RESEARCH LETTER

Identification of the transcription factor responsible for L-malate-dependent regulation in the marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

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Abstract

Two-component systems are widespread in nature and constitute the most common mechanism of transmembrane signal transduction in bacteria. Recently, a functionally active two-component system consisting of *malS* and *malR* genes possibly involved in the expression of a C4-dicarboxylate transporter system (*dctAB* operon) was identified in the marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125. In this paper, we performed a functional analysis of the two-component system and demonstrated its involvement in the regulation of the expression of C4-dicarboxylate transporter genes. The expression of the C4-dicarboxylate transporter genes was induced by L-malate with the promoter element located upstream of the *dctA* gene being active only in the presence of the inducer. A σ^{54} promoter responsible for the L-malate dependent transcription regulation was identified and functionally characterized. The molecular mechanism involves an inverted repeat sequence located upstream the σ^{54} promoter that was shown to bind regulatory proteins only in the presence of L-malate. The protein factor responsible for the induction of the *dctAB* operon expression was eventually identified as the transcriptional regulatory protein MalR. MalR is the first transcriptional factor identified in *P. haloplanktis* TAC125 and one of the few transcriptional modulators reported so far in cold adapted bacteria.

Introduction

Two-component regulatory systems are widespread in nature and exist not only in nearly all prokaryotes and many Archaea but also in eukaryotes such as plants, fungi and yeasts (West & Stock, 2001). A typical two-component regulatory system consists of a signalling histidine kinase (sensor kinase) and a cytoplasmic response regulator, usually a transcription factor acting as activator or repressor. Binding of an environmental signal ligand to the sensory domain of the histidine kinase induces autophosphorylation of the transmitter domain that in turn serves as phosphodonor for its cognate response regulator, thus activating the control protein and leading to transcriptional regulation (Wolanin *et al.*, 2002). In bacteria, these regulatory systems are involved in many signal-processing mechanisms, from chemotaxis and flagellar movement to internal nitrogen availability or pathogenicity, from the control of gene expression for nutrient acquisition and virulence to antibiotic resistance (Galperin, 2004). Two-component regulatory systems are often used by free-living bacteria that have to adapt to frequent changes in nutritional availability and, more generally, in environmental conditions in which they need to finely tune gene expression. By contrast, bacteria that live in stable niches (e.g. symbionts of aphids, rickettsias, extremophiles or some marine hydrocarbon-degrading bacteria) seem to have less-regulated promoters and less regulatory factors (Cases *et al.*, 2003; Cases & de Lorenzo, 2005).

The Antarctic *Pseudoalteromonas haloplanktis* TAC125 is the cold-adapted bacterium, so far characterized, endowed with the highest specific growth rate at low temperature. It was considered a likely candidate to study the molecular basis of physiological adaptation with the added value of having interesting biotechnological features. This microorganism was, in fact, suggested as a promising novel host system for the recombinant protein production at low

temperatures (Duilio *et al.*, 2004a, b). Using genome sequencing, corroborated by *in silico* and *in vivo* analyses, exceptional genomic and metabolic features have been uncovered (Medigue *et al.*, 2005). Annotation of the genome highlighted the presence of a large number of regulatory mechanisms including typical two-component systems, although *P. haloplanktis* TAC125 usually lives in stable environmental niches. Using a proteomic approach, we identified a functionally active two-component system consisting of two Coding DNA Sequences (CDSs) *PSHAb0361* and *PSHAb0362* (Papa *et al.*, 2006). This regulatory element was suggested to be involved in the expression of a C4-dicarboxylate transporter system comprising CDSs *PSHAb0363* and *PSHAb0364*, coding for an outer membrane porin and a putative periplasmic transporter of dicarboxylic acids, respectively (Papa *et al.*, 2006). *Pseudoalteromonas haloplanktis* TAC125 metabolizes L-malate very efficiently. *In silico* analyses, revealed the presence of a gene presumably coding for malic enzyme (PSHAa2725, EC No. 1.1.1.40). These enzymes play a key role in allowing cellular growth on L-malate, a key intermediate of Kreb's cycle (Iwakura *et al.*, 1979). In this paper, we performed a functional analysis of the two-component system with the aim to investigate its involvement in the regulation of the expression of C4-dicarboxylate transporter.

Materials and methods

Methods

Bacterial strains, DNA constructs and media

Pseudoalteromonas haloplanktis TAC125 (Birolo *et al.*, 2000) was collected in 1992 from seawater near the French Antarctic Station Dumont d'Urville (60°40′; 40°01′E). It was routinely grown in aerobic conditions at 150 r.p.m. at 15 °C in minimal medium, containing 1 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4NO_3, 10 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4 \times 7H_2O$, 10 mg L^{-1} $FeSO_4$, 10 mg L^{-1} $CaCl_2 \times 2H_2O$, supplemented with 0.5% casamino acid and 0.2% L-malate as carbon source (Papa *et al.*, 2006) and 100 μg mL^{-1} ampicillin when transformed.

Reverse transcriptase (RT)-PCR

Total RNA was extracted from *P. haloplanktis* TAC125 cells grown up to 3 $OD_{600\,nm}$ as described previously (Tosco *et al.*, 2003) in minimal medium in the presence and in the absence of 0.2% L-malate. RNA was reverse transcribed using SuperScript II RNAse H^- Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA was amplified using specific oligonucleotides designed on *malRS* and *dctAB* respectively as primers (Table 1), and *Taq* polymerase (Promega, Madison, WI) according to the manufacturer's instructions. The reaction mixture was amplified (95 °C for 45″; 58 °C for 45″; 72 °C for 60″, 35 cycles), and the products were detected by gel electrophoresis. As a control of the experiment, PCR amplifications on genomic DNA with the same oligonucleotide pairs used for RT-PCR, were performed. Densitometric analysis of the bands for the quantification of transcription levels in the presence and in the absence of L-malate was performed using QUANTITY ONE Biorad software.

Primer extension analysis

Total RNA for primer extension analysis was extracted from recombinant P(PSHA0363) *P. haloplanktis* TAC125 cells (Papa *et al.*, 2006) grown in minimal medium in the presence of L-malate. Primer extension experiments were performed as described previously (Tosco *et al.*, 2003), using the specific PSHAb0363PE Rev oligonucleotide as primer (Table 1).

Preparation of protein extracts

Protein extracts were performed as described previously (Papa *et al.*, 2006).

Construction of promoter and UP (upstream) deletion mutants

Two mutants P(PSHAb0363σ^{70*}) and P(PSHAb0363σ^{54*}) were constructed from P(PSHAb0363) vector applying the one-step overlap extension PCR strategy (Urban *et al.*, 1997). Either the fw/rev σ^{70*} or fw/rev σ^{54*} primers were designed to introduce two single-point mutations, thereby destroying the respective putative σ^{70} −10 box and σ^{54} −24 box (Table 1). Further, a vector containing a mutated version of the upstream sequence containing only the proximal portion of the upstream region was generated and named P(PSHAb0363)UP*. The P(PSHAb0363) vector was hydrolysed with ClaI and EcoRV enzymes. ClaI is located about 1600 bp upstream of the inverted repeat sequence, while EcoRV is located within the inverted repeat sequence (Fig. 2a and c). This enzymatic digestion separates the distal upstream region from the proximal region. A PCR-amplified fragment was obtained using an oligonucleotide containing ClaI in its original position and a mutated oligonucleotide containing EcoRV site immediately upstream the inverted repeat sequence (Table 1). This fragment, opportunely controlled by sequencing, was then inserted into the P(PSHAb0363) hydrolysed vector to generate the P(PSHAb0363)UP* vector.

Table 1. Plasmids and oligonucleotides

	Description	References
Plasmids		
P(PSHAb0363)	pPLB containing the PSHAb0363 promoter region (3600 bp)	Papa *et al*. (2006)
Oligonucleotides		
PSHAbMalS RT-PCR Fw	5′-GGTTTGGTCGAATACATGACTTAGAGC-3′	This work
PSHAbMalS RT-PCR Rv	5′-CATCAACCATAACAACCGAGGTAACTGC-3′	This work
PSHAbMalR RT-PCR Fw	5′-GATGAGGCGATGATCCGCGATTCG-3′	This work
PSHAbMalR RT-PCR Fw	5′-CTAAACCCACCTTCATTCACCAATGCC-3′	This work
PSHAbdctA RT-PCR Fw	5′-CCGATACAGGCGTTATGCGCTACGC-3′	This work
PSHAbdctA RT-PCR Rv	5′-CACTCTGAGTGTTGATACAAAGCACC-3′	This work
PSHAbdctB RT-PCR Fw	5′-GTAGCCGCGCCTTATGCTCATCC-3′	This work
PSHAbdctB RT-PCR Rv	5′-CCACTAAACTCGCCTAGCAAAGGT-3′	This work
PSHAbMalSR RT-PCR Fw	5′-CTAAGGCCGCACCCTTTGTCGGC-3′	This work
PSHAbMalSR RT-PCR Rv	5′-GTAACGTGCAACCAGCTCTTTGC-3′	This work
P(PSHAb0363) PE Rev	5′-CGTTAAGTGGGCTATGTGC-3′	This work
PSHAbdctA bandshift Fw	5′-GGGCATGTAGTAGTGTGCG-3′	This work
PSHAbdctA bandshift Rv	5′-GATGGCCAACTACTAAATAAAC-3′	This work
dctA bandshift control Fw	5′-CTTACCTAATATGGTATTAGC-3′	This work
dctA bandshift control Rv	5′-GGCGCTTCTATTTGTGTGCCTTC-3′	This work
PSHAb0363σ^{70}* Fw	5′-GGGTGTACAACAAAGAGTAGATTC-3′	This work
PSHAb0363σ^{70}* Rv	5′-GAATCTACTCTTTGTTGTACACCC-3′	This work
PSHAb0363σ^{54}* Fw	5′-GTAGTTAACCATCATTTTGCTTAATGG-3′	This work
PSHAb0363σ^{54}* Rev	5′-CCATTAAGCAAAATGATGGTTAACTAC-3′	This work
PSHAb0363UP* Fw	5′-CGTAAAACTATCGATATTAGATACAGG-3′	This work
PSHAb0363UP* Rv	5′-CACTTTTATTTAAAGATATCACCTTTATCG-3′	This work
PSHAb0363C Fw	5′-GATACTTCCTGCAGATATATTAATTG-3′	Papa *et al*. (2006)
PSHAb0363C Rev	5′-CCTGTGTCCCGGGTATCATCATGTGTCC-3′	Papa *et al*. (2006)
PSHAb0363 PE Rv	5′-GCTCTAAGTCATGTATTCGACC-3′	This work

Underlined sequences represent restriction sites inserted.

Transcriptional fusion assays

Recombinant strains were grown either in the presence or in the absence of L-malate up to midstationary phase (24 h). Reporter assays of transcriptional fusions were performed by measuring β-galactosidase activity as described by Duilio *et al*. (2004a, b).

Electromobility shift assay (EMSA)

A 120-bp DNA fragment containing the inverted repeat sequence located upstream *dctA* gene (UP*dctA*) was obtained by PCR amplification using the specific oligonucleotide pairs indicated in Fig. 2a and Table 1. The obtained fragment was ^{32}P labelled with the random primed DNA labelling kit (Roche) according to the manufacturer's instructions. EMSAs were performed in 20-μL reaction volume, in binding buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.1 mM PMSF). Total protein extract (10 μg) was incubated with the ^{32}P-labelled DNA (2 ng, 50 000–100 000 c.p.m.) in binding buffer in the presence of 8 μg of poly(dI–dC) as a nonspecific competitor for the binding reaction, for 20 min at room temperature. A 120-bp PCR-amplified fragment of the intragenic region of *dctA* was used as control (for oligonucleotide pairs, see Table 1). Mixtures were then analysed by electrophoresis on 6% native polyacrylamide gel (29 : 1 cross-linking ratio) in TBE buffer (45 mM Tris-HCl, pH 8.0, 45 mM boric acid, 1 mM EDTA). Electrophoreses were performed at room temperature at 200 V. The gels were dried and analysed by autoradiography. In competition experiments, incubations were performed after the addition of five- to 50-fold molar excesses of unlabelled competitor DNA to the reaction mixture, containing proteins and poly(dI-dC).

Western blot analysis

An antiserum against MalR was raised in rabbit (Primm, Milan, Italy), using the synthetic peptide comprising the region 122–136 of the protein (NH_2-CDKRSLVMENRALKR-COOH) as antigen. Protein extracted from *P. haloplanktis* TAC125 cells grown both in the presence and in the absence of L-malate, were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using standard procedures (Sambrook & Russell, 2001). The proteins were transferred on to a polyvinylidene difluoride (PVDF) membrane using an electroblotting transfer apparatus (Trans-Blot Semi-Dry Transfer Cell, Bio-Rad). MalR protein was detected using the

anti-rabbit polyclonal antibody produced on the 122–136 target peptide of the protein (1 : 500) and peroxidase-conjugated anti-rabbit secondary antisera (1 : 20 000) (A9169, Sigma). The membrane was developed using SuperSignal West Femto Maximum Sensitivity Substrate detection kit (Pierce) according to the manufacturer's instructions.

Southwestern blot and cross-interaction analyses

Proteins extracted from *P. haloplanktis* TAC125 cells grown both in the presence and in the absence of L-malate, were resolved by SDS-PAGE and transferred on to a PVDF membrane as described previously. The UP*dctA* ^{32}P-labelled fragment used for EMSA was also used for the Southwestern experiment. The PVDF membrane was first washed three times in 10 mM Tris-HCl, pH 7.5, containing 5% nonfat dry skimmed milk, 10% glycerol, 2.5% Triton X-100, 0.1 mM dithiothreitol and 150 mM NaCl at 25 °C. The membrane was then soaked in binding buffer (10 mM Tris-HCl, pH 7.5, 0.125% milk, 8% glycerol, 1 mM dithiothreitol, 1 mM EDTA and 40 mM NaCl). Incubation with UP*dctA* fragment (500 000 c.p.m. mL^{-1}) was performed in 3 mL of binding buffer containing 5 mM $MgCl_2$ at 25 °C for 16 h with gentle agitation. The membrane was then washed three times in 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl for 15 min. The membrane was then exposed for autoradiography. The PVDF membrane preincubated with the anti-MalR sera was then incubated in the presence of the radiolabelled UP*dctA* fragment in binding buffer for 16 h with gentle agitation at 25 °C. The membrane was then washed three times in 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl for 15 min and exposed for autoradiography.

Results

Functional analysis of the two-component regulatory system

We identified four genes possibly involved in the uptake of C4-dicarboxylic acids (Papa *et al.*, 2006). These genes, schematically shown in Fig. 1c, are located on *P. haloplanktis* TAC125 chromosome II. *malS* and *malR* encode for a putative C4-dicarboxylate sensor kinase and a putative C4-dicarboxylate response regulator, respectively. Downstream the *malR* gene, *dctA* and *dctB* are coding for an outer membrane porin and a putative transporter of tri-dicarboxylic acids, respectively. The transcriptional regulation of *malRS* and *dctAB loci* under different conditions was

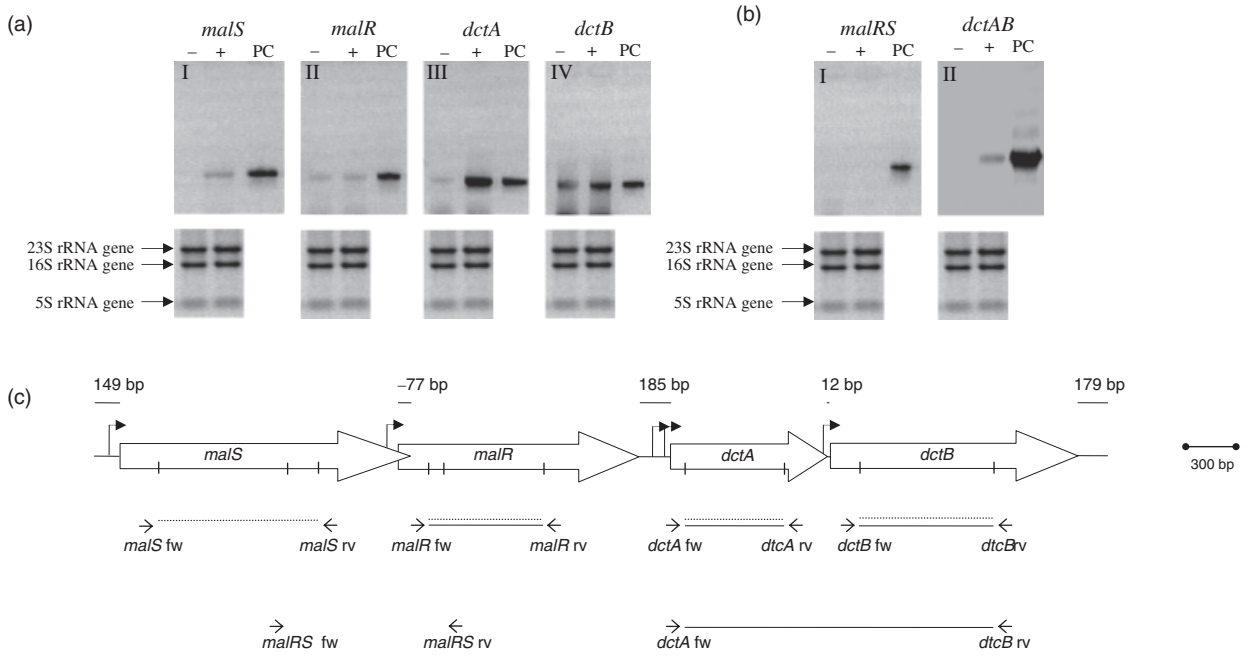

Fig. 1. Schematic organization and transcriptional analysis of *malRS* two-component regulatory system. (a) L-Malate-dependent transcriptional induction of the *malRS* and *dctAB* genes, monitored by RT-PCR (top panels) and visualization of the same samples in an agarose–formaldehyde gel of total RNA (bottom panels). Total RNA samples were extracted from cells grown in minimal medium in the presence (+) and in the absence (−) of L-malate. *Pseudoalteromonas haloplanktis* TAC125 genomic DNA was used as positive control (PC). (b)Transcriptional analysis of *malRS* and *dctAB loci* both in the presence and in the absence of L-malate (top panels) and visualization of the same samples in an agarose–formaldehyde gel of total RNA (bottom panels). *Pseudoalteromonas haloplanktis* TAC125 genomic DNA was used as positive control (PC). (c) A schematic representation of the *malRS* and *dctAB locus* and their transcriptional organization. The position of the oligonucleotides used for the amplifications (←) and the corresponding transcripts are also indicated. ↱, putative promoter element; ——, transcript detected in the absence of L-malate; ·····, transcript detected in the presence of L-malate.

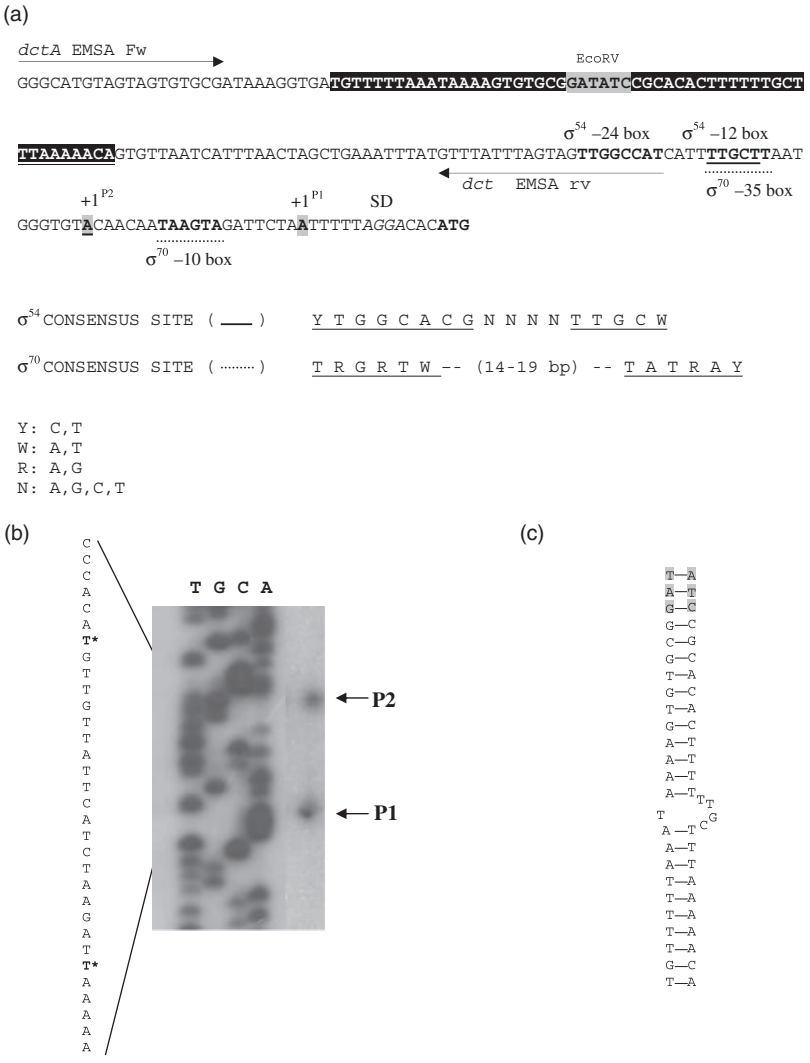

Fig. 2. Transcriptional analysis of *dctAB* operon by primer extension analysis. (a) The nucleotide sequence upstream of the *dctA* gene. The putative P1 and P2 promoters are underlined and highlighted in bold. The corresponding –10 and –35 regions of σ^{70} *core* promoter (indicated with broken lines) and the corresponding –12 and –24 regions of σ^{54} *core* promoter (indicated with continuous lines) are in bold. The transcriptional start sites are underlined and highlighted in grey. The ribosome-binding site (SD) is indicated in italics, while the dctA start codon is indicated in bold. Specific oligonucleotide pairs used to PCR amplify the region are shown. The putative binding site recognized by MalR is highlighted in black (EcoRV endonuclease site is shown). (b) Primer extension analysis of the *dctA* transcript. A 19-bp oligonucleotide was annealed to recombinant P(PSHAb0363) *Pseudoalteromonas haloplanktis* TAC125 total RNA and extended using reverse transcriptase. The nucleotide sequence of the upstream region was determined using the same oligonucleotide as primer (lane T, G, C and A). (c) Putative hairpin structure within the 53-bp-long inverted repeat sequence (EcoRV endonuclease site is shown in grey).

investigated by RT-PCR. RT-PCR analyses were performed on the RNA extracted from *P. haloplanktis* TAC125 cultured in minimal medium both in the presence and in the absence of L-malate. The results are shown in Fig. 1a. *malS* was not detected in the absence of L-malate, whereas a transcription product was weakly detected in the presence of the inducer (Fig. 1a). The light bands in panel II seem to show that transcription of the *malR* gene occurred essentially at the same level under both conditions. Analogously, a *dctA* transcription product was detectable in both conditions although it appeared strongly induced by the presence of L-malate (Fig. 1a). By contrast, transcription of the *dctB* gene did not seem to be strongly affected by the presence of L-malate, showing a band under both conditions (Fig. 1a). However, in the absence of malate, the band in panel IV of Fig. 1a appeared weaker than the band detected when malate was added. A densitometric analysis of the transcripts was then performed using the QUANTITY ONE Biorad software and the data are reported in Table 2. A slight but clear increase in the intensity of the transcript band was observed following malate addition.

We also analysed the possible presence of bicistronic transcripts for both *malRS* and *dctAB loci* (Fig. 1b, Table 1). A cotranscriptional regulation for *malS* and *malR* genes could not be detected, although previous sequence analysis had revealed that these two genes are partially overlapped (7 bp). This result could be probably due to the very low expression level of this locus. As shown in Fig. 1b, a very small amount of the bicistronic transcription product encompassing the *dctAB* locus could only be detected when L-malate was added to the minimal medium. A schematic view of the transcriptional regulation of *malRS* and *dctAB* loci is reported in Fig. 1c.

The occurrence of both the single *dctA* and the *dctA-dctB* bicistronic messengers was further investigated by primer extension experiments. Figure 2b shows the occurrence of

Table 2. Densitometric analysis of *malRS* and *dctAB* transcripts and ribosomal RNA, in the presence and in the absence of L-malate, as reported in Fig. 1

malS		*malR*		*dctA*		*dctB*		*dctAB*	
− malate	+ malate	− malate	+ malate	− malate	+ malate	− malate	+ malate	− malate	+ malate
13.45	308.97	141.36	190.12	208.31	2004.22	523.93	888.59	14.23	381.94

23S rRNA gene		16S rRNA gene		5S rRNA gene	
− malate	+ malate	− malate	+ malate	− malate	+ malate
1667.66	1735.55	1579.79	1590.87	699.20	673.78

To calculate the intensity of the band, the same area was used for each transcript. The value reported for each condition corresponds to the total intensity of all the pixels in the volume divided by the area of the volume (density), as reported in user guide of the QUANTITY ONE software. Estimated errors are c. 5% of the value.

two different transcription initiation sites. A proximal start site, corresponding to an adenine base, was identified only 13 bp upstream of the *dctA* start codon (P1), while a distal start site (adenine) was identified 33 bp upstream of the translational start site (P2). The nucleotide sequence upstream of the *dctA* gene was then carefully examined. A putative σ^{70} promoter element was identified upstream of the proximal start site, according to the *P. haloplanktis* TAC125 consensus sequence previously defined (Duilio *et al.*, 2004a, b). Moreover, a putative σ^{54} promoter element was detected upstream of the distal transcriptional start site. The consensus sequence required to define the σ^{54} -dependent core promoter was derived from a collection of 186 promoters from 47 different bacterial species (Barrios *et al.*, 1999). In contrast to σ^{70}, it is well known that σ^{54} can originate a transcriptionally competent open complex only in the presence of a transcriptional activator, known as bacterial enhancer-binding protein (EBP), usually bound at least 100 bp upstream of the promoter site (Studholme & Dixon, 2003). The DNA region located upstream of the σ^{54} *core* promoter was then examined revealing the presence of a 53-bp long sequence corresponding to a perfect inverted repeat element positioned 71-bp upstream of the P2 transcription initiation site (Fig. 2a–c).

To investigate which promoter is involved in the L-malate-dependent transcriptional regulation, the −10 box of the σ^{70} and the −24 box of the σ^{54} core promoters were individually mutated to destroy the respective promoter consensus sequence. P(PSHAb0363σ^{54}*) and P(PSHAb0363σ^{70}*) were obtained by mutating the nucleotide residues mainly conserved within the corresponding consensus sequences (Barrios *et al.*, 1999; Paget & Helmann, 2003). The nucleotide sequences of the two mutated promoters are shown in Fig. 3a. The activity of the promoters was investigated by transcriptional fusion experiments. The two mutated DNA sequences were individually fused to a promoter-less *lacZ* gene contained in the pPLB plasmid (Duilio *et al.*, 2004a, b). Recombinant cells harbouring the mutated and wild-type promoters were grown in minimal medium either in the absence or in the presence of L-malate and recombinant cells were collected in midstationary growth phase, as the maximum production of β-galactosidase was observed during this growth phase (Papa *et al.*, 2007). As expected, mutation of each core promoter sequence resulted in a clear decrease of β-galactosidase activity in comparison with the corresponding value obtained with the wild-type promoter (Fig. 3b). This result very likely indicates a cooperative effect of two promoters that could be disturbed by the introduction of a mutation in one of two core promoters. However, the transcriptional efficiency of the two mutated promoters was very differently affected by the presence of L-malate. The β-galactosidase activity remained unchanged when L-malate was supplied to cells containing the σ^{54}-mutated vector (PSHAb0363σ^{54}*). On the contrary, cells harbouring the σ^{70} mutated promoter (PSHAb0363σ^{70}*) showed an increase in the β-galactosidase activity when L-malate was added. These findings clearly indicated that only the σ^{54} promoter is involved in the transcriptional regulation mediated by L-malate. Moreover, a transcriptional *lacZ* fusion construct containing a deletion of the distal upstream region (UP) was also prepared. The deletion destroys the inverted and repeat sequence, thus preventing the formation of the hairpin structure (Fig. 2c). Cells harbouring (PSHAb0363)UP* were grown in the presence and in the absence of L-malate. As shown in Fig. 3b, the β-galactosidase activity remained unchanged when L-malate was supplied, suggesting that an intact UP element is needed for transcription regulation.

Identification of a *cis*-regulatory element

Functional analysis of the inverted repeat element located upstream of the P2 transcriptional start site, was carried out by EMSA to investigate whether this region might be able to interact with *P. haloplanktis* TAC125 proteins. A 120-bp DNA fragment encompassing the inverted repeat sequence and the −24 box of the σ^{54} promoter (UP*dctA*) (Fig. 2a), was incubated with the entire protein extracts from cells grown in minimal medium either in the absence or in the presence of L-malate. A clear gel mobility shift band could

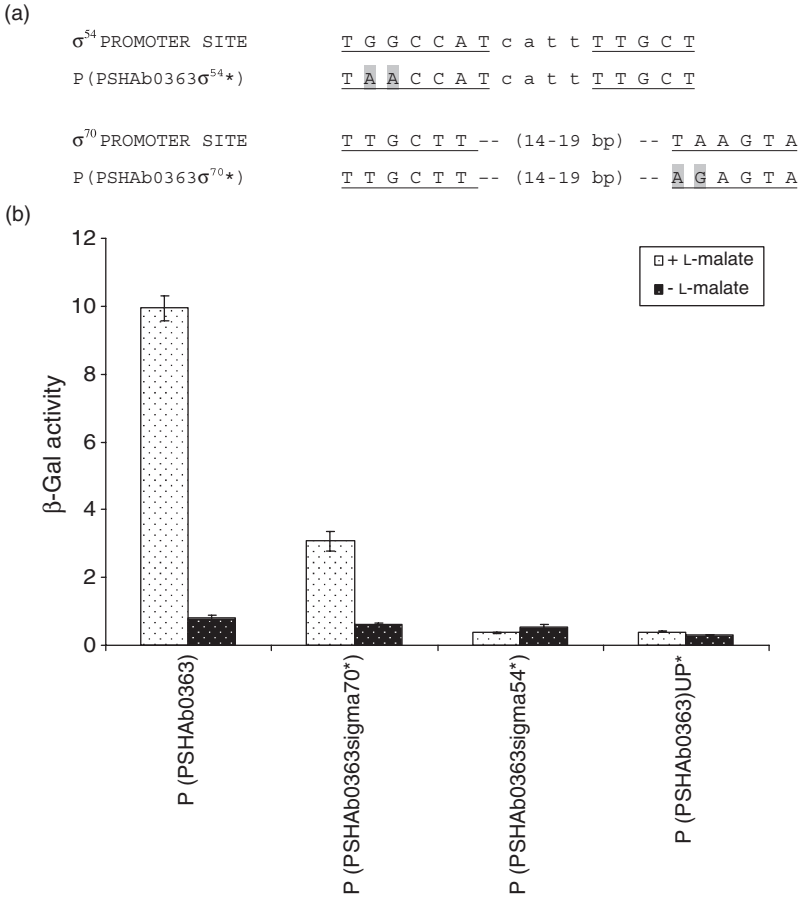

Fig. 3. Functional characterization of σ^{54} and σ^{70} *core* promoters by two single-point mutations. (a) The two *core* promoter sequences are underlined. Single-point mutations for each promoter are highlighted in grey. (b) Profiles of β-galactosidase activity of P(PSHAb0363), P(PSHAb0363σ^{70}*), P(PSHAb0363σ^{54}*) and P(PSHAb0363)UP* transcriptional fusion constructs measured in midstationary phase in *Pseudoalteromonas haloplanktis* TAC125 cells grown in minimal medium in the presence and in the absence of L-malate.

only be detected when the inverted repeat fragment was incubated with the protein extract from cells grown in the presence of L-malate, whereas no binding was observed in the absence of the inducer. A 120-bp DNA fragment of the intragenic region of *dctA* was incubated with the entire protein extracts from cells grown in minimal medium either in the absence or in the presence of L-malate, as control. The free probe is shown for comparison (Fig. 4a). The specificity of the binding was further demonstrated by competition EMSA experiments (Fig. 4b). The specific labelled probe was incubated with total protein extracts in the presence of increasing amounts (five-, 20- and 50-fold) of unlabelled specific and nonspecific DNA competitors.

Identification of the transcriptional regulatory factor

Bacterial EBPs typically consist of three domains, the N-terminal regulatory domain acts either positively or negatively on ring formation and ATPase activity while the central domain with ATPase activity contains the signature GAFTGA motif, which mediates interactions with σ^{54} (Bose *et al.*, 2008). Finally, the C-terminal DNA-binding domain enables specific promoter recognition by allowing EBPs to bind to enhancer-like sequences located *c.* 100–150 bp upstream of the transcription start site. An *in silico* INTERPROSCAN analysis of MalR revealed domain architecture similar to the nitrogen assimilation regulatory protein NtrC, with an N-terminal response regulator receiver domain that contains the phosphorylation site, a central output domain directly responsible for the interaction with the σ^{54} holoenzyme form of RNAP and a C-terminal DNA-binding domain. Mal R also presents the motif that mediates interactions with σ^{54} at residues 223–228, but with a tyrosine replacing the phenylalanine (GAYTGA). For this reason, MalR was suggested to be the likely candidate for the regulation of *dctAB* via σ^{54} activation.

Southwestern blot analysis was then designed to investigate the ability of MalR to specifically recognize the *cis*-regulatory element. The radiolabelled UP*dctA* was incubated with protein extracts from cells grown in the presence and in the absence of L-malate. Figure 5 shows that a single hybridized band with an apparent molecular mass of about 50 kDa was clearly detected in the presence of the inducer. The tiny band observed in the absence of L-malate indicated that recruitment of the regulatory factor also occurred in the

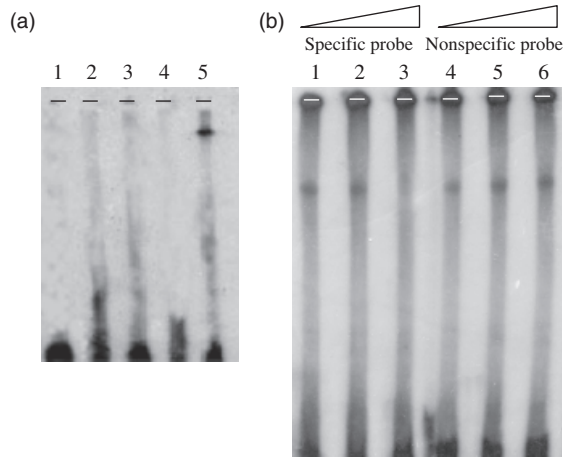

Fig. 4. EMSAs performed using UP*dctA* region and total protein extract of *Pseudoalteromonas haloplanktis* TAC125 cells grown in the presence and in the absence of L-malate. (a) Lane 1, free probe; lane 2, nonspecific probe incubated with proteins extracted from cells grown in the absence of L-malate; lane 3, specific probe incubated with proteins extracted from cells grown in the absence of L-malate; lane 4, nonspecific probe incubated with proteins extracted from cells grown in the presence of L-malate; lane 5, specific probe incubated with proteins extracted from cells grown in the presence of L-malate. (b) Competition assays performed by incubating specific radiolabelled probe with total protein extract of *P. haloplanktis* TAC125 grown in the presence of L-malate, and increasing quantity (five-, 20- and 50-fold) of unlabelled nonspecific and specific DNA competitors.

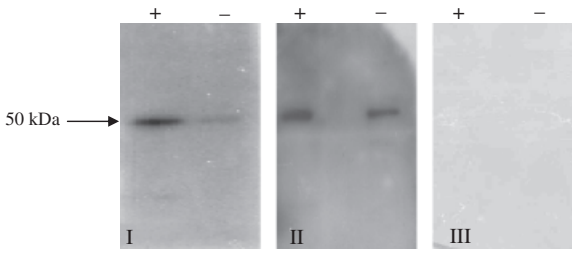

Fig. 5. Southwestern blot analysis of UP*dctA* region and total protein extract of *Pseudoalteromonas haloplanktis* TAC125 cells grown in the presence and in the absence of L-malate. Panel I, Southwestern assay showing the interaction between the UP*dctA* used as probe and a 50-kDa protein in the cellular extract of cells grown both in the presence (+) and in the absence (−) of L-malate. Panel II, Western immunoblot with anti-MalR antibodies showing the presence of MalR protein in the cellular extract of cells grown both in the presence (+) and in the absence (−) of L-malate. Panel III, Cross-competition assay. The PVDF membrane containing the protein extracts from cells grown in the presence (+) and in the absence (−) of L-malate was first incubated with anti-MalR serum and then hybridized with the radiolabelled UP*dctA* fragment.

absence of the inducer, although it was strongly induced when L-malate was added to the medium. The identity of the 50-kDa protein involved in the binding to the regulatory element was univocally assessed by two further experiments. First, the same membrane was developed with antibodies raised against the MalR protein. A clear immunostained band perfectly matching the radiolabelled signal was detected by the anti-MalR antibody when the nucleotide probe was incubated with the protein extract from cells grown in the presence of L-malate. According to the transcriptional data, an identical immunostained band could also be detected when L-malate was omitted from the medium. Second, the specificity of the interaction between the MalR and the DNA *cis*-regulatory element was confirmed by a specific cross-competition experiment. The membrane containing the protein extracts from cells grown in the presence and in the absence of L-malate was first incubated with anti-MalR serum and then hybridized with the radiolabelled DNA fragment. No hybridization bands could be detected following interaction between the radiolabelled DNA fragment and the cell extract, as it was clearly observed in the previous experiment (Fig. 5), suggesting that anti-MalR antibodies recruited MalR preventing its interaction with the DNA fragment. As a whole, these findings demonstrated that MalR is able to specifically recognize the *cis*-regulatory element located upstream of *dctA* gene and to regulate the L-malate dependent expression of the downstream genes.

Discussion

A functionally active two-component system was recently identified in the Antarctic bacterium *P. haloplanktis* TAC125 by differential proteomic experiments (Papa *et al.*, 2006). This regulatory element consists of *PSHAb0361* and *PSHAb0362* and was suggested to be involved in the expression of a C4-dicarboxylate transporter system comprising *PSHAb0363* and *PSHAb0364* (Papa *et al.*, 2006). The proteins encoded by *PSHAb0361* and *PSHAb0362*, hereby indicated as *malS* and *malR*, exhibited 31.7% and 58.3% identity with a C4-dicarboxylate transport sensor protein (Q87R82) and a C4-dicarboxylate transport transcriptional regulatory protein (Q8ECK1) from *Vibrio parahaemolyticus* and *Shewanella oneidensis*, respectively. *PSHAb0363* encoded a putative protein sharing 33.1% identity with an outer membrane porin from *S. oneidensis* (Q8EGP2). *PSHAb0364* coding for a putative protein sharing 46.3% identity with a di-tricarboxylate transporter from *Corynebacterium glutamicum* (Q8NTS7) was found 12 bp downstream of the outer membrane porin gene, thus suggesting the involvement of these two CDSs in the transport and the uptake of C4-dicarboxylic acids. Accordingly, these CDSs were renamed *dctA* and *dctB* (C4-dicarboxylate transport), respectively. Functional analysis of the *malRS* and *dctAB* loci seems to indicate that each gene is independently transcribed by its own promoter in both loci. However, functional differences exist among the various promoters. The promoter elements located upstream of the *malS* gene and, particularly, the *dctA* gene were strongly affected by L-malate with the former

being active only in the presence of the inducer. On the contrary, the promoter located upstream of the *malR* and *dctB* genes seem to be insensitive or slightly responsive to the presence of L-malate, as in the case of *dctB*. The malate dependence of the promoter located upstream of the *dctA* gene confirmed the involvement of the corresponding porin in the L-malate uptake, as reported previously (Papa *et al.*, 2006). The occurrence of two different transcription initiation sites upstream of the *dctAB* locus was demonstrated. A putative σ^{70} promoter element was identified upstream of the proximal start site. Moreover, a putative σ^{54} promoter element was inferred upstream of the distal transcriptional start site (Barrios *et al.*, 1999). The σ^{54} core promoters seem to be remarkably highly conserved both in sequence and in structure as compared with the more variable σ^{70} promoters. This conservation very likely reflects the strict requirements for promoter recognition and function needed for a highly controlled regulation. Functional analysis of the two promoters demonstrated that only the activity of the σ^{54} promoter was affected by L-malate. However, the *dctA* gene was found to be expressed from both promoters, the constitutive σ^{70} promoter active also in the absence of L-malate and responsible for the basal level of the *dctA* transcript, and the inducible σ^{54} promoter, which assures high levels of transcript in the presence of the inducer.

In contrast to σ^{70} RNAP bound at its cognate promoter sites, σ^{54} RNAP is unable to spontaneously isomerize from a closed complex to a transcriptionally competent open complex (Studholme & Dixon, 2003). To proceed with initiation of transcription, the closed complex must participate in an interaction with transcriptional activators known as bacterial EBPs, involving nucleotide hydrolysis. Transcriptional activators usually bind at least 100 bp upstream of the promoter site, and DNA looping is required for the activator to contact the closed complex and catalyse the formation of the open promoter complex. Accordingly, the molecular mechanism exerted by L-malate on the expression of the *dctAB* operon involves the inverted repeat sequence located upstream of the σ^{54} promoter. This DNA sequence represents a *cis*-acting region able to bind *P. haloplanktis* TAC125 regulatory proteins only when the cells were grown in the presence of L-malate. The protein factor responsible for the L-malate dependent induction was identified as the transcriptional regulatory protein MalR.

In silico analysis revealed a close similarity of MalR sequence with NtrC, a typical bacterial EBP. Two-component systems have evolved to allow bacteria to sense and respond to a wide range of stresses and environmental cues, using specialized EBPs. Examples include the nitrogen assimilation regulatory protein NtrC and the dicarboxylic acid transport regulator DctD (Studholme & Dixon, 2003). Bacterial EBPs show a classical architecture consisting of three domains, the N-terminal regulatory domain acting either positively or negatively on ring formation and ATPase activity, the central AAA+domain endowed with ATPase activity and the C-terminal DNA-binding domain enabling specific promoter recognition.

Binding of MalR to the inverted repeat sequence produced a very highly retarded shift band in the EMSA experiment, suggesting something very large binding to it, either RNAP or a multimer of MalR. Indeed, upon phosphorylation, NtrC forms oligomers (possibly an octamer) that can hydrolyse ATP and couple the energy available from ATP hydrolysis to the formation of an open complex with σ^{54} holoenzyme (Lee *et al.*, 2001). Interaction of MalR with the DNA inverted repeat sequence very likely stabilizes the interaction of the RNAP on the σ^{54} core promoter thus activating the transcription of downstream genes. However, transcription of *malR* gene and production of MalR protein seem to occur at the same level both in the absence and in the presence of L-malate. A possible explanation for this apparent discrepancy is that L-malate induces the expression of the MalS histidine kinase that can act as phospho-donor for its cognate MalR response regulator increasing the amount of the active form of the control protein and leading to transcriptional activation.

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