Molecular characterization of *Pseudomonas putida* KT2440 rpoH gene regulation

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The *rpoH* gene of *Pseudomonas putida* KT2440 encoding the heat-shock sigma factor $\sigma^{32}$ was cloned and sequenced, and the translated gene product was predicted to be a protein of 32.5 kDa. The unambiguous role of the gene as a sigma factor was confirmed because the cloned *P. putida* gene complemented the growth defect, at 37 and 42 °C, of an *Escherichia coli* rpoH mutant strain. Primer extension analysis showed that in *P. putida* the *rpoH* gene is expressed from three promoters in cells growing at 30 °C. Two of them, P1 and P3, share homology with the $\sigma^{70}$-dependent promoters, while the third one, P2, shows a typical $\sigma^{70}$-consensus sequence. The pattern of transcription initiation of the *rpoH* gene did not change in response to different stresses, i.e. a sudden heat shock or the addition of aromatic compounds. However, the predicted secondary structure of the 5′ region of the mRNA derived from the three different promoters suggests regulation at the level of translation efficiency and/or mRNA half-life. An inverted repeat sequence located 20 bp downstream of the *rpoH* stop codon was shown to function as a terminator *in vivo* in *P. putida* growing at temperatures from 18 to 42 °C.

Keywords: $\sigma^{32}$, stress response, aromatic hydrocarbons, heat-shock response, downstream box

INTRODUCTION

The ability of micro-organisms to transiently induce the synthesis of a set of proteins in response to an increase in growth temperature is known as heat-shock (HS) response. Initially, this response was believed to be specific for situations in which the cells were exposed to a heat shock. However, during the last decade, it has become evident that heat shock is only one of the stressors able to induce the HS response. A number of other conditions, such as the addition of ethanol, oxygen peroxide or some metals, extreme values of pH, and, in general, the presence of abnormal proteins in the cytoplasm, are known to induce the HS response in *Escherichia coli* (Bukau, 1993; Gross, 1996; van Dyk et al., 1995; Tomayasu et al., 1998; Yura et al., 1993). The HS response has been best characterized in *E. coli*, in which transcription of the so-called HS genes is strongly stimulated after transfer to a high growth temperature. The functions of this set of proteins are the folding, transport and degradation of proteins, especially under stress conditions that cause protein denaturation.

*Pseudomonas putida* is a soil-borne bacterium with significance in bioremediation and biodegradation. It is the natural host for several plasmids that confer the ability to mineralize toluene and other aromatic compounds (Worsey & Williams, 1975; Kunz & Chapman, 1981; Ramos et al., 1997). Bacteria have evolved adaptive mechanisms to survive various environmental stresses. In its natural habitat, *P. putida* is subjected to frequent changes in growth conditions—probably periods of stress and starvation cycles. Exposure to aromatic hydrocarbons could be considered an environmental stress, and this bacterial strain has developed a stress-responsive mechanism to degrade and use these compounds as carbon sources for growth. Nothing is known about the HS response in *P. putida*, but it seems to play a key role in the performance of the strain when it is exposed to contaminants (Marqués et al., 1999). We have identified, cloned and sequenced the *rpoH* gene from *P. putida*, and we have investigated its regulation at the transcriptional level. In addition, we
have studied the effect of aromatic compounds on α2 transcription in *P. putida*.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used are listed in Table 1. *E. coli* K165 selected by plating this strain on plates containing rifampicin (50 µg ml⁻¹). *E. coli* strains were routinely grown at 30 °C in liquid LB medium (Sambrook *et al.*, 1989). *P. putida* strains were grown at 30 °C in liquid LB medium or in modified M9 minimal medium with succinate (25 mM) as the sole carbon source (Abril *et al.*, 1989). When required, antibiotics were used at the following final concentrations (µg ml⁻¹): ampicillin, 100; kanamycin, 50; tetra-cycline, 10; rifampicin, 50; and streptomycin, 100. To identify *rpoH* complementation of *E. coli* mutants on solid media, the growth temperature was increased to 37 or 42 °C.

Plasmid pMPR was constructed by inserting the 580 bp Smal–BglII fragment from pIC551 (Macian *et al.*, 1994), containing the α-phage late promoter *p*ₘ, into the broad-host-range vector pMP220 (Spanik *et al.*, 1987) digested with *Kpn*I, made blunt with the T4 polymerase and then digested with BglII. Plasmid pMPRH was obtained by cloning the linker 5'-AGAAAAAGCGCTGGCCCTATGGGGTGAGCGGCTTTTTGTGGCTTTCTGCA-3' downstream hairpin structure (Fig. 1) into the PsI site of pMPR. Plasmid pMPRA, downstream of the α₂ promoter. Plasmid pMPRA was obtained by cloning the linker containing the random sequence 5’-GCTACCGCTCGCTCGGATTTAGGTCGTGGGGCGGTGA-3’ in the PsI site in pMPR. All plasmids were introduced into *P. putida* by electroporation.

**RNA extraction and analysis.** Samples of 1–10 ml were harvested by centrifugation in disposable plastic tubes precooled in liquid N₂ and were kept at −78 °C until use. RNA was extracted using a modification of the hot guanine isothiocyanate and phenol/chloroform extraction method (Chomczynski & Sacchi, 1987). A 1:4 ml aliquot of lysis solution [1 M guanidium isothiocyanate, 12 mM sodium citrate (pH 7), 48 mM β-mercaptoethanol, 0.25% (w/v) sarcosyl, 0.1 M sodium acetate and 50% (v/v) phenol] was added to each frozen cell pellet. Each mixture was incubated at 60 °C for 10 min before the addition of 0.28 ml chloroform. After gentle shaking and centrifugation at 9000 r.p.m. for 10 min, 0.66 ml 2-propanol was added to the supernatant. The samples were incubated at −20 °C for 15 min and then centrifuged at 14000 r.p.m. for 15 min. The pellets were then rinsed with 70% (v/v) ethanol and dried. After resuspension in 75 µl water [previously treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC)], the samples were subjected to standard treatments with deoxyribonuclease and proteinase K, followed by extraction with phenol:chloroform:isoamylic alcohol and chloroform:isoamylic alcohol (Sambrook *et al.*, 1989). The aqueous phase was precipitated with a 1:10 volume 3 M sodium acetate (pH 4.8) and 2.5 vols absolute ethanol. The samples were then kept at −78 °C for at least 30 min, centrifuged, and washed with 70% ethanol. Finally, the precipitates were dried and resuspended in 20–40 µl DEPC-treated water. Primer extension analysis of 20–60 µg RNA was carried out as described previously (Marques *et al.*, 1993), using a 32P-end-labelled oligonucleotide complementary to the *P. putida* rpoH coding region (5’-GCTACATTAGT-AGACGTCCGCCC-3’). A dideoxy sequencing reaction (Sambrook *et al.*, 1989) of plasmid pSH27 primed with the same labelled oligonucleotide was run in parallel. Gels were exposed to Amersham RPN-8 films for autoradiography.

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**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype and/or relevant characteristics</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<td><em>P. putida</em></td>
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<tr>
<td>KT2440</td>
<td>bdsMR</td>
<td>Franklin <em>et al.</em> (1981)</td>
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<tr>
<td>KT2440-rpoN</td>
<td>Km’, RpoN⁻ derivative from KT2440</td>
<td>Kohler (1989)</td>
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<td><strong>E. coli</strong></td>
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<td>K165</td>
<td>rpoH Sm’</td>
<td>Cooper &amp; Ruettinger (1975)</td>
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<tr>
<td>K165R</td>
<td>rpoH Sm’, Rif’ derivative of K165</td>
<td>This study</td>
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<tr>
<td>HB101</td>
<td>supE44 ara-14 galK2 lacY1 rpsL20 (Sm’), xyl-5 mtl-1 recA13 proA2 hsdS20 (M’)</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
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<td><strong>Plasmids</strong></td>
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<td>pLAFR3</td>
<td>pLAFR1 cosmid derivative (Friedman <em>et al.</em>, 1982) modified to include multiple cloning sites and the Plac promoter fused to lacZ &amp; peptide; Te’</td>
<td>Ramos-González &amp; Molin (1998)</td>
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<td>pMP</td>
<td>pMP220 derivative; <em>ip’₄</em>:galK’::lacZ</td>
<td>This study</td>
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<td>pMPRA</td>
<td>pMP derivative carrying a random sequence between <em>ip’₄</em> and lacZ.</td>
<td>This study</td>
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<td>pMPRH</td>
<td>pMP derivative carrying the rpoH terminator between <em>ip’₄</em> and lacZ</td>
<td>This study</td>
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<td>pSH27</td>
<td>Ap’; derivative of pUN419 with a 3 kb fragment of the <em>P. putida</em> KT2440 chromosome containing rpoH cloned at the PsI site</td>
<td>Yanish-Perron <em>et al.</em> (1985);</td>
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<td>pUN419</td>
<td>Ap’; pUC19 derivative in which the Ndel site was changed to NotI; a 0.7 kb fragment containing the oriT of RP4 was inserted into the new NotI site</td>
<td>S. Marquès (unpublished)</td>
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**RESULTS AND DISCUSSION**

**Isolation and sequencing of the** $\rho$**-independent terminator**

We used a complementation strategy to isolate the $\rho$**-independent terminator** from $P$. *putida* KT2440. A $P$. *putida* KT2440 gene bank generated in pLAFR3 (M. I. Ramos, unpublished) was mobilized into $E$. *coli* K165R, an $\rho$**-deficient strain unable to grow at 37 °C (Cooper & Ruettinger, 1975; Table 1), and tetracycline-resistant clones able to grow at 37 and 42 °C were selected. Plasmid DNA from 12 candidates was transferred to K165 and the growth of tetracycline-resistant transformants at 37 and 42 °C was checked. Only nine out of the 12 initially selected cosmids conferred the ability to grow at high temperatures. Southern hybridization of a $Pst$I restriction band that was also present in the $P$. *putida* chromosome (not shown). One of the cosmids was chosen for further studies. This plasmid was subjected to partial restriction with $Pst$I and ligated to pUN619 digested with the same restriction enzyme (Table 1). An ampicillin-resistant clone able to grow at 37 and 42 °C was selected and its plasmid was named pSH27. The nucleotide sequence of the $P$. *putida* DNA fragment cloned in pSH27 showed the high G+C content expected for $P$. *putida* (60%). The sequence was analysed with the algorithm of Fickett (1982) to detect ORFs. The longest ORF (855 nucleotides) corresponded to a putative protein of 285 amino acids (32.5 kDa). The translated amino acid sequence of this ORF was compared with all entries in the databases, as described in the **BLAST** (Alschul et al., 1990) and FASTA (Pearson & Lipman, 1988) programs. The sequences showing the highest levels of similarity were the $\rho$**-independent terminator** genes of *Pseudomonas aeruginosa* (accession no. U09650), *Xanthomonas campestris* pv. *campestris* (AF042156), *Citrobacter freundii* (X14960), *Enterobacter cloacae* (D50829) and *E. coli* (M20668), which showed protein identities of 88, 61, 61 and 60%, respectively. The alignment of the six proteins revealed that all the structural and functional regions conserved in the $\sigma^{54}$-family members are found in the $\rho$**-independent terminator**.

**β-Galactosidase assays.** Overnight cultures grown on LB supplemented with tetracycline were diluted 1:100 in fresh medium and grown until the exponential phase was reached. Samples were taken to determine β-galactosidase activity as described previously (Miller, 1972). Data are the mean of three independent assays.

**Other techniques.** Standard molecular biology techniques were used for DNA manipulations (Sambrook et al., 1989). The $\rho$**H mRNA** DNA probe used for Southern blots was plasmid pAN4 (Calendar et al., 1989), which contains the $E$. *coli* $\rho$**H gene cloned into pBR322. DNA sequencing of the $P$. *putida* $\rho$**H gene** was carried out on both strands by using the dideoxy sequencing termination method involving dichlororhodamine (Drho Terminators TAQ FS 100 RXN kit; Perkin Elmer).

**Prediction of RNA secondary structure.** Potential mRNA secondary structures for the 5′-ends of the different $\rho$**H transcripts** were predicted using MFOLD (Zucker et al., 1999), and the resulting structures were visualized with RNADRAW version 1.1 (Ole Matzura; available via an anonymous ftp at ftp.itc.univie.ac.at).

**Peptide alignment.** Alignment of peptides was performed using the CLUSTALW program.
segment of the protein implicated in the regulation of σ^{32} as a DnaK-binding domain (Arsene *et al*., 1999; McCarty *et al*., 1996; Nagai *et al*., 1994).

A potential RBS (GGAGGU) was found six bases upstream of the potential initiation codon (Fig. 1a). In addition, a 15 bp region showing 80% complementarity with the *P. putida* 16S rRNA sequence between nucleotides 1456 and 1471 was found six bases downstream of the first base of the coding region (Fig. 1a). The segment, called the downstream box (the DS box), has also been found in other Gram-negative bacteria, and seems to play a role in translation regulation (Nakahigashi *et al*., 1995). It is worth noting that in *P. putida*, the anti-DS box sequence in the 16S rRNA is slightly shifted from that in other Gram-negative bacteria, as is the case in *P. aeruginosa* (Nakahigashi *et al*., 1995).

Downstream of the stop codon, an inverted repeat sequence followed by a T(U) track was found (Fig. 1b). A similar hairpin sequence with the structure of a typical ρ-independent transcription terminator was also found downstream of the rpoH genes analysed so far (Naczynski *et al*., 1995; Sahu *et al*., 1997), although no experimental evidence for such a function has been reported. To analyse the possible role of this sequence in termination, we constructed pMPR, a terminator-probe vector able to replicate in *Pseudomonas*, in which lacZ gene expression was under the control of ρ^{P}_{R} (see Methods). We inserted a 50 bp linker, with the inverted repeat and the T-track sequence located downstream of rpoH, between the ρ^{P}_{R} promoter and the lacZ reporter gene, to produce pMPRH. As a control, we used pMPRA, the equivalent construction bearing a random sequence cloned in the same site. Fig. 2 shows that the presence of the inverted repeat downstream of ρ^{P}_{R} reduced the β-galactosidase activity to less than 10% of that of the construction without the insert; the presence of the random sequence had no effect. Similar results were obtained at 18, 30 and 42 °C (not shown). We therefore suggest that this sequence functions as a terminator for the rpoH transcription in *P. putida*.

**Three promoters control the expression of the rpoH gene in *P. putida***

Transcription of rpoH was analysed using reverse primer extension. Total RNA was isolated from *P. putida* KT2440 grown at 30 °C, and primer extension analysis was carried out with a labelled oligonucleotide complementary to the coding region (nucleotides 116–139 of the ORF; 5′-GCTACATTAATAGAGACGCT-CGCC-3′). Electrophoresis of the resulting cDNAs showed four major bands (Fig. 3a). The 5′-end of the RNA corresponding to three of them mapped upstream of the translation initiation codon of rpoH, whereas the 5′-end of the strongest band was located within the coding region, downstream of the DS box (Fig. 1a). The DNA sequence upstream of the rpoH coding region was searched for possible promoter sequences homologous to the consensus for the different types of promoters. The transcription initiation site furthest upstream was located 154 bp from the A of the first ATG codon. A sequence with good homology to the consensus for σ^{32} dependent promoters of *P. putida* was found at the appropriate distance upstream of this site (Fig. 4). This promoter was called P1. The second transcription initiation site was located 42 bp upstream of the translation start codon, and a sequence with good homology with *E. coli* σ^{32}-dependent promoters was found 6 bp upstream of this point in the DNA sequence. This promoter was called P2. The last transcription initiation point was located 23 bp upstream of the first ATG, and a putative σ^{70} promoter sequence was found starting 8 bp upstream of the transcription start site, which was called P3.

The fourth band present in the primer extension assays described above (Fig. 3a, b) mapped 25 nucleotides downstream of the start codon, five bases downstream of the DS box (Fig. 1a). Since its location within the coding sequence rules out the possibility of a fourth promoter, we suggest two alternatives to explain the presence of this strong signal: (i) the band could be the consequence of the stalling, *in vitro*, of the reverse transcriptase, because of the presence of a putative very stable mRNA secondary structure; (ii) alternatively, it could reflect the presence, *in vivo*, of a recognition site for endonucleases, just downstream of the DS box (Fig. 5), that would render an mRNA molecule inactive for translation. Whether this band originates from the decay of the mRNA synthesized specifically from one of the three promoters or non-specifically from any of them remains to be elucidated.

It has been suggested for *E. coli* that rpoH could also be transcribed by RNA polymerase with σ^{34} (Pallen, 1999).
This prompted us to test if rpoH transcription in P. putida was dependent upon σ^{54}. Total mRNA was isolated from an rpoN mutant of P. putida (Köhler et al., 1989), and from its isogenic wild-type strain, P. putida KT2440, grown at 30 °C; primer extension analyses were carried out using the same oligonucleotide primer as above (Fig. 3b). No differences were found in the banding patterns of the two strains. We can therefore conclude that σ^{54} does not influence rpoH expression in P. putida, in contrast with the situation in E. coli (M. Manzanera and others, unpublished).

To test if the presence of aromatic compounds could modify the transcription of rpoH, cultures of P. putida 2440(pWW0) growing on succinate were supplemented with either 3-methylbenzoate (to concentrations up to 10 mM) or toluene in the vapour phase, and total RNA was isolated after 2 h induction. In a control experiment, a culture of the same strain growing on succinate was subjected for 5 min to a heat shock at 42 °C. The rpoH-derived mRNAs were analysed as above. No difference in the pattern of bands was observed under any conditions (not shown), suggesting that in P. putida, rpoH transcription is not regulated by the presence of aromatics. In addition, it does not change after a heat shock; this is similar to what happens in most Gram-negative bacteria, in which σ^{32} transcription is not autoregulated. Caulobacter crescentus is the only strain described so far in which σ^{54} is autoregulated at the level of transcription (Wu & Newton, 1997).

The presence, in P. putida, of three promoters controlling rpoH gene expression contrasts with the situation in E. coli, in which four promoters have been found upstream of rpoH. Three of them (P1, P4 and P5) are transcribed by RNA polymerase Eσ^{70}, while the fourth (P3) is transcribed by Eσ^{24} (Erickson & Gross, 1989; Nagai et al., 1990; Wang & Kaguni, 1989). In P. aeruginosa, only two promoters have been found, one of them being σ^{54}-dependent (Naczynski et al., 1995).

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**Fig. 4.** Upstream sequence of the rpoH gene and location of transcription start sites (single letter shown in bold, with a vertical arrow) and the potential −10 and −35 sequences (bold and underlined for P1 and P3; bold and double-underlined for P2) of rpoH gene promoters. The first amino acids of the ORF are shown, the DS box is underlined, and the RBS is indicated.
**Fig. 5.** Possible secondary structure for the 5′-end of the mRNA originating from the three promoters P1, P2 and P3. The structures presented were the most stable predicted by Mfold (Zucker *et al.*, 1999) and were visualized with RNADRAW version 1.1 (O. Matzura; see Methods). The first AUG codon is boxed in black; the RBS is boxed in white; and the DS-box sequence is indicated by a line running parallel to the sequence. Because of its size, the folded sequence of P1 is shown schematically, and the region including the first nucleotide of the messenger and the most relevant features is magnified. The arrows show the positions of the 5′-end detected in primer extension analysis and located within the coding sequence (indicated by an asterisk in Fig. 3a).
Analysis of rpoH mRNA secondary structure

The secondary structure of the 5'-proximal region of rpoH mRNA from different Gram-negative bacteria was predicted to contain conserved patterns (Nakahigashi et al., 1995; Morita et al., 1999). The secondary structure is assumed to repress translation of the mRNA at low temperatures, through base pairing that would block access to the initiation codon and the DS box (Yuzawa et al., 1993). We have analysed the potential secondary structure of rpoH mRNAs produced from the three promoters described above, using the algorithm of Zuker et al. (1999). The structures obtained (Fig. 5) were different from the structure predicted for the E. coli rpoH mRNA. It is worth noting that although this structure was supported by analysis of point mutations (Nagai et al., 1991; Yuzawa et al., 1993), these authors did not consider, in their prediction, the different 5'-ends of the mRNA produced from the four promoters described for E. coli.

In the predicted structure of mRNAs originating from P2 and P3 of P. putida rpoH, some important features are conserved: (i) the first codon is blocked by the DS box in a similar folded structure (Fig. 5), and (ii) the RBS is partially blocked by base pairing. However, the situation predicted for the mRNA derived from the P1 promoter is different: the 5'-end of the ORF is completely blocked by the DS box in an almost perfect base pairing, whereas the first codon of the ORF is located within a loop. The RBS in this last folded structure has a level of obstruction by base pairing similar to that of P2- and P3-derived mRNAs. This suggests that translation efficiency could be a key factor in the regulation of σ32 synthesis in P. putida. Although the strength of the three promoters detected was very similar in all conditions tested, the translation of the three resulting mRNAs is probably different, according to the mRNA structures predicted. In addition, the half-lives of the different mRNAs could be an additional step in P. putida σ32 regulation.

Concluding remarks

Our results show that the rpoH gene of P. putida conserves the structural features of the rpoH genes described previously. Experimental data indicate that transcription of the rpoH gene in P. putida remains unchanged under the stress conditions assayed. Therefore, control may take place at a post-translational level because of the structural features found in the rpoH mRNAs, as observed in E. coli (Yura et al., 1993). It has been shown previously that the addition of aromatics provokes a typical HS response (van Dyck et al., 1995; Marqués et al., 1999); this response is not due to an increase in rpoH transcription in P. putida.

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