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D-Amino-acid oxidase gene from *Rhodotorula gracilis* (*Rhodosporidium toruloides*) ATCC 26217

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The complete nucleotide sequence of the *DAO1* gene encoding D-amino-acid oxidase (DAAO) in the yeast *Rhodotorula gracilis* (*Rhodosporidium toruloides*) ATCC 26217 has been determined. The primary structure of DAAO was deduced from the nucleotide sequence of a cDNA clone that covered the entire amino acid coding sequence. Comparison of cDNA and genomic sequences of *DAO1* revealed the presence of five introns. Because this is the first gene of strain ATCC 26217 that has been cloned so far, the nucleotide sequences of these introns were compared to those from other fungi. Upstream of the structural gene there was a stretch of C T-rich DNA similar to that found in the promoter region of a number of yeast genes. The cDNA gene, which encoded a protein of 368 amino acids (molecular mass 40 kDa), was overexpressed in *Escherichia coli* under the control of the strong lipoprotein promoter. Interestingly, a significant fraction (13–62%) of the total DAAO activity was recovered in its apoenzyme form, the percentage depending on the culture conditions. This fact allowed a rapid purification of the recombinant DAAO by affinity chromatography. The high level of expression achieved in *E. coli* and the possibility of modifying its catalytic properties by protein engineering provide a new model for the study of this enzyme.

Keywords: D-amino-acid oxidase, *Rhodotorula gracilis*, gene expression, yeast, *Escherichia coli*

INTRODUCTION

D-Amino-acid oxidase [DAAO; D-amino-acid:oxygen oxidoreductase (deaminating); EC 1.4.3.3] is a flavoenzyme containing FAD as the prosthetic group. It catalyses stereospecifically the oxidative deamination of D-amino acids, producing the corresponding 2-oxoacid and ammonia with a concomitant reduction of molecular oxygen to hydrogen peroxide. DAAOs have been reported in a wide variety of organisms, including animals and micro-organisms (Curti *et al*., 1992). Although it was first detected 60 years ago in pig kidney, and is considered as a marker enzyme for peroxisomes, the physiological role of the enzymes remains obscure (Angermüller, 1989).

Abbreviation: DAAO, D-amino-acid oxidase.

The GenBank accession number for the sequence reported in this paper is Z71657.

Although some *DAO1* genes have been cloned and sequenced (Fukui *et al*., 1987; Furuya & Matsuda, 1993; Isogai *et al*., 1990; Jacobs *et al*., 1987; Momoi *et al*., 1988, 1990; Tada *et al*., 1990), only four enzymes have been isolated to homogeneity, i.e. those from pig kidney (Jacobs *et al*., 1987), *Rhodotorula gracilis* (*Rhodosporidium toruloides*) (Pilone *et al*., 1987), *Trigonopsis variabilis* (Pollegioni *et al*., 1993) and *Fusarium solani* (Isogai *et al*., 1990). More recently, the primary structure of the DAAO from *R. gracilis* has been determined by Edman degradation (Faotto *et al*., 1995). In addition, during the preparation of this manuscript the cDNA sequence encoding the DAAO from *R. gracilis* has been submitted to GenBank (U60066).

DAAO has considerable biotechnological importance because it is used for the deamination of cephalosporin C on the two-step enzymic route to 7-aminocephalosporanic acid (Fig. 1). This compound is the starting material for producing several cephem antibiotics

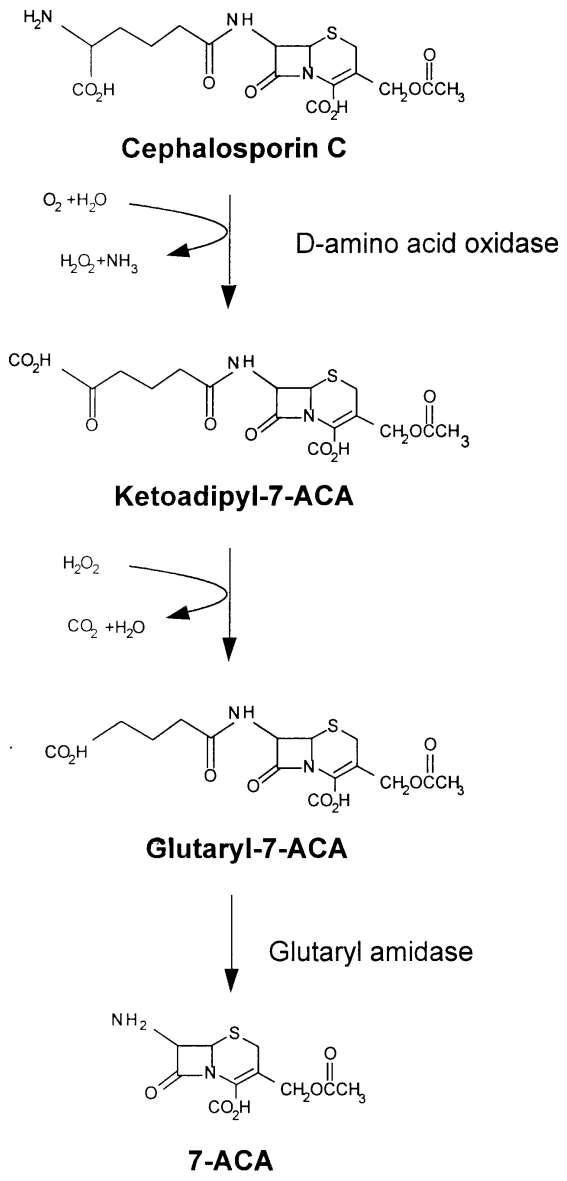

Fig. 1. Two-step enzymic synthesis of 7-aminocephalosporanic acid (7-ACA) from cephalosporin C. Cephalosporin C is transformed to 7-β-carboxy-5-oxopentanamido-cephalosporanic acid (ketoadipyl-7-ACA) by DAAO, which results in glutaryl-7-ACA after treatment with hydrogen peroxide. Finally, glutaryl-7-ACA is hydrolysed to 7-ACA by glutaryl amidase.

(Furuya & Matsuda, 1993). Because the mammalian enzyme is not suitable for biotechnological applications, much effort has been directed towards the preparation of yeast enzymes. In this sense, the DAAO enzyme from the archetypal oleaginous yeast *R. gracilis* has been extensively characterized (Faotto *et al.*, 1995; Gadda *et al.*, 1994; Pilone *et al.*, 1987; Pollegioni *et al.*, 1995). This enzyme is a dimer of molecular mass 79000 Da that is produced through induction by D-alanine (Pilone *et al.*, 1987; Perotti *et al.*, 1991). It has crucial properties that are different from those of other known DAAOs, such as the tightness of FAD binding and a high turnover number with various substrates that render it a suitable biocatalyst for industrial exploitation (Pollegioni *et al.*, 1995). However, the major drawback of using this enzyme on an industrial scale has been the low level of activity in the yeast strain. Because of the requirement of a highly productive process, our efforts were directed toward the cloning and expression in *Escherichia coli* of the *DAO1* gene from *R. gracilis*.

This paper deals with the characterization of the *DAO1* gene from *R. gracilis*. We present here the nucleotide sequence of the genomic gene and its corresponding cDNA as well as its overproduction in *E. coli*.

METHODS

Bacterial strains, plasmids and media. *R. gracilis* ATCC 26217 was provided by the Colección Española de Cultivos Tipo. *E. coli* strains used were DH5α (Sambrook *et al.*, 1989), NM538 (Promega) and NM539 (Promega). Plasmids pINIII-A3 (Inouye & Inouye, 1985) and pBC KS(+) (Stratagene) were used for cloning and sequencing. The phage λGEM-12 (Promega) was used for the construction of the genomic library. *R. gracilis* was grown at pH 5·6 in a basal medium supplemented with 30 mM D-alanine for 24 h at 30 °C with shaking (Perotti *et al.*, 1991). *E. coli* cells were cultured in LB broth (Sambrook *et al.*, 1989) at 25 or 37 °C with shaking. Ampicillin (100 μg ml^{-1}) or chloramphenicol (34 μg ml^{-1}) was added to the culture medium when required.

Molecular cloning and sequencing procedures. Plasmid DNA was isolated by the alkaline extraction procedure or by CsCl–ethidium bromide equilibrium density-gradient centrifugation (Sambrook *et al.*, 1989). The selected plasmids were sequenced by the dideoxy-mediated chain-termination method using double-stranded plasmids as templates and universal or synthetic oligonucleotides as primers. *R. gracilis* chromosomal DNA was isolated by the method of Sherman *et al.* (1986). Southern blot analysis was carried out as described by Sambrook *et al.* (1989). Labelling of the DNA probe was performed using the polar PLFX chemiluminescent blotting kit (Millipore). Competent cells for transformation were prepared using the rubidium chloride treatment (Sambrook *et al.*, 1989). Oligonucleotides RG1 [5′-GACCT(C/G)CC(C/G)-GAGGACGT(C/T/G)(T/A)(C/G)(T/A)(C/G)(G/C)CAG-AC-3′], RG2 [5′-GC(C/G)GG(G/C/T)CG(A/C/G)AG(G/-A/C)CC(G/A/C)ACGTTGTG-3′], RT1 (5′-GGAGGAAT-TCATATGCACTCTCAGAAGCGCGTCG-3′) and RT2 (5′-CCATCGATAAGCTTACAACTTCGACTCCCGCGCCG-C-3′) were used for cDNA synthesis or PCR amplifications. Total RNA was extracted as previously described by Köhrer & Domdey (1991). cDNA was prepared using avian myeloblastosis virus (AMV) reverse transcriptase (Promega) according to the recommendations of the supplier.

In brief, annealing took place over 5 min at 70 °C followed by 5 min at 25 °C in a total volume of 10 μl containing 20 units rRNasin (Promega), 7·5 μg total RNA and 2 μM primer RT2. Extension occurred over 90 min at 42 °C followed by 5 min denaturation at 95 °C in a total volume of 40 μl of the buffer recommended by the manufacturer containing the annealing solution, 20 units rRNasin, 1 mM of each dNTP and 40 units AMV reverse transcriptase. Amplification of cDNA with primers RT1 and RT2 was achieved with 30 cycles of 1 min denaturation at 95 °C, 2 min annealing at 55 °C and 2 min of

polymerase extension at 72 °C, using 2·5 μl cDNA solution, 2·5 units *Taq* polymerase (Perkin Elmer Cetus), 1 μM of each synthetic oligonucleotide primer, 250 μM of each dNTP, 13 % glycerol and 2 mM $MgCl_2$, in 100 μl of the buffer recommended by the manufacturer. Amplification with primers RG1 and RG2 was achieved with 5 cycles of 1 min denaturation at 98 °C, 2 min annealing at 55 °C and 2·5 min of polymerase extension at 72 °C, followed by 30 cycles of 1 min denaturation at 95 °C, 2 min annealing at 55 °C and 2·5 min of polymerase extension at 72 °C, using 1 μg chromosomal DNA, 2·5 units *Taq* polymerase (Perkin Elmer Cetus), 0·25 μM of each synthetic oligonucleotide primer, 250 μM of each dNTP, 13 % glycerol and 2 mM $MgCl_2$, in 100 μl of the buffer recommended by the manufacturer. PCR amplifications were done using Gene-ATAQ equipment (Pharmacia). PCR fragments were purified using *β*-agarase according to the recommendations of the supplier (New England Biolabs).

Construction of a genomic library. Total DNA of *R. gracilis* was partially digested with *Sau*3AI and fragments of about 20 kb were isolated in a sucrose gradient (10–40 %). These fragments were ligated to purified *λ*GEM-12 (Promega) arms, *Bam*HI digested, and the ligation mixture was packaged using the Packagene System (Promega). The complete genomic library (60000 p.f.u.) was transferred to nitrocellulose filters (BA85, 0·45 μm; Schleicher & Schuell) and hybridized using standard methods (Sambrook *et al*., 1989).

DAAO activity assay. The standard assay was done using a Shimadzu UV-260 spectrophotometer to follow the increase in A_{252} using 25 mM D-phenylglycine as substrate (Fonda & Anderson, 1967). Incubation was carried out at 30 °C in 50 mM sodium phosphate buffer, pH 8·0, containing 1 μM FAD. One unit of the enzyme activity was defined as the amount of enzyme transforming 1 μmol substrate min^{-1}. To compare the enzyme activities obtained by this method with previous data reported in the literature, the DAAO activity was occasionally assayed by polarography at 37 °C with a Hansatech oxygen electrode using D-alanine as substrate (Pilone *et al*., 1987). In this case, one unit of activity corresponds to the uptake of 1 μmol oxygen min^{-1}. Note that the values determined with the standard assay were about 10–14-fold lower than that determined polarographically using D-alanine as substrate. The percentage of the holoenzyme form contained in the cell extract was determined by comparing the DAAO activity in the presence and absence of exogenous FAD. The protein concentration was determined by the method of Bradford (1976).

Purification of recombinant DAAO. Cells of *E. coli* DH5α-(pCDAAO20) were grown at 25 °C with shaking (250 r.p.m.) for 12 h in a 2 l flask containing 200 ml LB medium plus ampicillin (100 μg ml^{-1}) in a shaking incubator (New Brunswick Scientific). Approximately 50 % of the DAAO produced under these culture conditions is recovered in the apoenzyme form (Table 1, see below). Cells were collected by centrifugation, washed and resuspended in 20 ml sodium phosphate buffer (20 mM, pH 8·0) containing 20 % glycerol, 5 mM 2-mercaptoethanol and 2 mM EDTA (buffer A), before disruption by passage through a French press (Aminco) operated at a pressure of 20000 p.s.i. (138 MPa). The cell debris was removed by centrifugation at 18000 r.p.m. for 20 min in an SS-34 rotor (Sorvall). The clear supernatant fluid [0·5 U (mg protein)$^{-1}$] was loaded in a DEAE-cellulose column (6 × 2·5 cm) equilibrated and eluted with buffer A. The active fractions [50 ml, 1·3 U (mg protein)$^{-1}$] were combined and applied to a Cibacron Blue 3GA-Sepharose column (4 × 1 cm) equilibrated with buffer A. The column was washed with 30 ml sodium phosphate buffer (1 M, pH 8·0). This wash fraction contained the holoenzyme form of DAAO and many contaminant proteins that were not retained in the matrix. The DAAO retained on the column (apoenzyme form) was eluted in 10 ml sodium phosphate buffer (20 mM, pH 8·0) containing 20 % glycerol, 5 mM 2-mercaptoethanol, 2 mM EDTA and 50 μM FAD. Note that because of the presence of FAD in the elution buffer, the purified DAAO was recovered in the holoenzyme form. The purified enzyme showed a specific activity of 13 U (mg protein)$^{-1}$ on D-phenylglycine using the standard assay and 194 U (mg protein)$^{-1}$ on D-alanine when it was determined by the polarographic method.

Western blot analysis. Western blot analysis was performed according to the procedure previously described by Sánchez-Puelles *et al*. (1992). Rabbit antibodies against DAAO of *R. gracilis* were prepared as described by Sánchez-Puelles *et al*. (1992), using the purified yeast enzyme supplied by Dr M. P. Castillón (Complutensian University of Madrid).

RESULTS AND DISCUSSION

Cloning of the genomic *DAO1* gene of *R. gracilis*

A 1 kb fragment containing a segment of the *DAO1* gene of *R. gracilis* was isolated by PCR using the degenerate primers RG1 and RG2. These primers were designed according to the sequences of the peptides DLPEDV-SSQT and HNVGLRPA, which had already been determined as part of the DAAO enzyme (Gadda *et al*., 1994), before the complete amino acid sequence was available (Faotto *et al*., 1995). The resulting PCR fragment was cloned into the *Eco*RV site of the vector pBC KS(+), producing the recombinant plasmid pPCR20. The high similarity of the deduced amino acid sequence encoded by this fragment with that of other DAAOs strongly suggested that it corresponded to the *DAO1* gene of *R. gracilis*. Thereafter, the 1 kb *Hin*dIII–*Eco*RI fragment of pPCR20 was used as a probe to screen a *Sau*3AI genomic library of *R. gracilis* constructed in the *Bam*HI-digested bacteriophage *λ*GEM-12. Sixteen of 54 positive phages were isolated and analysed by Southern blotting, which showed that all of them contained the putative *DAO1* gene in 8·5 kb *Eco*RI and 3·5 kb *Hin*dIII fragments. These fragments were subcloned in both orientations into the vector pBC KS(+), digested with *Hin*dIII or *Eco*RI, producing the plasmids pALR90 and pALR91 (3·5 kb *Hin*dIII fragment) and pALR92 and pALR93 (8·5 kb *Eco*RI fragment). The complete genomic sequence of the *DAO1* gene was determined using these plasmids as DNA templates and specific oligonucleotides as primers (Fig. 2). A preliminary analysis of this sequence revealed the existence of several truncated open reading frames as well as different putative lariat sequences, suggesting that the gene might contain a large number of introns.

Cloning and sequencing of the cDNA encoding the DAAO of *R. gracilis*

Although the analysis of the genomic *DAO1* gene did not allow us to determine precisely the amino acid sequence of the protein, its comparison to other DAAOs provided an excellent clue about the regions that might encode the N- and C-terminal segments of the protein.

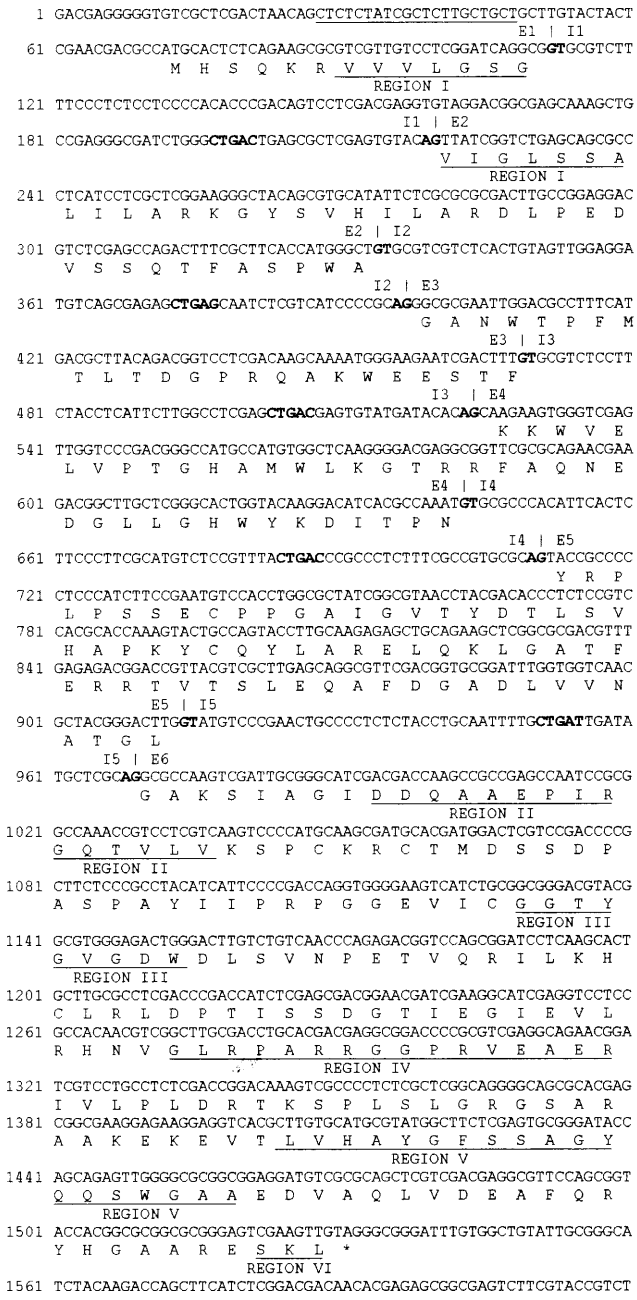

Fig. 2. Nucleotide and deduced amino acid sequence of the genomic clone for the *R. gracilis DAO1* gene. The introns (I1–I5) and exons (E1–E6) are indicated. The sequences of the lariats and the 5′- and 3′-ends of the introns are shown in bold letters. The C+T-rich region is underlined. Regions I–VI indicate the amino acids that are conserved in all DAAOs (Faotto *et al.*, 1995).

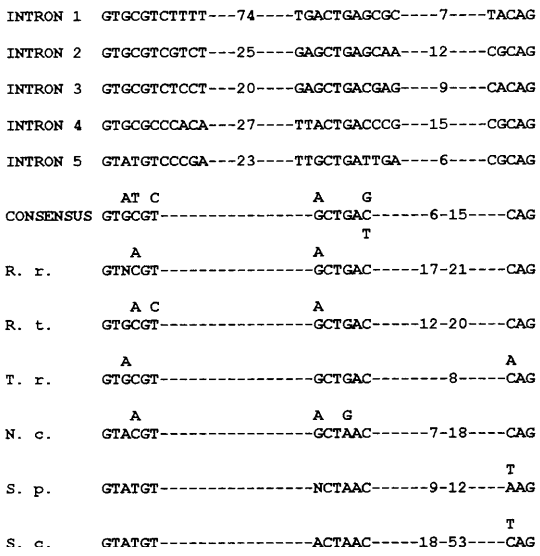

Fig. 3. Analysis of the *DAO1* gene introns. The intron sequences of *R. rubra* (R. r), *Rhodosp. toruloides* IFO 0559 (R. t.), *Trich. reesei* (T. r.), *Neurospora crassa* (N. c.), *Schizosaccharomyces pombe* (S. p.) and *Saccharomyces cerevisiae* (S. c.) have been published (Anson *et al.*, 1987; Filpula *et al.*, 1988; Gallwitz *et al.*, 1987). Dashes represent non-consensus nucleotides.

To clone the cDNA of the *DAO1* gene, the mRNA complementary strand was prepared using the oligonucleotide RT2 corresponding to the 3′-end of the gene. Then, a mixture of oligonucleotides RT1 and RT2 was used to amplify the resulting complementary strand by PCR. The RT1 primer also contains the sequence GGAGG (ribosome-binding site) to facilitate the translation of the *DAO1* gene in *E. coli*. The resulting 1·1 kb fragment was subcloned into the *Eco*RV site of the vector pBC KS(+), producing the plasmids pCDAAO10 and pCDAAO11.

The sequence of the cDNA insert of pCDAAO10 revealed the presence of five introns within the genomic *DAO1* gene (Fig. 2). Although the existence of introns has been described in yeast, they are very much the exception, relatively short and mainly located in the 5′-end of the coding sequence (Gallwitz *et al.*, 1987). Hence, the high number of introns detected in the *DAO1* gene of *R. gracilis* appears to be one of these rare exceptions. Because this is the first genomic gene of *R. gracilis* ATCC 26217 that has been sequenced so far, no data on the intron structure in this yeast are available for comparison. Nevertheless, the genomic *PAL* (phenylalanine ammonia-lyase) genes of the related microorganisms *Rhodotorula rubra* and *Rhodosp. toruloides* IFO 0559 contain five and six introns, respectively (Anson *et al.*, 1987; Filpula *et al.*, 1988). In common with a number of genes from yeast and filamentous fungi, the *DAO1* introns were relatively small with sizes ranging from 56 to 108 bp. All five introns contained the nucleotides CAG at their 3′-ends, demonstrating perfect agreement with the consensus intron acceptor sequence observed in eukaryotic genes. The sequences at the 5′-end also exhibited a good overall agreement with the consensus donor sequences (Fig. 3). The internal intron sequence needed for the splicing mechanism (lariat formation) was also conserved in the case of the *DAO1* gene (Fig. 3). However, note that the consensus sequence is more similar to that of *R. rubra*, *Rhodosp. toruloides* IFO 0559 or *Trichoderma reesei* than to that of other yeasts.

Table 1. DAAO activity of cell extracts of *E. coli* recombinants cultured under different conditions

Recombinant *E. coli* DH5α cells were cultured for 12 h at 25 or 37 °C with shaking (250 r.p.m.) in 100 ml flasks containing 10 ml (high aeration) or 50 ml (low aeration) LB medium plus ampicillin or chloramphenicol. Flasks were inoculated with a final ratio of 1/10 of an overnight culture grown at 25 or 37 °C. Enzyme activities [U (mg protein)$^{-1}$] were determined with the standard assay. Values in parentheses indicate the percentage of the holoenzyme form of DAAO present in the cell extracts. This value was calculated by comparing the enzyme activity in the absence and presence of exogenous FAD (1 μM).

Plasmid	Aeration at:			
	25 °C		37 °C	
	High	Low	High	Low
pCDAAO10	0·09 (47%)	0·14 (70%)	0·01 (38%)	0·26 (59%)
pCDAAO20	0·24 (66%)	0·31 (87%)	0·03 (51%)	0·60 (74%)

The analysis of the 5′ non-coding sequence of the *DAO1* gene revealed that there is a C+T-rich region just upstream of the ATG start codon (Fig. 2). Although the functional significance of these sequences remains to be determined, equivalent C+T-rich regions have been observed in a number of highly expressed genes, including the *PAL* genes of *R. rubra* and *Rhodosp. toruloides* IFO 0559 (Anson *et al.*, 1987; Filpula *et al.*, 1988). We have not been able to identify a consensus TATA box; however, the existence of a CGAA box downstream from the C+T-rich block is remarkable because, in efficiently expressed yeast genes, transcription start sites are often located in this sequence (Brown & Lithgow, 1987). Moreover, the C+T-rich regions are located immediately upstream of the transcription start point, particularly in highly expressed genes lacking apparent TATA boxes.

The amino acid sequence of DAAO from *R. gracilis* deduced from the cDNA and genomic clones revealed that the enzyme contains 368 amino acid residues (40 kDa). This sequence was identical to that previously determined by automated Edman degradation (Faotto *et al.*, 1995). The nucleotide sequence of the cDNA was almost identical to that reported in GenBank under the accession number U60066, because only two differences in the third nucleotide of the codons for Ser_3 (TCT for TCG) and Gly_{199} (GGC for GGG) were observed. As in other fungal genes, the codon utilization is biased and those codons ending in A are used infrequently. This phenomenon does not seem to be a consequence of the high G+C content as a similar selection for codons ending in T is not observed. The deduced amino acid sequence showed a remarkable similarity to the sequences of other DAAOs (Faotto *et al.*, 1995). The six highly conserved regions of the protein that have been postulated to play important roles in FAD binding (regions I and III), in active site topology (regions II, IV and V) and in peroxisomal targeting (VI) are located in exons E1, E2 and E6 (Fig. 2) (Faotto *et al.*, 1995). Note that the first intron of the *R. gracilis DAO1* gene divides the consensus FAD-binding sequence, Gly-X-Gly-X-X-Gly, into two exons as occurs in the *DAO1* gene of *Trig. variabilis* (Furuya & Matsuda, 1993), whereas the other conserved regions of the enzyme are encoded by the last exon.

Overexpression of the *DAO1* gene in *E. coli*

When DAAO production was analysed by the standard assay in the recombinant *E. coli* DH5α cells harbouring plasmid pCDAAO10, a considerable enzyme activity was detected [0·26 U (mg protein)$^{-1}$] (Table 1). Nevertheless, because this plasmid expresses the *DAO1* gene under the control of the *lac* promoter we tried to increase this production by subcloning the *Xba*I–*Hin*dIII fragment of plasmid pCDAAO11 containing the *DAO1* gene into the *Xba*I–*Hin*dIII-digested vector pINIII-A3 that carries the stronger *lpp–lac* promoter. As expected, we observed that clones carrying the resulting plasmid pCDAAO20 produced a higher amount of DAAO [0·60 U (mg protein)$^{-1}$] (Table 1).

Analysis of *DAO1* expression under several aeration conditions revealed that DAAO activity increased when cells were cultured with low aeration (Table 1). These results correlate with the intensities of the bands observed in SDS-PAGE as well as with the intensities of the signals detected by Western blot analysis (data not shown), suggesting that the activity matches the amount of protein produced by the cells. Although the reasons for such behaviour are still unknown, it can be argued that DAAO is probably lethal for the cells because it might drastically reduce the intracellular pool of D-alanine, an essential component of the cell wall. In this sense, we have observed that some clones segregate into two different phenotypes, one exhibiting a normal colony morphology that is unproductive, and the other showing small, transparent and flat colonies firmly bound to the agar, producing a higher amount of

DAAO. Hence, we propose that low aeration should favour DAAO production because a low availability of oxygen might reduce the enzyme activity and consequently its toxicity.

Taking into account that the *lpp–lac* promoter is inducible by IPTG, we tested the effect of this substance in DAAO production and observed that the addition of 5 mM IPTG to the culture medium increased DAAO production [0·99 U (mg protein)$^{-1}$]. To investigate the effect of D-alanine on DAAO production, this compound was added to the culture medium at different concentrations showing the maximal effect at a concentration around 10 mM [1·14 U (mg protein)$^{-1}$]. Because the *lpp–lac* promoter is not inducible by D-alanine, this effect cannot be ascribed to a direct increase of the *DAO1* transcription. However, as pointed out above, the addition of exogenous D-alanine should increase the internal pool of this critical amino acid, reducing the toxicity of DAAO activity and favouring the accumulation of the enzyme. Note that when the activity produced by *E. coli* DH5α(pCDAAO20), under these fermentation conditions, was determined by the polarographic method using D-alanine as substrate, we obtained a value of 15·6 U (mg protein)$^{-1}$. This production is about 20-fold higher than the value of 0·6 U (mg protein)$^{-1}$ reported for *R. gracilis* under optimal fermentation conditions (Pilone *et al.*, 1989).

In contrast, we have observed that the DAAO extracted from the recombinant micro-organism requires the exogenous addition of FAD to reach maximum activity, suggesting that an important fraction of the enzyme is obtained in an inactive apoenzyme form. Depending on the culture conditions, between 13 and 62 % of the total DAAO produced is recovered as apoenzyme (Table 1). In this context, it is worth noting that the amount of apoenzyme increased under high aeration conditions. Whether the presence of this inactive form reflects a low level of intracellular FAD that is insufficient to transform all the DAAO produced in *E. coli* into the holoenzyme form or whether it can be ascribed to folding/unfolding problems requires further investigation. Nevertheless, because the apoenzyme form of DAAO can be purified by affinity chromatography in Cibacron Blue-Sepharose, we were able to purify the recombinant DAAO from *E. coli* DH5α(pCDAAO20) (Fig. 4). The purified enzyme showed a specific activity of 194 U (mg protein)$^{-1}$ on D-alanine, which is similar to the value reported for the DAAO purified from *R. gracilis* (Pilone *et al.*, 1989), suggesting that the enzyme produced in *E. coli* is identical to that produced in the yeast. In this sense, the biochemical and spectrophotometrical data obtained with the recombinant DAAO protein were indistinguishable from those obtained with the enzyme prepared from *R. gracilis* (data not shown).

The results presented here not only show for the first time the structure of a gene of the yeast *R. gracilis* ATCC 26217 but also demonstrate the possibility of overproducing its DAAO in a heterologous host. Consequently, these studies should facilitate the use of this enzyme on an industrial scale and open the possibility of analysing its structural and biochemical properties using genetic engineering approaches.

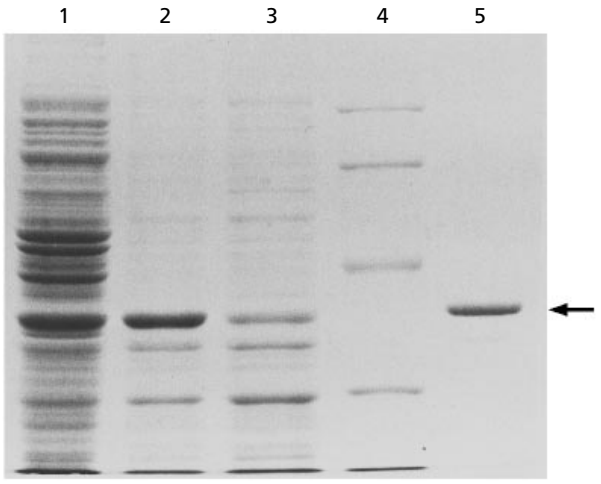

Fig. 4. SDS-PAGE of the purified recombinant DAAO. *E. coli* DH5α(pCDAAO20) cells were cultured at 25 °C with a 1/10 aeration ratio (200 ml LB medium in a 2 l flask). Lanes: 1, cell extract; 2, proteins after DEAE-cellulose chromatography; 3, proteins eluted in 1 M sodium phosphate buffer from Cibacron Blue-Sepharose; 4, molecular mass markers; 5, purified DAAO eluted in FAD-containing buffer from Cibacron Blue-Sepharose. The gel was stained with Coomassie blue.

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