The *Pseudomonas aeruginosa* hscA gene encodes Hsc66, a DnaK homologue

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Under heat-stress conditions bacteria induce, among other heat-shock proteins, the Hsp70 molecular chaperone (DnaK), which is involved in protein stabilization. It has been shown in *Escherichia coli* that an Hsp70 homologue called Hsc66, which is widespread in bacteria, functions as a chaperone *in vitro*. This paper reports the isolation of a *Pseudomonas aeruginosa* W51D mutant (W51M22) by insertion of the mini-Tn5-Hg transposon, which was unable to grow on ethanol and other short-chain alcohols as sole source of carbon. The transposon insertion in this mutant was shown to be located in the hscA gene encoding Hsc66. The inability of mutant W51M22 to use ethanol was complemented by the *E. coli* hscBA–fdx operon. The authors characterized the transcriptional arrangement of hscA, showing that it forms part of an operon with the upstream hscB gene, and that it is also expressed from its own promoter. These results are compatible with the *P. aeruginosa* Hsc66 protein being a functional molecular chaperone involved in the stabilization, in the presence of ethanol, of some proteins required for bacterial growth on short-chain alcohols.

**Keywords:** *Pseudomonas aeruginosa*, chaperone, DnaK

INTRODUCTION

The 70 kDa heat-shock proteins (Hsp70) make up a ubiquitous multigene family of highly conserved proteins. Hsp70 is the most conserved protein known to date which is found in all biota (Gupta & Golding, 1993). Hsp70 is known to act as a molecular chaperone under normal and stress conditions, stabilizing protein folding intermediates, and to play a major role in thermotolerance (Parsell & Lindquist, 1993). Eukaryotic organisms have been found to contain multiple Hsp70 family members, while bacteria contain only two (Gupta & Golding, 1993), and among these, only one, DnaK, has been reported to be functional as a molecular chaperone *in vivo*. The chaperone activities of DnaK and other Hsp70 chaperones are regulated by DnaJ and Hsp40 accessory proteins, respectively, which stimulate the ATPase activity of the chaperone (Liberek et al., 1991). The second bacterial DnaK homologue, called Hsc66, is encoded by the hscA gene. The existence of an *hscA* gene has been documented in the following bacteria: *Actinobacillus actinomycetemcomitans* (Actinobacillus Genome Sequencing Project, http://www.genome.ou.edu/act.html), *Azotobacter vinelandii* (Zheng et al., 1998), *Buchnera aphidicola* (Clark et al., 1998), *Escherichia coli* (Seaton & Vickery, 1994), *Haemophilus influenzae* (Fleischmann et al., 1995), *Neisseria gonorrhoeae* (Genome Sequencing Project, http://www.genome.ou.edu/act.html), *Neisseria meningitidis* (Neisseria meningitidis Sequencing Group at the Sanger Centre, ftp://ftp.sanger.ac.uk/pub/pathogens/nm) and *Pseudomonas aeruginosa* (Pseudomonas Genome Project http://www.pseudomonas.com). The only case in which it has been shown that the Hsc66 protein actually functions as a molecular chaperone is *E. coli*, where it has been purified and shown to have a characteristic ATPase activity (Vickery et al., 1997) and to solubilize protein aggregates (Silberg et al., 1998). In *E. coli* it was also shown that the Hsc20 protein, which is homologous to the DnaJ N-terminal domain and is encoded by *hscB*, functions as its co-chaperone *in vitro* (Silberg et al., 1998). The *hscB* gene is found adjacent to *hscA* in all the bacteria listed above. In *E. coli*, the *hscBA* genes form an operon (Lelivelt & Kawula, 1995), although there is

The GenBank accession number for the sequence of the W51D chromosomal region including the *hscB*, *hscA* and *fdxA* genes is AF096864.
evidence of promoters transcribing hscA alone (Kawula & Lelivelt, 1994).

The hscA gene is also found in a wide range of eukaryotic organisms, including yeast, Drosophila and mammals (Konstantopolou et al., 1995; LéJohn et al., 1994). In all organisms where the hscA gene is found, its complementary DNA strand presents an open reading frame that could encode a protein homologous to the enzyme glutamate dehydrogenase (Kawula & Lelivelt, 1994; Konstantopolou et al., 1995; LéJohn et al., 1994). The functionality of this unusual genetic structure has not been determined. In the case of bacteria, a ferredoxin is encoded downstream of hscA as sole carbon source, and the characterization of the hscA

et al encoded downstream of hscA determined. In the case of bacteria, a ferredoxin is used for P. aeruginosa strain W51D were: carbencillin (Cb) 50, gentamicin (Gm) 30, kanamycin (Km) 100, tetracycline (Tc) 50. Chloramphenicol was used at 50 µg ml⁻¹ in the experiment to test hscA mRNA induction. For quantification of hscA mRNA, strain W51D was cultivated on LB medium at 30 °C and when the culture reached an OD₆₀₀ of 0.6, different treatments were applied for an additional 3 h. Alcohols used as carbon sources were used at a concentration of 0.5% (v/v).

Nucleic acid procedures. DNA isolation, cloning and sequencing, Southern blotting, nick translation and PCR procedures were carried out as described by Sambrook et al. (1989). Primer extension analysis was done with primer R3 (see Fig. 1) located in the 5’ region of the hscA gene from P. aeruginosa W51D, using a Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Science). Other sequencing reactions were done using Taqfs DNA polymerase and fluorescent dyeoxy terminators in a cycle sequencing method; the resultant DNA fragments were separated by electrophoresis and analysed using an automated Applied Biosystems 377 DNA sequencer. To sequence the region flanking the mini-Tn5-Hg insertion in mutant W51M22, oligonucleotides derived from the transposon’s insertion sequences were used as primers. Total RNA was purified with a DNA-RNA isolation kit (US73750; Amersham Life Science) according to the manufacturer’s instructions.

To perform the combined reverse transcriptase (RT) and PCR reactions, total RNA was extracted from a saturated LB culture, and samples were further treated with DNase from Boehringer as instructed by the manufacturer. For the RT reaction 1 µg total RNA was used as template of the RAP2 reverse transcriptase (Amersham) reaction, using 20 pmol of the corresponding primer oligonucleotide (see Fig. 5). The reaction was incubated for 1 h at 37 °C and inactivated at 70 °C for 5 min. One-tenth of the product of this reaction was used as template for the PCR reaction using the oligonucleotides described in Fig. 5 as templates.

**METHODS**

**Microbiological methods.** Strains and plasmids used in this work are shown in Table 1. *P. aeruginosa* strains were grown at 30 °C on LB medium, Pseudomonas Isolation Agar (PIA; Difco) or modified M9 minimal medium (Abril et al., 1989) supplemented with one of the following carbon sources: glucose 0.2% (w/v), ethanol 1% (v/v). HgCl₂ (Hg) was used at a concentration of 12 µg ml⁻¹. Antibiotic concentrations, in µg ml⁻¹, used for *P. aeruginosa* strain W51D were: carbencillin (Cb) 50, gentamicin (Gm) 30, kanamycin (Km) 100, tetracycline (Tc) 50. Chloramphenicol was used at 50 µg ml⁻¹ in the experiment to test hscA mRNA induction. For quantification of hscA mRNA, strain W51D was cultivated on LB medium at 30 °C and when the culture reached an OD₆₀₀ of 0.6, different treatments were applied for an additional 3 h. Alcohols used as carbon sources were used at a concentration of 0.5% (v/v).

**Table 1. P. aeruginosa strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W51D</td>
<td>Strain able to degrade surfactants</td>
<td>Soberón-Chávez et al. (1996)</td>
</tr>
<tr>
<td>W51M22</td>
<td>W51D hscA::mini-Tn5-Hg mutant</td>
<td>This work</td>
</tr>
<tr>
<td>W51M23</td>
<td>W51D fdxA::Km mutant</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pUT mini-Tn5-Hg</td>
<td>Suicide vector for mutagenesis with mini-Tn5-Hg unable to replicate in <em>P. aeruginosa</em>, Hg’</td>
<td>de Lorenzo &amp; Timmis (1994)</td>
</tr>
<tr>
<td>pJQ20-0mp18</td>
<td>Cloning vector Gm’, unable to replicate in <em>Pseudomonas</em></td>
<td></td>
</tr>
<tr>
<td>pCP13</td>
<td>RK2-derived cosmid vector, Tc’</td>
<td>Quandt &amp; Hynes (1993)</td>
</tr>
<tr>
<td>pJC7</td>
<td>Cosmid containing 20 kb of W51D DNA, including hscB, hscA and fdxA, Tc’</td>
<td>Darzins &amp; Chakrabarty (1984)</td>
</tr>
<tr>
<td>pJC8</td>
<td>pJQ20mp18 with 1223 bp of W51D DNA, including the fdxA gene and flanking regions</td>
<td>This work</td>
</tr>
<tr>
<td>pJC9</td>
<td>pJC8 with a Km’ cassette cloned in the fdxA gene</td>
<td>This work</td>
</tr>
<tr>
<td>pUCP20</td>
<td>pUC19 derived <em>E. coli–Pseudomonas</em> shuttle vector, Cb’</td>
<td>West et al. (1994)</td>
</tr>
<tr>
<td>pJC10</td>
<td>pUCP20 with 5·4 kb of <em>E. coli</em> DNA including the hscBA and fdxA genes</td>
<td>This work</td>
</tr>
</tbody>
</table>
Genetic manipulations. *P. aeruginosa* matings (Soberón-Chávez et al., 1996) and transformation (Olsen et al., 1982) were done as reported previously. The W51D hscA::mini-Tn5-Hg insertion was constructed using the pUT-Hg plasmid (de Lorenzo & Timmis, 1994). The fdxA::Km mutant was constructed by selection of double recombination events with plasmid pJC9. This plasmid is a derivative of plasmid pJC8, constructed by selection of double recombination events with plasmid pJC9. This plasmid is a derivative of plasmid pJC8, which contains 1223 bp of W51D DNA amplified by PCR using oligonucleotides L2 (5'-GTGCTGCAAGGCCAGGT-GAG-3') and R5 (5'-CAGGCGGCGACTGG AAATCC-3') (see Fig. 3), with a Km' cassette (Alexeyev et al., 1995) cloned in the StuI site within the fdxA gene. These plasmids include the entire fdxA gene and its flanking sequences. The *E. coli* hscB, hscA, fdxA operon was subcloned from plasmid pTHK100 (Kawula & Lelivelt, 1994) into the vector pUCP20 (West et al., 1994), yielding plasmid pJC10.

Computer analysis of the DNA and protein sequences. The sequences were analysed by using the *GENE* WORKS program (IntelliGenetics) and the University of Wisconsin Genetics Computer Group (UWGGC) programs. The sequences of *P. aeruginosa* PAO1 contigs were obtained from the *Pseudomonas* Genome Project website (http://www.pseudomonas.com) released on 15 March 1999.

RESULTS AND DISCUSSION

Selection and characterization of mutant W51M22

We have previously reported the selection of a *P. aeruginosa* strain (W51D) which is able to mineralize at least 70% of a commercial branched-chain alkylbenzene sulfonate mixture and is resistant to high concentrations of these surfactants (Campos-García et al., 1999; Soberón-Chávez et al., 1996). In order to determine the degradation pathway of the surfactants, we selected a mutant (W51M22), created by insertion of transposon mini-Tn5-Hg, which was unable to grow on 2-phenylpropionate, a presumed surfactant degradation intermediate. Further characterization showed that neither the parental strain W51D nor the W51M22 mutant was able to use 2-phenylpropionate as a growth substrate; the apparent growth of strain W51D was due to the consumption of ethanol, the solvent used to solubilize 2-phenylpropionate. We concluded that mutant W51M22 was impaired in ethanol utilization (Table 2) and was not ethanol-sensitive, since it was able to grow in the presence of this alcohol on LB medium at a concentration of 2.5%, the same concentration as tolerated by wild-type strain W51D. The inability of mutant W51M22 to degrade ethanol was studied further (see below).

Sequence analysis of the region adjacent to the transposon insertion

The nucleotide sequence of the W51D genome adjacent to the mini-Tn5-Hg transposon insertion (see GenBank accession no. AF096864) allowed the identification of the *hscA* gene and, further downstream, apparently forming part of the same transcriptional unit, the *fdxA* gene (see Fig. 3). The deduced *P. aeruginosa* Hsc66 amino acid sequence shows that it belongs to the family of Hsp70 proteins and shows 64% sequence identity with its *E. coli* homologue.

We found the *hscBA* and *fdxA* genes in the PAO1 genome sequence as a part of contig 52 (from nucleotide 445243 to 448125) of the *Pseudomonas* Genome Project. The sequences of the W51D and PAO1 *hscA* genes are 91% identical, and both present the antisense ORF encoding a putative glutamate dehydrogenase. In *E. coli* (Lelivelt & Kawula, 1995) and *A. vinelandii* (Zheng et al., 1998), *hscA* is the second gene of an operon, with *hscB*, encoding a DnaJ homologue, being the first transcribed gene. These genes are also clustered in the *P. aeruginosa* genome and, as shown below, are also transcribed as an operon, although their transcriptional regulation is somewhat different. The PAO1 genomic structure in this region is very similar to that reported for *A. vinelandii* (Zheng et al., 1998), containing.
**Table 2. Ability of W51D and its mutants to grow on different media**

<table>
<thead>
<tr>
<th>Strain</th>
<th>LB</th>
<th>M9 medium supplemented with*:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Glu</td>
</tr>
<tr>
<td>W51D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W51M22</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W51M23</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W51M22/pJC7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>W51M22/pJC10</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* M9 minimal medium was supplemented with: Glu, glucose; EtOH, ethanol, PrOH, propanol; ButOH, butanol; DecOH, decanol; or NonOH, nonanol. Glucose was added at 0.2% and the alcohols at 0.5%.

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**Construction of the fdxA::Tc mutant W51M23**

Theoretically a ferredoxin could be involved in the mechanism of alcohol degradation by *P. aeruginosa*, and the mini-Tn5-Hg insertion within the *hscA* gene could have a polar effect on the expression of the *fdxA* gene. To determine whether the W51M22 phenotype was due to the lack of *fdxA* expression we constructed an *fdxA::Km* mutant (W51M23) according to the strategy shown in Fig. 3(a). Mutant W51M23 had no detectable growth defect (Table 2); thus the inactivation of *hscA* seems to be responsible for the inability of mutant W51M22 to use ethanol as a carbon source. Strain W51D possesses multiple ferredoxins as evidenced by Southern blot hybridization using the *fdxA* gene as a probe (Fig. 3b, panel I).

**Phenotypic characterization of mutant W51M22**

The proposed role of the *P. aeruginosa* Hsc66 protein, based on its homology with other Hsp70 proteins, is the stabilization of protein folding intermediates. We therefore hypothesized that the inability of mutant W51M22 to use ethanol as sole source of carbon was due to the instability in the presence of this alcohol of a protein or proteins absolutely required for ethanol consumption. In accordance with this hypothesis the inability of mutant W51M22 to degrade ethanol was dependent on the alcohol concentration (Fig. 4).

The enzyme alcohol dehydrogenase, which is required for alcohol degradation, is a candidate for being the critical Hsc66 substrate in this growth condition. To obtain additional evidence on the possible deficiency of an alcohol dehydrogenase activity in mutant W51M22, we determined the ability of this mutant to use different alcohols as growth substrates. Strain W51M22 was unable to grow with C2–C6 alcohols, but could readily use decanol and nonanol (Table 2). These results suggest...
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that an alcohol dehydrogenase which is necessary for short-chain alcohol degradation is deficient in mutant W51M22, due to the lack of Hsc66 activity.

It has been suggested that in A. vinelandii the proteins encoded by the hscA and hscB genes are involved as chaperone and co-chaperone in the maturation of proteins containing iron–sulfur clusters (Zheng et al., 1998). Some alcohol dehydrogenases contain an iron–sulfur cluster (Gutheil et al., 1992). The genetic arrangement of the PAO1 iscSUA and hscB, hscA and fdxA genes is consistent with the proposed role of Hsc66 in the stabilization of Fe–S-containing proteins.

It was also apparent that the hscA gene product plays a role in the stabilization of proteins involved in ethanol resistance, since the growth of mutant W51M22 on M9 medium supplemented with glucose as carbon source was decreased by the presence of lower concentrations of ethanol than the growth of strain W51D (Fig. 4b). As mentioned above, the Hsc66 effect on ethanol resistance was not apparent on LB medium.

**Complementation of mutant W51M22 by the E. coli hscBA–fdx operon**

The E. coli Hsc66 and Hsc20 proteins have been shown to have chaperone activity in vitro. In order to obtain additional evidence to support the hypothesis that the W51M22 phenotype was due to the instability of a protein or proteins involved in short-chain alcohol degradation caused by the lack of Hsc66 chaperone activity, we transferred plasmid pJC10, containing the E. coli hscBA–fdx operon (Table 1), to strain W51M22 and determined the complementation of the mutant phenotype.

Strain W51M22/pJC10 was able to grow on C2–C4 alcohols and it grew even better than the wild-type

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**Fig. 3.** Molecular characterization of the W51D fdxA mutant W51M23. (a) Schematic representation of the strategy to construct this mutant. (b) Southern blot hybridization of W51D DNA using as probes the W51D fdxA internal fragment (panel I), the W51D chromosomal region cloned in plasmid pJC7 (panel II), or the Km R resistance cassette (panel III). Lanes in panels II and III correspond to DNAs from: 1, mutant W51M23; 2, W51D; and 3, the plasmid used as probe in each experiment.

**Fig. 4.** Bacterial growth on different ethanol concentrations after 12 h incubation at 30°C, in the absence of Hg, (a) using ethanol as sole source of carbon, and (b) in the presence of 0.2% glucose. ●, W51D; ○, W51M22; ▲, W51M22/pJC10. The results are means of duplicate experiments.
strain W51D on ethanol concentrations in the range 2–3.5% (Table 2, Fig. 4). These results strongly suggest that Hsc66 chaperone activity is the limiting factor in the assimilation of short-chain alcohols by W51M22. The presence of plasmid pJC10 renders mutant W51M22 more ethanol resistant than the wild-type strain (Fig. 2b), suggesting, as mentioned above, that the Hsc66 chaperone also plays a role in ethanol resistance.

Transcriptional regulation of the P. aeruginosa W51D hscA gene

To obtain evidence on the regulatory circuits involved in hscA transcriptional regulation we measured, by RNA–DNA hybridization in dot-blot, the level of its expression in different culture conditions. hscA transcription was not induced by 3 h treatments with ethanol, heat, cold or chloramphenicol (data not shown). These results show that the E. coli and P. aeruginosa hscA genes are regulated differently, since E. coli hscA has been shown to be induced by cold-shock and chloramphenicol treatment (Lelivelt & Kawula, 1995). It is interesting, however, that the E. coli hscBA–fdxA genes contained in plasmid pJC10 complemented the inability of mutant W51M22 to use ethanol as carbon source (Table 2, Fig. 4), thus suggesting that the E. coli and P. aeruginosa Hsc66 proteins have a similar function.

The pattern of expression of the W51D hscB gene is identical to that of hscA as evidenced by the level of its hybridization on RNA dot blots (data not shown), thus suggesting that the two genes form a single transcriptional unit. To further analyse this possibility, we did RT-PCR analysis using as primers oligonucleotides (R1, R2 and L1; Fig. 1) which would only give a PCR product if hscB and hscA were co-transcribed, yielding a polycistronic mRNA. The results shown in Fig. 5 clearly show the existence of mRNA molecules containing both hscB and hscA sequences.

Primer extension analysis of the region upstream of hscA revealed the presence of a major mRNA start site (Figs 1 and 6), corresponding to promoters recognized by RNA polymerase containing σ70 (Fig. 1). The appearance of this transcriptional start site was highly reproducible, and could be detected when RNA was extracted from cultures grown at different temperatures or using ethanol as substrate (data not shown). These data show that hscA can be transcribed both from its own promoter and from a promoter upstream of hscB (Fig. 5). No sequences with homology to known transcriptional activator binding sites were detected in the hscA or hscB 5′ regions.

Taken together, the results reported herein are compatible with the P. aeruginosa Hsc66 protein being a functional molecular chaperone important for the stabilization in the presence of ethanol of a protein, or proteins, involved in short-chain alcohol degradation. It is tempting to speculate that Hsc66 is specific for the stabilization of proteins containing an Fe–S cluster, but this hypothesis remains to be experimentally validated.
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