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Expression of gentisate 1,2-dioxygenase (*gdoA*) genes involved in aromatic degradation in two haloarchaeal genera

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Abstract Gentisate-1,2-dioxygenase genes (*gdoA*), with homology to a number of bacterial dioxygenases, and genes encoding a putative coenzyme A (CoA)-synthetase subunit (*acdB*) and a CoA-thioesterase (*tieA*) were identified in two haloarchaeal isolates. In *Haloarcula* sp. D1, *gdoA* was expressed during growth on 4-hydroxybenzoate but not benzoate, and *acdB* and *tieA* were not expressed during growth on any of the aromatic substrates tested. In contrast, *gdoA* was expressed in *Haloferax* sp. D1227 during growth on benzoate, 3-hydroxybenzoate, cinnamate and phenylpropionate, and both *acdB* and *tieA* were expressed during growth on benzoate, cinnamate and phenylpropionate, but not on 3-hydroxybenzoate. This pattern of induction is consistent with these genes encoding steps in a CoA-mediated benzoate pathway in this strain.

Keywords Haloarchaea · Aromatic · Catabolism · Haloferax · Benzoate · 3-Hydroxybenzoate

Introduction

Aerobic aromatic acid degradation pathways have been reported in two haloarchaeal strains: *Haloferax* sp. D1227 (*Hfx*. D1227; Emerson et al. 1994) and *Haloarcula* sp. D1 (*Haa*. D1; Fairley et al. 2002). Unusually, rather than free aromatic acids such as benzoic acid (BA), aryl-coenzyme A (CoA) thioesters serve as substrates for ring-hydroxylating oxygenases in haloarchaea (Fu and Oriel 1999), and also in *Azoarcus evansii*, *Bacillus stearothermophilus* and *Thauera aromatica* (Mohamed et al. 2001; Zaar et al. 2001; Gescher et al. 2002; Schuhle et al. 2003; Barragan et al. 2004; Shinoda et al. 2004). It now appears that gentisate is not an intermediate during BA metabolism by these strains, in contrast to some earlier reports (Buswell and Clark 1976; Clark and Buswell 1979; Altenschmidt et al. 1993; Kiemer et al. 1996), although there is good evidence from physiological and biochemical studies that gentisate is an intermediate in the biodegradation of 3-hydroxybenzoic acid (3HBA) in *Hfx*. D1227 (Fairley et al. 2002; Fu and Oriel 1999) and 4-hydroxybenzoic acid (4HBA) in *Haa*. D1 (Fairley et al. 2002). Gentisate is also an intermediate in salicylate degradation (involving salicyl-CoA) by *Streptomyces* strain WA46 (Daisuke Ishiyama et al. 2004). Apart from gentisate 1,2-dioxygenase (*gdoA*) from *Hfx*. D1227 (Fu and Oriel 1998), no other genes that encode aromatic metabolism in haloarchaea have been identified to date. We report here the cloning and characterisation of *gdoA* and related genes from two haloarchaeal genera and the expression profiles of these genes during growth on various aromatic substrates.

In the opinion of the authors, D.J. Fairley and G. Wang should be regarded as joint first authors.

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Materials and methods

DNA isolation, primer design, PCR, sequencing and cloning

Hfx. D1227 and *Haa*. D1 were grown and maintained as described previously (Fairley et al. 2002). Genomic DNA for PCR was extracted using the method of Pitcher et al. (1989), and internal *gdoA* primers were designed by reference to the published *gdoA* sequence from *Hfx*. D1227 (Fu and Oriel 1998). Note that two corrections must be made to the sequence in GenBank (AF069949) to obtain the published sequence: (1) insert G at position 835 (622 in coding sequence) and (2) insert G at position 1016 (802 in coding sequence). PCR reactions (25 μl) contained PCR buffer, deoxyribonucleotide triphosphates (200 μM each), primers (3.5 pmol each), genomic DNA (~5 ng) and 0.25 U *Taq* polymerase (Amersham Biosciences, Piscataway, NJ, USA). The following primers were used (obtained from this study unless indicated; all 5′–3′): PO92f, GCG GAA AGC TTT GGG AGT AC (Fu and Oriel 1998); PO93r, TAG GTA CCT ACC CGG CCT GG (Fu and Oriel 1998); 139 f, TGG AAG TGG GAA GAC ATC GAG GC; 625 f, GAG GAC ACG AAG GAA GAC GG; 876r, GCG TTG TGG AAG TGC GGG TC; 993r, TCG TGG TGG TGA ATC TCG TC; Haa_tie_f, ATG AGC TAC GAA CGC GTC TG; Haa_tie_r, TCA CGG TTC GGC GTA CGC C; Haa_acdB_f, ATG ACT GAC GAC CCC ATC GCG; Haa_acdB_r, TCG CTG ACC CTC CAG TTC GGC; Hfx_tie_f, ATG ACC GGC TTC ACG CGC AC; Hfx_tie_r, TCA CCC CTC GGT GTA CGG TT; Hfx_acdB_f, TGA CCC ACA AAA GCG AGT GGA T; Hfx_acdB_r, ATT TCG GCG ATC TCC TCC CGC T; 16S_338 f, CTC CTA CGG GAG GCA GCA G (Gentry et al. 2004); 16S_784r, GGA CTA CCA GGG TAT CTA ATC C (Gentry et al. 2004). Thermal cycling was performed as follows: 94 °C (3 min), then 35 cycles of 94 °C (45 s), 55 °C (30 s) and 72 °C (90 s), followed by 72 °C (10 min). PCR products were purified with a Sephaglas BandPrep kit (Amersham Biosciences) and sequenced directly (ABI-3700; Applied Biosystems, Foster City, CA, USA). PCR products were cloned in *Escherichia coli* JM109 with the pGEM-T Easy vector system (Promega, Madison, WI, USA).

Construction and screening of cosmid libraries

High molecular weight genomic DNA (~150 kb) was prepared as described previously (DasSarma and Fleichmann 1995). Cosmid libraries (30–40 kb inserts) were constructed using the SuperCos1 Cosmid Vector Kit (Stratagene, La Jolla, CA, USA) and screened by colony hybridisation with ^{32}P-labelled *gdoA* gene probes (amplified from clones using primers 139f and 993r) using standard methods (Sambrook et al. 1989). Positive cosmids were isolated (QIAprep Spin Miniprep Kit; Qiagen, Hilden, Germany) and sequenced by primer walking.

RNA isolation, Northern and Southern blot analysis

Cultures (50 ml) were grown on either pyruvic acid (PA; 10 mM) or aromatic acid substrates (4 mM). When culture OD_{600}≈1.0, additional substrate (500 μM) was added, cultures were re-incubated for 3 h before cells were harvested, and total RNA isolated as described by Dyall-Smith (2001). RNA and DNA samples (restriction fragments from *Eco*RI/*Pst*I, *Not*I and *Not*I/*Pst*I digests) were transferred to nylon membranes and probed by standard methods (Sambrook et al. 1989) using gene probes amplified from genomic DNA, or from cloned PCR products. For positive control probes, 16S rDNA gene fragments were amplified from *Haa*. D1 and *Hfx*. D1227 using primers 16S_338f and 16S_784r and hybridised to the same membranes.

Sequence alignments and phylogenetic analysis

Nucleotide and translated basic local alignment search tool (BLAST) searches used the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/BLAST) and the University of Maryland Biotechnology Institute BLAST server (http://halo.umbi.umd.edu/cgi-bin/blast/blast_hvo.pl). Sequences were aligned using ClustalW (Higgins at al. 1994; http://www.ebi.ac.uk/clustalw) and phylogenetic analysis used the programmes GeneDoc (Nicholas and Nicholas 1997) and TreeCon (Van de Peer and De Wachter 1993).

Results

Screening of cosmid libraries (600 clones) yielded a single *gdoA*$^+$*Haa*. D1 cosmid (Haa312; ~30 kb insert) which contained a *gdoA* gene (1,110 bp) with 78% nucleotide homology to the *Hfx*. D1227 gene, encoding a protein with 76% homology (85% similarity). Four *gdoA*$^+$*Hfx*. D1227 cosmids (Hfx75, 131, 160 and 179; ~30 kb inserts) were found to contain *gdoA* (1,077 bp), with identical sequence to the gene reported previously (Fu and Oriel 1998).

Additional open reading frames, encoding a putative CoA-synthetase β-subunit (*acdB*), and a CoA-thioesterase (*tie*A) were located immediately upstream of the *gdoA* genes in both *Hfx*. D1227 and *Haa*. D1 (Fig. 1). These displayed identical order, orientation and substantial sequence identity in both strains, although a region of very high G+C content prevented sequencing the upstream end of the *Hfx*. D1227 *acdB* gene. Excluding this region, *acdB* genes from both strains were similar at the nucleotide (66%) and amino acid

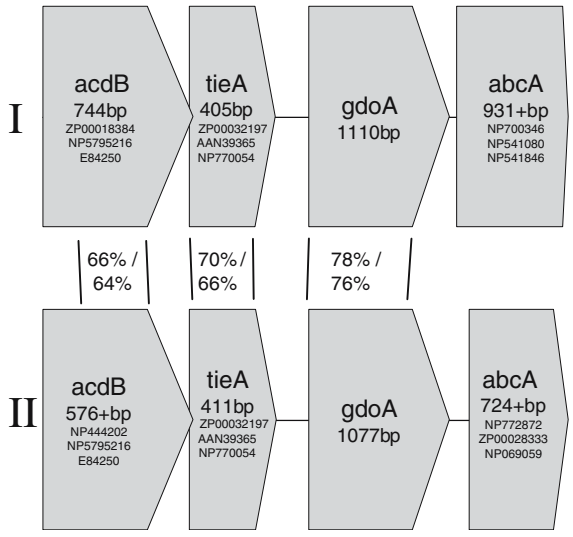

Fig. 1 Gene order and homology in the *gdoA* region in *Haa*. D1 (*I*) and *Hfx*. D1227 (*II*). Nucleotide/amino-acid similarity between the genes investigated in this study (*centre*) and GenBank accession numbers for the closest database matches (tblastx) are also shown. Transcription start sites (TTAT) were present 25 and 30 bp upstream of the *gdoA* start codon in *Hfx*. D1227 and *Haa*. D1, respectively

(64%) levels, and homologous to archaeal 'isoform II' aryl-CoA synthetases (Musfeldt and Schönheit 2002). The *acdB* stop codon overlapped with the *tieA* start codon in both strains, indicating that these genes are co-transcribed. The *tieA* genes were similar at the nucleotide (70%) and amino acid (66%) levels, and belong to the thioesterase superfamily [GenBank Conserved Domain Database (CDD) accession: pfam03061], which includes both acyl- and aryl-CoA thioesterases.

A *gdoA* probe from *Haa*. D1 hybridised to a single restriction fragment in all three of the restriction enzyme combinations tested by Southern blotting (data not shown) indicating that a single copy of *gdoA* is present in this strain. However, a *Hfx*. D1227 *gdoA* probe hybridised with two restriction fragments, suggesting that at least two similar *gdoA* genes are present in *Hfx*. D1227. Northern blotting was used to establish whether the genes identified during this study were induced during growth on various aromatic acid substrates (Table 1). There was clear induction of *gdoA* expression in *Haa*. D14 cells when grown on HBA. However, no induction of *gdoA* was detected in BA-grown cells. No expression of either *tieA* or *acdB* was detected in *Haa*. D1 at any time following growth on 4HBA, BA or PA. In contrast, expression of *gdoA* was observed in *Hfx*. D1227 following growth on BA, 3HBA, phenylpropionic acid (PPA) and cinnamic acid (CA), but not PA.

Discussion

None of the haloarchaeal *gdoA* genes under study here were similar at the nucleotide level to any other sequences in GenBank. However, a related gene (80 and 74% identical to *gdoA* from *Hfx*. D1227 and *Haa*. D1, respectively) was identified in the unfinished genome of *Haloferax volcanii* DS2. The predicted product of this gene (bases 10273–11346; contig3151) was homologous to the *gdoA* gene products from *Hfx*. D1227 (82%) and *Haa*. D1 (77%). A number of bacterial gene products also showed similarity at the amino acid level (up to 28/44% conserved/similar residues; data not shown). Although a GDO protein family (COG3435) has been identified in the GenBank CDD database (Marchler-Bauer et al. 2002), no proteins from the euryarchaeota are currently included. Phylogenetic analysis (not shown) indicated that the products of the haloarchaeal *gdoA* genes considered here clearly belong to this family, as do several bacterial 1-hydroxy-2-naphthoate dioxygenases (Iwabuchi and Harayama 1998).

Table 1 Expression of genes during growth of two haloarchaeal strains on aromatic substrates (Northern blot analysis)

Strain & gene	Primer pair (probe length/name)	Expression[a] in cells grown on:					
		4HBA	BA	3HBA	PPA	CA	PA
Haloarcula sp. D1							
Gentisate 1,2-dioxygenase (*gdoA*)	139f/993r (854 bp/Haa-gdo)	+	−	na	na	na	−
CoA-thioesterase (tieA)[b]	Haa_tie_f/Haa_tie_r (405 bp/Haa-tie)	−	−	na	na	na	−
CoA-synthetase subunit (acdB)[b]	Haa_acdB_f/Haa_acdB_r (741 bp/Haa-acdB)	−	−	na	na	na	−
16S rRNA (positive control)	16S_338f/16S _784r (346 bp/Haa-16S)	+	+	na	na	na	+
Haloferax sp. D1227							
Gentisate 1,2-dioxygenase (*gdoA*)	139f/993r (854 bp/Hfx-gdo)	na	+	+	+	+	−
CoA-thioesterase (tieA)[b]	Hfx_tie_f/Hfx_tie_r (411 bp/Hfx-tie)	na	+	−	+	+	−
CoA-synthetase subunit (acdB)[b]	Hfx_acdB_f/Hfx_acdB_r (430 bp/Hfx-acdB)	na	+	−	+	+	−
16S rRNA (positive control)	16S_338f/16S _784r (346 bp/Hfx-16S)	na	+	+	+	+	+

4HBA 4-hydroxybenzoic acid, *BA* benzoic acid, *3HBA* 3-hydroxybenzoic acid, *PPA* phenylpropionic acid, *CA* cinnamic acid, *PA* pyruvic acid
[a]+, induced; −, not induced; na, not applicable (no growth on this substrate)
[b]Putative gene function, assigned on the basis of sequence homology

Not surprisingly, there was induction of *gdoA* expression in *Haa*. D14 cells grown on HBA, which was consistent with involvement of gentisate in 4HBA metabolism in this strain (Fairley et al. 2002). However, no induction of *gdoA* was detected in BA-grown cells, although GDO activity (3.7±0.1 nmol O_2/min/mg protein) was noted previously (Fairley et al. 2002), suggesting either that a second *gdoA* gene with low homology is present in this strain (and regulated independently of the 4HBA pathway), or that the gentisate-dependent oxygen uptake measured previously in cell extracts was due to co-oxidation.

In the gentisate-independent 'box' pathway (Zaar et al. 2001), GDO activity is induced in BA-grown cells, but this is attributed to the formation of 3HBA as a metabolic side-product in these pathways (Gescher et al. 2002). In strains which can also metabolise 3HBA by 6-hydroxylation to yield gentisate (such as *Hfx*. D1227), coincidental induction of a 3HBA-induced *gdoA* during growth on BA is therefore expected, as observed here and which is consistent with metabolism of BA via a 'box' pathway in *Hfx*. D1227. Significantly, expression of *acdB* and *tieA* (encoding a putative CoA-synthtase subunit, and an aryl-CoA thioesterase, respectively) was observed during growth of *Hfx*. D1227 on BA, PPA and CA, but not on 3HBA. This suggests that these genes encode steps in a CoA-mediated benzoate pathway, as PPA, CA and BA are metabolised by a convergent route (Fu and Oriel 1999). Homology of the *tieA* gene products to a thioesterase identified in the *A. evansii* box pathway (Gescher et al. 2002) is also noteworthy, although the *A. evansii* gene (ORF1) was constitutively expressed, rather than being BA-induced.

In conclusion, we have found that closely related *gdoA* genes are present in two different haloarchaeal genera. The products of these genes belong to a protein family with members in both bacteria and archaea, and are distinct from other bacterial intra-diol and extra-diol dioxygenases. Additional genes, which are highly conserved in both strains, and display similar organisation and orientation, were located adjacent to the *gdoA* genes. These genes also had homologs in bacteria (in the case of *tieA*) and in other archaea (in the case of *acdB*). Induction patterns of these genes during growth on aromatic acids suggested that *acdB* and *tieA* are part of a BA-degradation pathway in *Hfx*. D1227, while the *gdoA* genes encode part of a 4HBA pathway in *Haa*. D1, and a 3HBA pathway in *Hfx*. D1227. This strain appears to metabolise BA by a similar route to *A. evansii*, which also explains the induction of *gdoA* during growth on BA, PPA and CA. Sequence data used in this study have been deposited in GenBank under the accession numbers AY293578 and AY297456.

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