have not any significant differences ($P>0.05$) nor BY neither RP

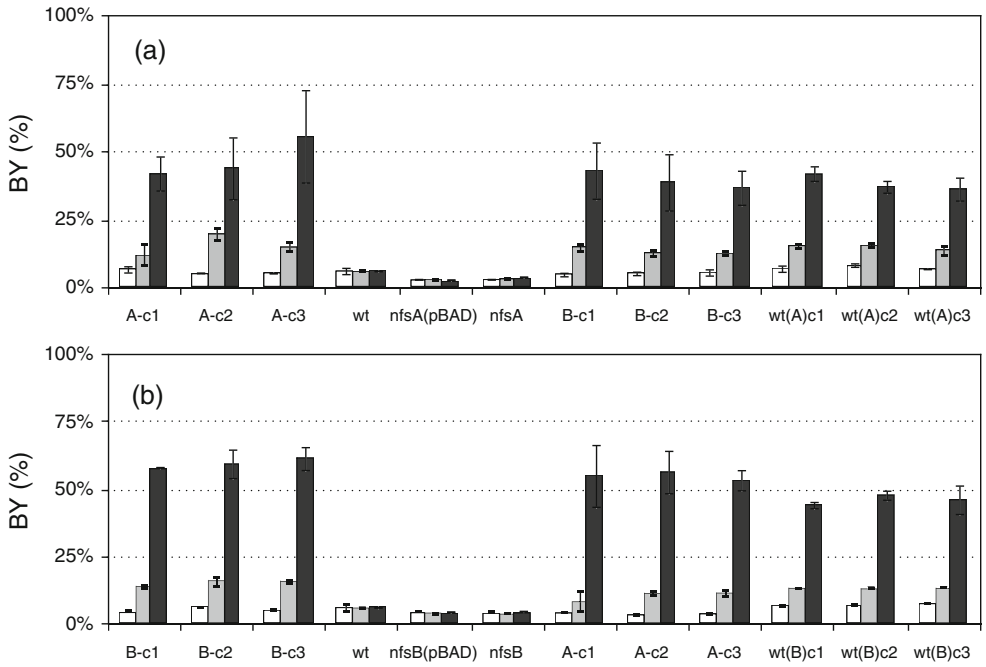

Fig. 4 Histograms of BY of three clones from the same transformant selected in LB-ampicillin. The three clones were named c1, c2 and c3. Prefix A indicate *nfsA* single mutant and prefix B indicate *nfsB* singles mutants. **a** Overexpression of NfsA protein in three clones of the mutants and wild-type strains; *nfsA*pBAD-NfsA (A-c1, A-c2 and Ac-3), *nfsB*pBAD-NfsA (B-c1, B-c2 and B-c3), wtpBAD-NfsA and the corresponding positive control of *nfsA* mutant and the negative control *nfsA*pBAD and **b** overexpression of NfsB protein in three clones of the mutants and wild-type strains; *nfsB*pBAD-NfsB (B-c1, B-c2 and B-c3), *nfsA*pBAD-NfsB (A-c1, A-c2 and Ac-3) and wtpBAD-NfsB with their corresponding positive control of *nfsB* mutant and the negative control *nfsB*pBAD. Both histograms show in *white bars* the assays tested with 0.0002%, in *grey* with 0.002% and in *black* 0.02% of L-arabinose. The *error bars* represent the SD of at least three independent replicates

due neither to the presence of the expression vector nor to the inductor since the BY of the single mutants *nfsA* and *nfsB* transformed with the pBAD/His A empty vector and using the same L-arabinose concentrations were very similar to the BY of the non transformed mutants (Fig. 4).

In order to explore the effect caused (yields of conversion observed) by an extra copy of NfsA or NfsB or both genes, the *nfsB* single mutant and the wild-type strain were transformed with pBAD-NfsA plasmid (named *nfsB*pBAD-NfsA and wtpBAD-NfsA, respectively); on the other hand, the *nfsA* single mutant and the wild-type strain were transformed with pBAD-NfsB construct (named *nfsA*pBAD-NfsB and wtpBAD-NfsB, respectively). The biotransformation ability of these strains was analysed in the same experimental conditions previously described. We found that there was no increment of the BY when the *nfsA*pBAD-NfsA strain was compared with *nfsB*(pBAD-NfsA) and wtpBAD-NfsA strains (Fig. 4a). A similar behaviour regarding the BY was observed when the *nfsB*pBAD-NfsB strain was compared with *nfsA*pBAD-NfsB and wtpBAD-NfsB strains (Fig. 4b).

All the assays previously described were carried out 16 h after inoculation because in the wild-type strain the maximum BY was reached at this time. However, in order to evaluate how the biotransformation capacity of these strains varies in time, clones c2 of *nfsA*pBAD-NfsA and c3 of *nfsB*pBAD-NfsB were supplemented with 0.02% of L-arabinose and the BY for both clones was monitored at several times (Fig. 5).

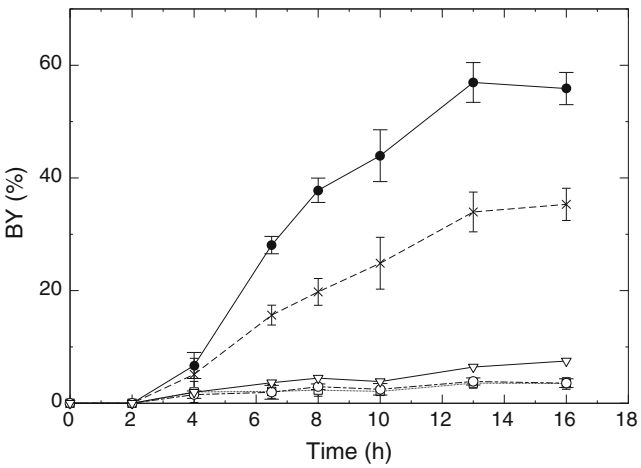

Fig. 5 Curves of BY with the clones; c3 of *nfsB*pBAD-NfsB (—●—) and c2 of *nfsA*pBAD-NfsA (– –×– –) and the control strains: wild-type (—▽—), *nfsA* mutant (···□···) and *nfsB* mutant (- -○- -). Both clones selected were induced with 0.02% (*w/v*) of L-arabinose. The samples were taken at 0, 2, 4, 6.5, 8, 10, 13 and 16 h. The average and SD were calculated of at least three independent replicates

The production of D-DIBOA in both strains increased linearly from 4 to around 13 h (Fig. 5) reaching a maximum BY of 35.31%±2.47% for *nfsA*pBAD-NfsA at 16 h and 55.87%±4.35% at 13 h for *nfsB*pBAD-NfsB. As expected, the wild-type strain presented a lower BY than the above strains but a higher BY than the *nfsA* and *nfsB* single mutants (Fig. 5).

Discussion

Nitroreductases have been identified and characterised in a variety of bacteria: Gram-negative and Gram-positive; symbionts, pathogens, and free living organsims; heterotrophs and phototrophs; mesophilic and thermophilic species; and aerobic and anaerobic facultative bacteria. In the last few years, these enzymes have received attention due to their environmental, biotechnological and clinical applications (Roldán et al. 2008). These enzymes are thought to participate in oxidative stress response (Liochev et al. 1994, 1999), although their physiological role has not been elucidated yet (Umezawa et al. 2008). Nitroreductases could also be involved in the reduction of non-natural substrates as xenobiotic compounds released by humans activities in the recent years (Rau and Stolz 2003).

In a previous work, we have demonstrated that *E. coli* is able to biotransform the precursor [4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one] to D-DIBOA (Valle et al. 2011), a compound with interesting biological properties (Macías et al. 2006a). Here, we studied the possible role of the NfsA and NfsB nitroreductases and the flavin reductase NemA in this biotransformation. In our approach, we generated the single and double mutants of these genes and then we analysed their BY and SP. However, we will only discuss the BY values because the behaviour of both parameters was very similar in all the strains. This would indicate that the differences in the biotransformation ability were not due to the difference in the biomass concentration (Fig. 3). On the other hand, these enzymes do not seem to be essential for central metabolism because biomass production was not affected in the mutants compared to the wild-type.

According to the BY obtained with all the mutants, only the NfsA and NfsB nitroreductases were involved in the conversion of the precursor to D-DIBOA in our experimental conditions. It has been reported that these enzymes catalyse similar biotransformations as the reduction of the nitro group of nitrofurazone. In this previous work, the double mutant *nfsA/nfsB* constructed by Rau and Stolz (2003) presented only a 10% of activity respect to the *E. coli* wild-type strain activity. Mutants inactivated in the *nfsB* gene presented higher BY than mutants inactivated in the *nfsA* gene (Fig. 3), indicating that NfsA was more active toward the precursor than NfsB, in vivo. It has been reported that in the reduction of the nitro group of 4-nitrophenol, tested in vitro, the specific activity of NfsA was 5-fold higher than for NfsB (Zenno et al. 1996a,b). Furthermore, the NfsA activities tested in other nitro compounds, like as nitrofurazone, nitrofurantoin, 4-nitrobenzoate or 4-nitrotoluene, were higher than for NfsB. Two reasons could explain these results. Firstly, the NfsB protein seems to be expressed at a lower rate than NfsA (Bryant et al. 1981; Kobori et al. 2001; Zenno et al. 1996a,b). Secondly, the K_m value reported for NfsB is twice that of NfsA, when chromate is used as the substrate, which would indicate that NfsA has more affinity than NfsB for this kind of substrates (Kwak et al. 2003). Therefore, the higher yields observed with the *nfsB* single mutant compared to the *nfsA* single mutant could be explained by enzyme concentration and/or substrate affinity effects (Fig. 3).

The BY of the *nfsA/nemA* double mutant was slightly higher than in the *nfsA* single mutant. This would indicate that there is indirect and unknown interaction between NemA activity and the biotransformation of the precursor by NfsB enzyme (Fig. 3).

In order to confirm that NfsA and NfsB were responsible for the biotransformation of the precursor to D-BIBOA, both ORF were cloned into pBAD/His A, a vector that allows different levels of expression of genes under the *araBAD* promoter by adding different concentrations of L-arabinose to the culture medium (Guzman et al. 1995). When the *nfsA* and *nfsB* single mutants were complemented with the corresponding genes cloned in pBAD, the loss of activity was recovered at the lowest inducer concentration tested in our experiments (0.0002% of L-arabinose). As the inductor concentration was increased, the BY considerably increased up to 9.6 for *nfsB*pBAD-NfsB and 6.5 for *nfsA*pBAD-NfsA (Fig. 4). These yields are close to the values obtained in the chemical process (77%) described in Macías et al. (2006b).

Regarding the question about which of these two genes (*nfsA* or *nfsB*) has a more active role in the biotransformation of the precursor, the results obtained from the gene inactivation or the overexpression experiments are somehow contradictory. On the one hand, mutagenesis experiments suggested that NfsA is the main contributor to the biotransformation, but on the other hand, when NfsB protein was overexpressed in the *nfsB* mutant (*nfsB*pBAD-NfsB strain), the BY was higher than when NfsA was overexpressed in the *nfsA* mutant (*nfsB*pBAD-NfsB strain). These experiments would reveal the presence of unknown limiting factor(s), being probably one of them the availability of the reduced cofactors NADH or/and NADPH within the cells. Whereas the NfsB may use either NADH or NADPH, NfsA can only use NADPH. The only use of NADPH and the lower concentration of this cofactor compared with the one of NADH (Rau and Stolz 2003) could represent a limiting

factor for NfsA. On the other hand, although NfsB could have lower activity levels than NfsA in normal physiological conditions, when the enzymes are overexpressed, the use of both NADH and NADPH by NfsB may represent an advantage that would explain the higher biotransformation activity observed in the overexpression of NfsB compared to NfsA. This hypothesis would be also supported by the data obtained from the experiments in which NfsA or NfsB were overexpressed in the wild-type strain (wtpBAD-NfsA and wtpBAD-NfsA strains, respectively). In these strains, there is an extra copy of *nfsA* (Fig. 4a) or *nfsB* (Fig. 4b) genes provided by the endogenous *nfsA* and/or *nfsB* genes. However, in both cases, the presence of an extra copy does not reveal an increment of the biotransformation ability respect to the recovery of the biotransformation capacity experiments with the *nfsA*pBAD-NfsA and nfsBpBAD-NfsB strains. These experiments would suggest that NfsA and NfsB concentration within the cells are not limiting the biotransformation in the overexpression experiments. In these cases, probably other factors like the redox cofactors availability are limiting the process. The importance of redox cofactors as limiting factor for these enzymes have been previously reported in an in vitro assay in which the reduction of TNT by *E. coli* nitroreductases significantly increased when redox cofactors were added to the reaction (Rau and Stolz 2003). In conclusion, taking together, the mutagenesis and overexpression experiments would reveal a higher efficiency of NfsA respect to NfsB but less versatility in the usage of redox cofactors in the synthesis of D-DIBOA from the precursor.

In summary, our results demonstrate the implication of the NfsA and NfsB nitroreductases in the biotransformation of the precursor to D-DIBOA by *E. coli*. Furthermore, we show that the genetic improvement of these bacteria can represent a plausible alternative to the second step of the chemical synthesis of D-BIBOA, which is a complex and expensive process. The aim of our future efforts will be to achieve an increase of the production yield of D-DIBOA by the optimisation of culture medium and the usage of several *E. coli* strains.

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