***Acinetobacter baumannii* has two genes encoding glutathione-dependent formaldehyde dehydrogenase: evidence for differential regulation in response to iron**

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The *adhC1* gene from *Acinetobacter baumannii* 8399, which encodes a glutathione-dependent formaldehyde dehydrogenase (GSH-FDH), was identified and cloned after mapping the insertion site of Tn3-HoHo1 in a recombinant cosmid isolated from a gene library. Sequence analysis showed that this gene encodes a protein exhibiting significant similarity to alcohol dehydrogenases in bacterial, yeast, plant and animal cells. The expression of the *adhC1* gene was confirmed by the detection of GSH-FDH enzyme activity in *A. baumannii* and *Escherichia coli* cells that expressed the cloned gene. However, the construction and analysis of an *A. baumannii* 8399 *adhC1*::Tn3-HoHo1 isogenic derivative revealed the presence of *adhC2*, a second copy of the gene encoding GSH-FDH activity. Enzyme assays and immunoblot analysis showed that *adhC2* encodes a 46.5 kDa protein that is produced in similar amounts under iron-rich and iron-limited conditions. In contrast, the expression of *adhC1*, which encodes a 45 kDa protein with GSH-FDH activity, is induced under iron limitation and repressed when the cells are cultured in the presence of free inorganic iron. The differential expression of *adhC1* is controlled at the transcriptional level and mediated through the Fur iron-repressor protein, which has potential binding sites within the promoter region of this *adhC* copy. The expression of both *adhC* copies is significantly enhanced by the presence of sub-inhibitory concentrations of formaldehyde in the culture media. Examination of different *A. baumannii* isolates indicates that they can be divided into two groups based on the type of GSH-FDH they produce. One group contains only the constitutively expressed 46.5 kDa protein, whilst the other produces this GSH-FDH type in addition to the iron-regulated isoenzyme. Further analysis showed that the presence and expression of the two *adhC* genes does not confer resistance to exogenous formaldehyde, nor does it enable it to utilize methylated compounds as a sole carbon source when cultured under iron-rich as well as iron-deficient conditions.

Keywords: *adhC* copies, formaldehyde metabolism, iron regulation, gene duplication

This paper is dedicated to the memory of Dr M. A. Vides, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina, who was a great mentor and colleague.

Abbreviations: EDDHA, ethylenediamine-di(o-hydroxyphenylacetic) acid; FURTA, Fur titration assay; GSH-FDH, glutathione-dependent formaldehyde dehydrogenase.

The GenBank accession number for the sequence reported in this paper is AF130307.

INTRODUCTION

The glutathione-dependent formaldehyde dehydrogenases are members of the superfamily of zinc-containing alcohol dehydrogenases found in animal, plant, yeast and bacterial cells (Uotila & Koivusalo, 1989). These ubiquitous enzymes belong to the class III alcohol dehydrogenases and catalyse the oxidation of S-(hydroxymethyl)glutathione, which is formed by the spontaneous reaction between formaldehyde and the SH group of glutathione. This enzymic reaction leads ultimately to the oxidation of highly toxic formaldehyde into formic acid (Uotila & Koivusalo, 1989) and it is thus considered an essential function for cell viability.

It was initially proposed that the primary function of bacterial glutathione-dependent formaldehyde dehydrogenase (GSH-FDH) is the metabolism of formaldehyde formed endogenously by oxidative demethylation of compounds such as monomethyl-L-amino acids (Ling & Tung, 1948). However, it was found later that this enzyme is essential in the oxidation of methanol and other methylated compounds when they are the sole carbon source in methylotrophic bacteria such as *Paracoccus denitrificans* (Ras *et al.*, 1995) and the facultative phototroph *Rhodobacter sphaeroides* (Barber *et al.*, 1996; Barber & Donohue, 1998a). It was also shown that GSH-FDH activity is involved in microbial resistance to exogenous formaldehyde (Kaulfers & Wollman, 1988; Sasnauskas *et al.*, 1992). In the case of *Escherichia coli*, this resistance activity is due to the GSH-FDH-mediated degradation of formaldehyde, which is encoded by a gene located within a large self-transmissible plasmid (Kummerle *et al.*, 1996).

In some bacterial species, the production of GSH-FDH is induced when the cells are growing in culture media containing C_1 substrates that are sources of formaldehyde (Barber *et al.*, 1996; Hanson & Hanson, 1996). Furthermore, examination of GSH-FDH activity in *E. coli* and *Haemophilus influenzae* showed that this enzymic activity is induced by formaldehyde at concentrations between 7.9 μ M and 264 μ M, whereas higher concentrations of this disinfectant were toxic to the bacterial cells (Gutheil *et al.*, 1997). The expression of GSH-FDH activity in these two bacteria is controlled at the transcriptional level. In addition, it was recently shown that the transcription of the *adhI* gene in *R. sphaeroides* is upregulated by exogenous formaldehyde and S-(hydroxymethyl)glutathione (Barber & Donohue, 1998b). The redox state of the cell is also an important signal controlling the expression of *adhI* in *R. sphaeroides* through the global regulator PrrA (Barber & Donohue, 1998b). This positive regulator controls the expression of bacterial photosynthetic genes in response to changes in oxygen tension, and it is required to fully stimulate the expression of this gene. However, this is not the only factor controlling *adhI* expression because its transcription is also increased by the *trans*-acting mutation *spd-7* (Barber & Donohue, 1998b). These data show that the expression of the gene encoding this activity in bacteria is indeed controlled by a complex

regulatory circuitry in which some of the components remain to be identified and characterized.

The genus *Acinetobacter* includes bacterial species that are widely distributed (Baumann, 1968). *A. baumannii*, previously known as *A. calcoaceticus* subsp. *anitratus*, is the most prevalent genospecies associated with human infections, mainly in hospitalized and ambulatory compromised patients (Bergogne-Berenzin & Towner, 1996). Although numerous reports describing different aspects related to this pathogen and the infections it causes in compromised patients have been published (Bergogne-Berenzin *et al.*, 1996; Bergogne-Berenzin & Towner, 1996), not much is known about the basic metabolic processes and the regulation of gene expression.

In this work, we provide evidence that all *A. baumannii* clinical isolates examined produce a 46.5 kDa protein immunologically related to GSH-FDH, which is encoded by a constitutively expressed gene named *adhC2*. However, some of these strains harbour an additional copy, identified as *adhC1*, that encodes a slightly smaller GSH-FDH whose expression is controlled by the iron concentration of the culture media. In addition, our data indicate that the presence and expression of two *adhC* copies does not confer resistance to exogenous formaldehyde, nor does it enable these strains to utilize some methylated compounds as sole carbon sources.

METHODS

Bacterial strains, media and plasmids. Bacterial strains and plasmids used in this work are shown in Table 1. All strains were routinely grown at 37 °C in Luria (L) broth or agar (Sambrook *et al.*, 1989), containing the appropriate antibiotics. A Tris/succinate minimal medium consisting of 12.1 g Tris l^{-1} , 3.7 g KCl l^{-1} , 1.1 g NH_4Cl l^{-1} , 0.27 g KH_2PO_4 l^{-1} , 0.001 g $CaCl_2 \cdot 2H_2O$ l^{-1} , 0.14 g Na_2SO_4 l^{-1} , 0.01 g $MgCl_2 \cdot 6H_2O$ l^{-1} , 5.0 g NaCl l^{-1} , 5.0 g sodium succinate l^{-1} , 1.0 g yeast extract l^{-1} (pH 7.4) was used to grow cells to determine β -galactosidase (Miller, 1972) and GSH-FDH (Barber *et al.*, 1996) enzymic activities and the secretion of catechol compounds (Arnow, 1937) by *E. coli* DH5 α . The production of these enzymes and catechol compounds by *A. baumannii* 8399 was tested using cells cultured in Tris/M9 minimal medium, which consisted of Tris/M9 salts (12.1 g Tris l^{-1} , 3.7 g KCl l^{-1} , 1.1 g NH_4Cl l^{-1} , 0.15 g $CaCl_2 \cdot 2H_2O$ l^{-1} , 0.14 g Na_2SO_4 l^{-1} , 0.1 g $MgCl_2 \cdot 6H_2O$ l^{-1} , 5.0 g NaCl l^{-1} , adjusted to pH 7.4) supplemented with 0.5 % (w/v) glucose, 0.2 % (w/v) Casamino acids and 20 μ M KH_2PO_4 (pH 7.4). Iron-rich conditions and iron-deficient conditions were obtained by adding $FeCl_3$ and the iron chelator ethylenediamine-di(o-hydroxyphenylacetic) acid (EDDHA; Sigma), respectively, to minimal media. *A. baumannii* 8399 cells cultured in Tris/M9 minimal medium supplemented with either 0.00125 % (v/v) or 0.0025 % (v/v) formaldehyde, concentrations that do not affect cell growth, were used to determine the effect of this C_1 compound on the expression of GSH-FDH activity. Resistance to exogenous formaldehyde was determined by the ability of bacterial cells to grow in the presence of increasing concentrations of formaldehyde as previously described (Kaulfers & Brandt, 1987), using *E. coli* VU3695 as a positive control. Growth assays in Simmons salts supplemented with either

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant properties	Source or reference
Strains		
<i>A. baumannii</i>		
Outbreak strains	Isolated from respiratory infections	Hartstein <i>et al.</i> (1988)
8399	Outbreak strain, <i>adhC1 adhC2</i>	Hartstein <i>et al.</i> (1988)
8399M6	As 8399 but <i>adhC1::Tn3-HoHo1</i>	This work
<i>E. coli</i>		
HB101	<i>recA13 r_B⁻ m_B⁻</i>	Boyer & Roulland-Doussoix (1969)
DH5 α	<i>recA1 r_K⁻ m_K⁻ lacZΔM15</i>	Sambrook <i>et al.</i> (1989)
BL21(DE3)	λ DE3, T7 RNA polymerase	Studier <i>et al.</i> (1990)
BN4020	<i>fur::Tn5</i>	Bagg & Neilands (1985)
H1717	FURTA strain, <i>fhuF-λplacMu53</i>	Hantke (1987)
RRJC1	As BN4020 but <i>fhuF-λplacMu53</i>	Wertheimer <i>et al.</i> (1994)
VU3695	pVU3695-mediated formaldehyde resistance	Kaulfers & Brandt (1987)
Plasmids		
pAB2001	Promoterless <i>lacZ</i> -Gm ^R cassette	Becker <i>et al.</i> (1995)
pACYC184	Cloning vector	ATCC
pBCSK ⁺	Cloning vector, T7 promoter	Stratagene
pCR2.1	TA cloning vector	Invitrogen
pHoHo1	Plasmid containing Tn3-HoHo1	Stachel <i>et al.</i> (1985)
pMH15	<i>E. coli fur</i>	Hantke (1984)
pPH1JI	Incompatible with pRK2073, Gm ^R	Hirsch & Beringer (1984)
pRK2073	Conjugation helper plasmid	Leong <i>et al.</i> (1982)
pSK ⁺	Cloning vector	Stratagene
pSShe	Plasmid containing <i>tnpA</i>	Stachel <i>et al.</i> (1985)
pVK102	Cosmid cloning vector	Knauf & Nester (1982)
pZErO-1	Cloning vector	Invitrogen
pPA6	pVK102 containing <i>adhC1</i>	This work
pM6	As pPA6 but <i>adhC1::Tn3-HoHo1</i>	This work
pMU6	2.1 kb <i>Clal</i> - <i>EcoRI</i> pBCSK ⁺ containing <i>adhC1</i>	This work
pMU6. Δ N	As pMU6 but with <i>NsiI</i> deletion encompassing most of <i>adhC1</i>	This work
pPE9	pSK ⁺ containing the promoter and the 5' end of <i>adhC1</i>	This work
pPE9- <i>lacZ</i>	As pPE9 but <i>adhC1-lacZ</i> , Gm ^R	This work
pMU45	pCR2.1 containing the 8399 <i>fur</i>	This work
pMU45.184	8399 <i>fur</i> subcloned into pACYC184	This work

sodium citrate (10 mM), methylamine (100 mM), ethanol (50 mM), methanol (50 mM), sodium succinate (25 mM), or choline (10 mM) were used to test the utilization of different carbon sources. Simmons salts consisted of 0.2 g MgSO₄ l⁻¹, 1.0 g NH₄H₂PO₄ l⁻¹, 1.0 g K₂HPO₄ l⁻¹ and 5.0 g NaCl l⁻¹, adjusted to pH 7.0.

Determination of GSH-FDH activity. Bacterial strains were grown for 16 h at 37 °C in 25 ml of either Tris/succinate or Tris/M9 minimal medium. Stationary-phase cells were collected, washed twice with 25 ml TBS (Tris/HCl 50 mM, NaCl 0.15 M, pH 7.5), suspended in 2 ml TBS and lysed by sonication at 4 °C. Supernatants obtained after centrifugation at 15000 g for 3 min were used as crude cell extracts to determine enzymic activity spectrophotometrically as described previously (Barber *et al.*, 1996). The enzymic activity of each sample, expressed as μ mol NAD reduced min⁻¹ (mg

protein)⁻¹, was determined at least twice using fresh samples each time. The assays were done in duplicate each time and the results are reported as the mean value $\pm$ 1SD of a representative experiment. GSH-FDH activity was also tested in proteins separated by polyacrylamide gel electrophoresis under non-denaturing conditions. The gels and samples were prepared and run under the conditions described previously (Actis *et al.*, 1985), but without the addition of SDS and 2-mercaptoethanol. The enzymic activity was revealed by staining the gels with NAD, reduced glutathione, phenazine methosulfate and nitro blue tetrazolium (Uotila & Koivusalo, 1979). Protein concentration was determined as described by Bradford (1976).

Recombinant DNA techniques. Standard DNA recombinant methods were done as described by Sambrook *et al.* (1989). DNA restriction fragments used as probes were either isolated

from agarose gels with the GeneClean kit (Bio101) or amplified by PCR using the conditions described previously (Barancin *et al.*, 1998). The probes were labelled with [α - 32 P]dATP or [α - 32 P]dCTP using the oligolabelling method (Feinberg & Vogelstein, 1983). The *adhC1.5'* (5'-CTTGCTTAGACGTA-TGTGAC-3') and *adhC1.3'* (5'-CCTACTCCAGTATGGG-TAATC-3') primers were used to amplify the 5' region of *adhC1*. DNA nucleotide sequences were determined manually (Sanger *et al.*, 1979) or by automated DNA sequencing with the BigDye terminator cycle sequencing kit (PE-Applied Biosystems). DNA and protein sequences were analysed using the BLAST network service at NCBI, the GCG University of Wisconsin Genetics Computer software package, and ProfileScan, which is available at <http://www.ch.embnet.org/software/PFSCAN>.

Construction of a genomic library and transposition mutagenesis. The library was constructed using pVK102 and 8399 chromosomal DNA partially digested with *Hind*III as described previously (Genti-Raimondi *et al.*, 1991). The Tn3-HoHo1 transposon (Stachel *et al.*, 1985) was used as described previously (Tolmasky *et al.*, 1988) to generate insertional derivatives of recombinant cosmids harbouring *A. baumannii* 8399 DNA. Recombinant cosmids and Tn3-HoHo1 insertional derivatives were introduced into *E. coli* DH5 α by electroporation. The *A. baumannii* 8399M6 *adhC1::Tn3-HoHo1* isogenic derivative was obtained using pM6 and the allelic-exchange method described previously (Tolmasky *et al.*, 1993). The cosmid pM6 was conjugated into the 8399 wild-type strain and then cured by incompatibility with pPH1J1. The 8399M6 transconjugant was selected on L agar containing 10 μ g chloramphenicol ml $^{-1}$, 500 μ g ampicillin ml $^{-1}$ and 50 μ g gentamicin ml $^{-1}$. Generation of the appropriate derivative was confirmed by Southern hybridization and nucleotide sequencing after PCR amplification and cloning into pCR2.1. DNA hybridization experiments were carried out under high- and low-stringency conditions as previously described (Graber *et al.*, 1998).

Cloning of promoter elements, RNA analysis and S1 mapping. The 500 bp *Clal*-*Eco*RI fragment, containing the left end of Tn3-HoHo1 and the predicted *adhC1* promoter region, was cloned into pSK $^{+}$ to generate pPE9. This derivative was used to create the reporter construct pPE9-*lacZ* by cloning at the *Eco*RI site the DNA cassette from pAB2001 (Becker *et al.*, 1995), which harbours a promoterless *lacZ* gene and encodes gentamicin resistance. Total RNA from *A. baumannii* 8399 cells, grown to mid-exponential phase at 37 °C in Tris/M9 containing either 50 μ M EDDHA or 300 μ M FeCl $_3$, was isolated using hot phenol (Barancin *et al.*, 1998). Northern blots were prepared and probed with the 32 P-labelled *Clal*-*Eco*RI fragment using standard techniques (Sambrook *et al.*, 1989). The *adhC1* transcription initiation site(s) was determined by S1 mapping as described previously (Bidinost *et al.*, 1999; Wu & Janssen, 1997). Primer #188 (5'-CGATAC-TACTCAAGCTCCAAATGGC-3') was end-labelled and used together with unlabelled primer #189 (5'-CCTGGAG-CAAAGGCGACAGCTGCACG-3') to PCR amplify the *adhC1* promoter region. The purified amplicon and total RNA were coprecipitated, dissolved in hybridization buffer and allowed to anneal at 53 °C. *E. coli* tRNA was used as negative control. The samples were digested with nuclease S1 and analysed by electrophoretic comparison under denaturing conditions to a ladder obtained by dideoxynucleotide sequencing using the primer #189 and the plasmid pMU6 as a template.

Overexpression of GSH-FDH. *E. coli* BL21(DE3) cells harbouring pMU6, a pBCSK $^{+}$ derivative with *adhC1* cloned

downstream of the T7 promoter, were grown in L broth at 37 °C until the OD $_{600}$ reached a value of 0.5–0.6. Gene expression was then induced by the addition of IPTG as previously described (Studier *et al.*, 1990). After washing with 1 vol. TBS, whole-cell lysates were prepared and analysed by SDS-PAGE (Actis *et al.*, 1985; Smoot *et al.*, 1998).

Protein electrophoresis and immunoblot analysis. Samples containing either 25 μ g or 50 μ g proteins were loaded onto mini (5 cm long $\times$ 8 cm wide separating gel) and large (16 cm long $\times$ 15 cm wide separating gel) polyacrylamide gels, respectively. GSH-FDH and the Fur repressor were detected by immunoblotting using standard protocols (Towbin *et al.*, 1979). The antiserum against the *E. coli* Fur was raised and absorbed as described previously (Tolmasky *et al.*, 1994). The anti-GSH-FDH serum was prepared using the protein isolated by SDS-PAGE and electroelution, and immunopurified with nitrocellulose strips containing the cognate antigen band as described previously (Olmsted, 1981). The immunocomplexes were detected using horseradish-peroxidase-labelled protein A and the SuperSignal CL-HRP substrate (Pierce Chemical).

Cloning of fur and detection of Fur boxes. The *A. baumannii* 8399 *fur* gene was PCR amplified using the primers #265 (5'-CCAAAGATTGAACAACAAGCTAGG-3') and #267 (5'-CGATTATTTCTTGCAGCAATGCTTCC-3'), which were designed using the nucleotide sequence reported by Daniel *et al.* (1999). The reaction and cycling conditions were as described previously (Barancin *et al.*, 1998) with the exception that 55 °C was used as the annealing temperature. The amplicon was cloned with the Invitrogen TA system using *E. coli* Top10F' competent cells. The presence of the *A. baumannii* *fur* homologue and the expression of Fur activity by recombinant clones were confirmed by determining the nucleotide sequence of the cloned amplicon and testing it using the *E. coli* RRJC1 reporter strain (Wertheimer *et al.*, 1994), respectively. The *Bam*HI-*Xba*I insert of one of these clones, named pMU45, was ligated into pACYC184 to create the plasmid pMU45.184 that was used to examine the iron-regulated expression of cloned *adhC1*. The presence of Fur (iron) boxes was assessed using the Fur titration assay (FURTA) (Stojiljkovic *et al.*, 1994). The FURTA phenotype was confirmed by determining the β -galactosidase activity (Miller, 1972) of cells cultured in liquid medium supplemented with 100 μ M FeCl $_3$ at least twice. Each time, the assays were done in duplicate and the results are reported as the mean value $\pm$ 1SD of a representative experiment.

RESULTS AND DISCUSSION

Cloning and sequencing of *adhC*

The presence of a gene encoding GSH-FDH in the genome of *A. baumannii* was initially detected during the screening of Tn3-HoHo1 insertion derivatives of a genomic library that expressed iron-regulated β -galactosidase activity. Analysis of one of these derivatives, which was named pM6, showed that Tn3-HoHo1 interrupted a potential ORF related to the human class III alcohol dehydrogenase and several bacterial glutathione-dependent formaldehyde dehydrogenases. The entire gene was cloned as part of a 2.1 kb *Clal*-*Eco*RI fragment, which was identified by colony and Southern blotting using an *A. baumannii* 8399 DNA fragment adjacent to the Tn3-HoHo1 site as a probe. Nucleotide sequence analysis showed that the ORF where the transposon inserted consists of 1110 nucleo-

tides followed by a GC-rich palindrome and a poly(T) tract that may function as a rho-independent transcriptional terminator. This ORF is preceded by the nucleotide sequence AGGAAA resembling a ribosome-binding site (Shine & Dalgarno, 1974) and encodes a polypeptide of 369 aa residues with a predicted molecular mass of 39267 Da. A comparison of the predicted amino acid sequence encoded by this ORF with the GenBank database confirmed the similarity of this protein to glutathione-dependent formaldehyde dehydrogenases found in prokaryotic and eukaryotic cells. Although these dehydrogenases have the same activity and share high amino acid sequence homology, they have been reported with different names such as FDH, GS-FDH, GSH-FDH, FALDH and GD-FALDH. Similarly, the genes encoding these proteins in different cell types were reported with various names such as *adhC*, *adhI*, *adh3*, *gd-faldh* and *flhA*. In this work, the *A. baumannii* gene encoding glutathione-dependent formaldehyde dehydrogenase activity and its cognate translation product are reported as *adhC* and GSH-FDH, respectively.

The predicted amino acid sequence of the *A. baumannii* GSH-FDH is highly similar to bacterial enzymes such as those found in *H. influenzae* Rd (83.7% identity, accession no. P44557), *P. denitrificans* (62.9% identity, accession no. L36327), *R. sphaeroides* (61.7% identity, accession no. L47326), *Methylobacter marinus* (28.0% identity, accession no. L33464), *E. coli* K-12 strain MG1655 (85.6% identity, accession no. AAC73459.1) and *E. coli* VU3695 (85.9% identity, accession no. X73835). The latter is a plasmid-encoded gene product that confers formaldehyde resistance (Kummerle *et al.*, 1996). The *A. baumannii* 8399 GSH-FDH also showed significant similarity to the glutathione-independent formaldehyde dehydrogenase produced by *Pseudomonas putida* (30.7% identity, accession no. D21201). The alignment of these predicted amino acid sequences showed that the *A. baumannii* GSH-FDH enzyme contains several conserved amino acids required for the structure and enzymic activity of this protein, such as the C-40, H-62 and C-169 ligands to the active Zn, the cysteine residues at positions 92, 95, 98 and 106 that bind to the second Zn atom, and the typical NAD⁺-binding site 193-GLGGIG-200 (Ras *et al.*, 1995; Whener *et al.*, 1993).

Generation of an *adhC* isogenic mutant

One of the metabolic pathways that detoxifies formaldehyde by using it as a carbon source and generates essential reducing power in the form of NADH includes the enzymic action of GSH-FDH (Hanson & Hanson, 1996; Large, 1983). To test whether this enzyme activity plays a similar role in *A. baumannii*, the pM6 cosmid harbouring the *adhC*::Tn3-HoHo1 copy was used to create the 8399M6 (*adhC*::Tn3-HoHo1) isogenic mutant by marker exchange (Tolmasky *et al.*, 1993). Southern blot analysis showed that the *adhC* probe hybridized with a 6.4 kb *Hind*III fragment present in the

Fig. 1. Detection of *adhC* copies in *A. baumannii* by Southern blot analysis. Total DNA isolated from the 8399 wild-type strain (lane 1) and the 8399M6 (*adhC*::Tn3-HoHo1) isogenic derivative (lane 4) and the recombinant cosmids pPA6 (lane 2) and pM6 (lane 3) were digested with *Hind*III and blotted onto nitrocellulose. The blots were probed with *adhC1* under low stringency. The size of the λ *Hind*III fragments (lane 5) is indicated in kb.

chromosome of the 8399 wild-type strain and the pPA6 cosmid clone (Fig. 1, lanes 1 and 2). As predicted, the pM6 *Hind*III fragment harbouring *adhC* increased its size to about 20 kb, a 14 kb increase due to the insertion of Tn3-HoHo1 within this gene (Fig. 1, lane 3). A *Hind*III fragment with a size similar to the latter was detected in the genome of the *A. baumannii* 8399M6 strain, with the concomitant disappearance of the 6.4 kb *Hind*III fragment detected in the wild-type strain (Fig. 1, lane 4). The Tn3-HoHo1 insertion site in the chromosome of *A. baumannii* 8399M6 was confirmed by Southern blot hybridization and by sequencing a PCR fragment encompassing the putative *adhC* promoter region and the 5' end of the leaderless β -galactosidase gene present at one of the ends of Tn3-HoHo1. This approach showed that the transposon inserted between the T₁₁₆ and the A₁₁₇ of the predicted *adhC* ORF.

The DNA hybridization analysis also revealed that the *adhC* probe recognizes an additional 4.3 kb *Hind*III fragment present in the genome of the 8399 wild-type strain, which is absent in the pPA6 recombinant cosmid (compare lanes 1 and 2 of Fig. 1). The same 4.3 kb *Hind*III fragment was also present in the genome of the 8399M6 (*adhC*::Tn3-HoHo1) derivative (Fig. 1, lane 4). These results demonstrate that the *A. baumannii* 8399 strain has two *adhC* genes located in different genomic regions. However, the nucleotide sequences of these two *adhC* copies appear to be somewhat different because the presence of the *adhC* copy located within the 4.3 kb *Hind*III fragment could be detected only when the hybridization experiments were conducted under low stringency conditions. The copy cloned and characterized in this work is identified as *adhC1*, whilst the

Fig. 2. Overexpression and detection of GSH-FDH. (a) *E. coli* BL21(DE3) cells harbouring either the cloning vector pBCSK⁺ (lanes 1 and 2) or the recombinant derivatives pMU6 (lanes 3 and 4) or pMU6.ΔN (lanes 5 and 6) were cultured as described in Methods. Lysates (25 µg per lane) of non-induced (lanes 1, 3 and 5) and IPTG-induced (lanes 2, 4 and 6) cells were size fractionated by SDS-PAGE and stained with Fast stain. The arrows indicate the protein band corresponding to GSH-FDH. (b) The samples shown in lanes 2, 4 and 6 of (a) were fractionated by PAGE under non-denaturing conditions and stained with NAD, reduced glutathione, phenazine methosulfate and nitro blue tetrazolium to detect GSH-FDH activity. (c) Immunological detection of GSH-FDH produced by the 8399 wild-type strain (lane 1) and the 8399M6 (*adhC1*::Tn3-HoHo1) isogenic derivative (lane 2). Total cell proteins (50 µg per lane) were size-fractionated in a large polyacrylamide gel under denaturing conditions before blotting and probing with anti-GSH-FDH serum.

copy detected by DNA hybridization is referred to as *adhC2*.

Growth studies in L broth revealed that strain 8399M6 (*adhC1*::Tn3-HoHo1) was viable and showed a growth rate slightly higher than the 8399 wild-type strain during the exponential phase (data not shown). However, both cultures reached similar cell density during stationary phase. Both strains also showed similar growth rate and final cell density when cultured in Tris/M9 minimal medium, although the final cell mass was not as high as that observed with L broth.

Analysis of the expression of *adhC*

GSH-FDH assays showed that the 8399 parental strain and the 8399M6 isogenic derivative produced 160.9 ± 6.9 and 59.7 ± 2.61 µmol NADH min⁻¹ (mg protein)⁻¹, respectively. These data demonstrate that the insertion of Tn3-HoHo1 in *adhC1* results in a 2.7-fold reduction but not complete elimination of enzyme activity, providing further support for the presence of *adhC* isogenes in the genome of *A. baumannii* 8399. It is noteworthy that no formaldehyde dehydrogenase activity was detected in either of these *A. baumannii* strains when glutathione was not added to the reaction mixture, suggesting that the 8399 strain does not express glutathione-independent formaldehyde dehydrogenase activity.

Enzyme levels similar to those detected in the 8399 parental strain were also measured in *E. coli* DH5α cells harbouring pPA6, in which *adhC1* was cloned into the cosmid pVK102, whilst the activity was increased almost

10-fold when the cells contained this gene cloned in the higher-copy-number recombinant plasmid pMU6. In contrast, DH5α cells that harboured the cloning vectors pVK102 or pBCSK⁺ produced 106.8 ± 29.2 and 116.0 ± 34.5 µmol NADH min⁻¹ (mg protein)⁻¹, respectively. These lower levels represent the enzyme activity encoded by the chromosome of this bacterium (Gutheil *et al.*, 1997). Comparable lower enzyme levels were also detected in *E. coli* DH5α cells containing pM6, the pPA6 derivative that has Tn3-HoHo1 inserted within the *adhC1* gene.

The *adhC1* gene product was characterized by PAGE under denaturing and non-denaturing conditions using cells that overexpressed this gene. For this purpose, we used the T7 promoter-RNA polymerase system (Tabor & Richardson, 1985) and exploited the fact that the *adhC1* cloned in pMU6 was inserted downstream and transcribed in the same direction as the T7 promoter present in the pBCSK⁺ vector. SDS-PAGE analysis showed the presence of a 45 kDa protein in the extract of *E. coli* BL21(DE3) cells harbouring pMU6 when induced with IPTG (Fig. 2a, lane 4). This protein was also detected, although at a lower amount, in the extract of non-induced cells (lane 3). In contrast, no significant changes of protein bands within this molecular size range were detected in the extracts of non-induced and induced *E. coli* BL21(DE3) cells harbouring either pBCSK⁺ (lanes 1 and 2) or pMU6.ΔN (lanes 5 and 6). Both these constructs were used as negative controls, with the latter being a derivative of pMU6 in which a 941 bp *Nsi*I fragment that contains most of *adhC1* was deleted. When the samples obtained from IPTG-induced

cells (Fig. 2a, lanes 2, 4 and 6) were analysed by PAGE under non-denaturing conditions and stained to detect GSH-FDH, the extract of cells containing pMU6 (Fig. 2b, lane 2) was the only sample that showed a protein band with enzymic activity. A faint band with similar mobility representing the GSH-FDH produced by the *E. coli* host strain was detected in the other two samples only after prolonged staining (data not shown).

The 45 kDa band produced after IPTG induction of *E. coli* BL21(DE3) cells harbouring pMU6 was used to raise polyclonal antibodies against GSH-FDH. Western analysis using immunopurified anti-GSH-FDH serum showed the presence of a protein band in the whole-cell extracts of *E. coli* DH5 α harbouring the vector pBCSK⁺ or the pMU6 plasmid that contains the 8399 *adhC1* copy, although the signal was much stronger in the sample containing the latter. As indicated before, the band detected in *E. coli* DH5 α (pBCSK⁺) most likely represents the protein encoded by the cognate gene present in the genome of this cloning strain. The same immunoblot analysis using mini-SDS-polyacrylamide gels showed the presence of a single and an apparent double band, which all migrated close to the 45 kDa molecular mass marker, in the whole-cell extracts of the 8399M6 *adhC1*::Tn3-HoHo1 isogenic derivative and the 8399 parental strain, respectively (data not shown). This observation was further confirmed by immunoblot analysis using large polyacrylamide gels which showed presence of two polypeptides that differ by 1.5 kDa in their sizes in the 8399 cell lysate (Fig. 2c, lane 1). In contrast, the 8399M6 whole-cell extract contained only the 46.5 kDa protein, the larger of the two protein bands reacting with the anti-GSH-FDH serum (Fig. 2c, lane 2).

These data, taken together with the results obtained using enzymic assays, confirm that *A. baumannii* 8399 carries and expresses two *adhC* isogenes, which encode proteins with molecular sizes similar to those detected in other bacteria that produce GSH-FDH activity. Cloning and sequencing experiments have shown so far that, generally, bacteria carry a single *adhC* gene, with the apparent exception of *E. coli* VU3695. This clinical strain contains a large self-conjugative plasmid that harbours an *adhC* copy encoding resistance to exogenous formaldehyde (Kummerle *et al.*, 1996). This observation, together with the fact that *E. coli* DH5 α (Gutheil *et al.*, 1997) and MG1655 (Blattner *et al.*, 1997) have an *adhC* chromosomal copy, suggest that *E. coli* VU3695 indeed carries chromosomal and plasmid *adhC* copies. Whether either *A. baumannii* 8399 *adhC* isogene is located on a plasmid element remains to be determined.

Regulation of the expression of *adhC*

It has been established that C₁ compounds such as formaldehyde control the production of GSH-FDH in bacteria like *E. coli* and *H. influenzae* (Gutheil *et al.*, 1997). Similarly, the enzyme activity produced by the *A. baumannii* 8399 wild-type strain and the 8399M6

Table 2. GSH-FDH activity in *A. baumannii* 8399 and *E. coli* DH5 α clones harbouring various plasmids cultured under iron-rich and iron-limiting conditions

Strain (plasmid)	Condition	GSH-FDH activity
<i>A. baumannii</i> 8399	300 μ M Fe	16.8 $\pm$ 1.0
	200 μ M Fe	72.0 $\pm$ 6.2
	50 μ M EDDHA	136.2 $\pm$ 12.9
	100 μ M EDDHA	164.5 $\pm$ 13.5
<i>E. coli</i> (pVK102)	100 μ M Fe	57.9 $\pm$ 6.4
	100 μ M EDDHA	69.4 $\pm$ 1.3
<i>E. coli</i> (pPA6)	100 μ M Fe	120.2 $\pm$ 1.7
	100 μ M EDDHA	242.3 $\pm$ 10.1
<i>E. coli</i> (pM6)	100 μ M Fe	49.8 $\pm$ 1.6
	100 μ M EDDHA	61.8 $\pm$ 5.4

adhC1::Tn3-HoHo1 isogenic derivative was increased between five- and tenfold when the cells were cultured in the presence of sub-inhibitory concentrations of formaldehyde. However, our initial observation that an *adhC1*-*lacZ* transcriptional fusion expressed iron-regulated β -galactosidase appears to be novel, and it prompted us to examine the effect of this metal on the expression of GSH-FDH in more detail. Spectrophotometric assays showed that GSH-FDH activity in *E. coli* DH5 α harbouring pPA6 was about twofold higher in cells cultured under iron limitation than in cells cultured under iron-rich conditions (see Table 2). A similar response to changes in iron concentration was observed in *E. coli* DH5 α cells harbouring pMU6, although the enzymic activity detected in iron-starved cells was much higher due to the higher copy number of this clone. This table also shows that the enzymic activity of cells harbouring either the vector pVK102 or pM6 (*adhC1*::Tn3-HoHo1) was comparable under iron-limiting or iron-rich conditions. The effect of iron on the expression of GSH-FDH was also tested in the *A. baumannii* 8399 wild-type strain. Table 2 shows that the GSH-FDH activity increased significantly as the minimal medium was supplemented with increasing concentrations of the iron chelator EDDHA, with an almost tenfold difference between the enzymic activity detected in 8399 cells cultured under iron-rich and iron-limiting conditions. The enzymic activity of *A. baumannii* 8399M6 (*adhC1*::Tn3-HoHo1) cells cultured in the presence of 300 μ M FeCl₃ was similar to that of the parental strain grown under the same conditions and was increased only about 1.5-fold when the cells were cultured under iron-limiting conditions. The iron regulation of *adhC1* transcription in the *A. baumannii* 8399 wild-type strain was further examined by Northern blot analysis. Fig. 3 shows the presence of an RNA band of 1400 nucleotides which coincides with the predicted size of the transcription product of *adhC1*. Scanning densitometry of the autoradiogram showed that the amount of *adhC1* transcripts in the total RNA sample obtained from iron-starved cells (lane 2) was six- to eightfold

Fig. 3. Northern blot analysis of *adhC1* expression in *A. baumannii*. Total RNA isolated from cells grown under iron-rich (lane 1) and iron-deficient (lane 2) conditions was fractionated by formaldehyde-agarose gel electrophoresis. (a) Gel stained with ethidium bromide. (b) Northern blot of samples shown in (a) probed with a ^{32}P -labelled fragment harbouring *adhC1*. The size of the transcript detected with the probe is indicated on the right.

higher than the amount present in total RNA isolated from iron-replete cells (lane 1). Taken together, the data indicate that although the expression of both *A. baumannii* 8399 *adhC* copies are regulated by for-

maldehyde, only the transcription of *adhC1* is affected by the iron concentration of the media.

The possibility that the regulation of the expression of *adhC1* by iron is mediated through the Fur repressor was investigated by testing for the presence of potential Fur or iron boxes within the promoter of *adhC1* using FURTA (Stojiljkovic *et al.*, 1994). The *E. coli* H1717 Fur-deficient reporter strain harbouring the pPE9 recombinant construct containing the *adhC1* promoter region produced 125 ± 19 Miller units of β -galactosidase when cultured in broth supplemented with inorganic iron. In contrast, only 16 ± 4 units of enzyme were detected when this reporter strain harboured just the cloning vector. These results suggested that the *adhC1* promoter region contains Fur-binding sites. This hypothesis was confirmed by the observation that cells of the *E. coli fur* mutant strain BN4020 harbouring pPE9-*lacZ* and the empty vector pACYC184 produced 1132 ± 20.7 Miller units of β -galactosidase, while this enzymic activity was reduced to 125.8 ± 7.1 and 11.2 ± 0.08 units when the reporter cells harboured pPE9-*lacZ* together with either pMH15 (*E. coli fur*) or pMU45.184 (*A. baumannii fur*), respectively. Immunoblot analysis and Arnow tests confirmed that the presence of pMH15 and pMU45.184 resulted in the biosynthesis of the Fur repressor protein and restored the iron-regulated biosynthesis of catechol compounds in the *E. coli* H1717 Fur-deficient strain (data not shown). Analysis of the *adhC1* region expressing promoter activity showed that it contains potential Fur-binding sites similar to those described in other iron-regulated genes. The nucleotide sequence GATAATGAT, which is located 198 nucleotides upstream of the AUG codon (Fig. 4a), shares 9 of the 19 nucleotides found in the GATAATGATAATCATTATC consensus sequence for the Fur-binding site of iron-

Fig. 4. Analysis of the *adhC1* promoter region. (a) Location of transcription start sites and predicted Fur boxes within the *adhC1* promoter region. Putative -35 and -10 promoter elements and a potential ribosome-binding site (RBS) are underlined. The three horizontal arrows indicate the cognate *adhC1* transcriptional initiation sites marked in (b). The first codon of *adhC1* is in bold italics and the boxed nucleotides identify potential Fur-binding sites. (b) *adhC1* transcription start site mapping using either total RNA (lane 1) or tRNA (lane 2). The DNA sequencing ladders represent the sense strand and the transcription initiation sites are marked with asterisks.

regulated genes (Calderwood & Mekalanos, 1988). This sequence is followed by the hexamer GATAAG that closely resembles the minimal interaction unit NAT(A/T)AT, which was recently reported as the nucleotide array that can explain the iron-regulated expression of AT-rich promoters that do not contain canonical Fur boxes (Escobar *et al.*, 1999). Furthermore, at least 10 other units very similar to those found in the aerobactin promoter region (Escobar *et al.*, 2000) can be identified upstream of the *adhC1* initiation codon. This region also includes the sequence TAGCATTACAT-TATCTATTTTGCT that matches 19 of the 24 nucleotides found in the non-template strand of the Fur-binding site I of the promoter region of the *fatDCBA* operon of the pJM1 plasmid (Chai *et al.*, 1998). The potential regulatory role of these sequences is further supported by the results obtained using S1 mapping, which showed three adjunct *adhC1* transcription initiation sites (Fig. 4b) located within a region that contains three adjacent Fur-binding units (Fig. 4a). Furthermore, the putative -10 *adhC1* promoter element was found nearby these units. These results are consistent with the presence of potential Fur-binding sites in the *adhC1* promoter region that can account for the iron-regulated expression of this gene via the Fur iron repressor protein. The precise interaction of this regulator with specific nucleotides located in the *adhC1* promoter remains, however, to be determined with approaches similar to those used in the characterization of other iron-regulated genes in different bacteria.

Detection of GSH-FDH in other clinical isolates of *A. baumannii*

A collection of 12 isolates typed by their plasmid content, which were obtained during an outbreak of respiratory infections caused by this pathogen (Actis *et al.*, 1993) and which includes the strain 8399, was tested by immunoblotting. Although all strains produced the 46.5 kDa protein reacting with the anti-GSH-FDH serum, only six of them contained the 45 kDa protein detectable by this approach. The growth of these 12 strains in broth was inhibited by as little as 0.005% formaldehyde. Conversely, *E. coli* strain VU3695, which is resistant to formaldehyde due to the expression of plasmid-mediated formaldehyde dehydrogenase activity (Kaulfers & Brandt, 1987), grew well in the L broth containing up to 0.02% of this disinfectant. Furthermore, in the case of *A. baumannii* 8399, the parental strain and its *adhC1* insertional derivative 8399M6 expressed similar formaldehyde-resistance levels under iron-rich and iron-limiting conditions. It was also determined that all the *A. baumannii* strains grew in minimal medium supplemented with either citrate, succinate or ethanol as a carbon source. However, no growth was detected when these 12 strains were cultured in minimal medium supplemented with either choline, methanol or methylamine. Further assays showed that the parental strain 8399 and the isogenic derivative 8399M6 could not grow in a chemically defined medium supplemented with either 0.00125% or 0.0025% for-

maldehyde as a sole carbon source. These two formaldehyde concentrations, which were the same as those used to test the induction of GSH-FDH activity by this compound, did not affect the growth of these two strains in minimal medium supplemented with succinate as a sole carbon source. The ability of the 8399 parental strain and its *adhC1::Tn3-HoHo1* isogenic derivative to use formaldehyde as a carbon source did not change by using iron-rich or iron-restricted culture conditions.

Concluding remarks

The data presented in this report show that some strains of *A. baumannii* contain two *adhC* isogenes, one of which is expressed in a Fur- and Fe-dependent manner. However, the presence of two *adhC* copies and the differential expression of one of them does not affect, at least under the conditions used in this study, the growth rate of the cells, the ability to resist exogenous formaldehyde, or the use of different methylated compounds as sole carbon sources. This gene duplication may represent simply an intermediate step or an incomplete gene transfer process that left two *adhC* isogenes in some strains of *A. baumannii*. However, the fact that only *adhC1* is iron regulated may suggest that the product of this gene plays a role additional to its well-established function in the detoxification of intracellular formaldehyde. It was recently reported that organic acids such as formate, which is produced by the successive enzymic action of GSH-FDH and S-formylglutathione hydrolase on formaldehyde, protect stationary-phase *E. coli* and *Salmonella* cells from killing by cationic peptides (Barker *et al.*, 2000). Thus, a potential protective function of increased expression of GSH-FDH, which would result in higher intracellular levels of formate, is in agreement with the fact that pathogens such as *A. baumannii* encounter antimicrobial peptides produced by the host. Furthermore, the Fur-regulated expression of *adhC1* is consistent with the well-known fact that iron-restricted conditions of the host control the expression of a large number of bacterial genes. Also, it has recently been proposed that Fur works more like a global regulator that controls different cell functions in addition to those involved in iron acquisition (Escobar *et al.*, 1999). Another aspect that should be considered is that other metals, besides iron, could be involved in the Fur-mediated regulation of the *adhC1* expression in *A. baumannii* 8399. *Bacillus subtilis* contains the *yqkL*, *yqfV* and *ygaG* fur homologues in its genome (Cummings & Connerton, 1997; Mizuno *et al.*, 1996), which encode distantly related regulatory proteins that control different regulons. The first two homologues encode a Fur iron repressor, which controls the transcription of siderophore biosynthetic and ferri-siderophore transport genes, while the PerR regulator controls the response of this bacterium to peroxide stress (Bsat *et al.*, 1998). The *ygaG* gene encodes the *B. subtilis* Zur homologue that controls the expression of two operons involved in zinc transport. The potential presence of such a homologue, which would control the biosynthesis of the GSH-FDH zinc

metalloenzyme, seems quite reasonable. However, the expression of more than one *fur* homologue in *A. baumannii* 8399 that can control the expression of unrelated regulons remains to be seen.

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