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ORIGINAL PAPER

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Promoters from a cold-adapted bacterium: definition of a consensus motif and molecular characterization of UP regulative elements

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Abstract Although low-temperature tolerant micro-organisms were discovered long ago, limited information is still available on the transcription machinery in cold-adapted bacteria. This knowledge represents a necessary background for the investigation of the adaptation of gene-expression mechanisms at low temperatures. The recent development of a shuttle genetic system for the transformation of the cold-adapted Gram-negative bacterium *Pseudoalteromonas haloplanktis* strain TAC125 has made possible the isolation of the psychrophilic promoters described in this paper. TAC125 genomic DNA fragments were cloned in the shuttle vector and the promoter-containing recombinant clones were selected for their ability to express a promoter-less *lac*Z gene. The nucleotide sequence of several selected inserts and the transcription start points of the transcribed m-RNAs were determined. A promoter consensus sequence for *Pseudoalteromonas haloplanktis* TAC125 was proposed on the basis of a sequence comparison between the various active promoters. Furthermore, the identification and the functional characterization of two UP elements from this cold-adapted bacterium are also reported.

Keywords Adaptation to cold · Promoter consensus sequence · *Pseudoalteromonas haloplanktis* · Psychrophilic bacteria · UP regulative sequence

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Introduction

Micro-organisms able to grow at temperatures as low as 0°C have been known for over 100 years in natural and manmade cold environments, such as marine and continental waters, soils, and foods. Bacteria are unable to insulate themselves against the effects of cold, and therefore all the cellular components of a cold-adapted bacterium must be adapted to allow the organism to grow successfully. Indeed, these organisms breed, grow, and move at rates similar to those achieved by closely related species living in temperate environments, despite the strong negative effect of low temperature on biochemical reactions. To compensate for the above deleterious effects, cold-active enzymes tend to have reduced activation energy in comparison with their mesophilic counterparts. This feature may result in a high catalytic efficiency at low temperature, which has sometimes been reported to be due to enhanced local or overall flexibility of the protein structure (D'Amico et al. 2002). Amongst other cellular components, membranes appear to be crucial for the adaptation to temperature fluctuation, and incorporate specific lipid constituents to maintain fluidity and the critical ability to transport substrates and nutrients under very cold and rigidifying conditions (Chattopadhyay and Jagannadham 2001).

Similarly, fundamental cellular processes of metabolism, replication, transcription, and translation must also be adapted to withstand the temperature decrease. Translation appears to be the most temperature-sensitive process, and the ribosome and associated proteins have been suggested to play a crucial role in temperature sensing (Thomas et al. 2001). On the other hand, less attention has been addressed to transcription in cold-adapted bacteria. The crucial step in gene expression and regulation is the initiation of transcription, a complex

process essentially consisting of two events: the recognition of the promoter sequences by RNA polymerase (RNAP) holoenzyme, and the formation of the open complex. Information so far available on the cold-adapted RNA polymerase concerns the molecular characterization of the holoenzyme isolated from the Antarctic bacterium *Pseudomonas syringae* (Uma et al. 1999). Although this enzyme proved to be structurally very similar to its mesophilic counterparts, it exhibited a remarkable activity even at a very low temperature (0–4°C). Only a few systematic studies have been carried out on the sequence of promoter regions in mesophilic prokaryotes, leading to the definition of consensus sequences for gene-expression signals in these organisms (Doree and Mulks 2001). Even less, and fragmentary, information is available on the structure of these regulatory elements in cold-adapted bacteria. This knowledge would be of the utmost importance to investigate the adaptation of gene-expression mechanisms at low temperatures.

The recent development of a shuttle genetic system for the transformation of the cold-adapted Gram-negative bacterium *Pseudoalteromonas haloplanktis* strain TAC125 (*Ph*TAC125) isolated from Antarctic seawater (Tutino et al. 2001) made it possible to randomly isolate several psychrophilic promoters and their molecular characterization.

In this paper, the isolation of DNA fragments with promoter activity by their ability to express a promoterless *PhlacZ* gene and the identification of their transcriptional start sites by primer extension analysis are reported. On the basis of a comparison between the various active promoters, a consensus sequence for the *Ph*TAC125 promoter region is proposed. Finally, two UP elements, a sequence of about 20 bp located upstream of the −35 region in several bacterial and phage promoters (Lisser and Margalit 1993; Ross et al. 1993; van Ulsen et al. 1997) were identified in this cold-adapted bacterium. The functional characterization of these elements demonstrated that they positively contribute to the efficiency of their respective downstream core promoter.

Materials and methods

Bacterial strains, media and growth conditions

Pseudoalteromonas haloplanktis TAC125 (*Ph*TAC125) (Birolo et al. 2000), a Gram-negative bacterium devoid of any detectable β-galactosidase activity, was grown in aerobic conditions at 4° or 15°C in TYP broth (16 g/l yeast extract, 16 g/l bacto tryptone, 10 g/l marine mix) or LB broth (Sambrook et al. 1989) at pH 7.5, supplemented with 100 μg/ml ampicillin if transformed.

Table 1 Bacterial strains, plasmids, and oligonucleotides used in this work

	Description	Reference
Bacterial strains		
TAC125	*Pseudoalteromonas haloplanktis* TAC125	Birolo et al. 2000
DH5α	*Escherichia coli* [*supE44*, Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1]	Hanahan et al. 1983
S17-1(λ*pir*)	*Escherichia. coli* strain S17-1(λ*pir*) [*thi, pro, hsd* (r^-m^+) *recA*::RP4-2-TC^r:: Mu Km^r::Tn7 Tp^rSm^rλ*pir*]	Tascon et al. 1993
Plasmids		
cloneQ	pGEM-4z containing the T/R box	Tutino et al. 2001
pPLB	cloneQ vector containing the termination region of the *P. haloplanktis*TAC125 *asp*C gene and a promoter-less version of the *Ph*TAE79 β-galactosidase	This work
pP#	pPLB recombinant vectors containing the P# promoter fragments	This work
pSP79β-gal	Genomic clone containing the P. haloplanktis TAE79 lacZ gene and its flanking regions	Hoyoux et al. 2001
Oligonucleotides		
T*asp*1	5′GACGTCTGCAGATCATAATAGTTAACC-3′	
T*asp*2	5′GGAAGCTT CTGAGGTCCGG-3′	
BG1	5′-CATTCTAGATATCGATTTATGCA AGGAATAAACATG-3′	
BG2	5′-CCCGGATCCAAT AACGACTCATCGACC-3′	
Pe1*lacZ*	5′-GTGCTGTAAAGAGGTCATG-3′	
Pe2*lacZ*	5′-GCCGTTAAGTGGGCTATG-3′	
PeP3	5′-CTTTTTGGAAGATTTAAAACCGAAG-3′	
P*edsb*	5′-GACAAAGAAGTAGTAGGCTCAG-3′	
P*easp*	5′-ATAGACACCCACACCTAAATC-3′	
FW2Δ	5′-AAAAATACTAAGGATCCGAATGAACTA-3′	
RV2	5′-TCCTGTTGCGATATCGAATCCAAGG-3′	
FW3Δ	5′-AAAAAGATCTAGGATCCGGCTTGATCTG-3′	
RV3	5′-GCAGGTAAAATAACTGATATCGAGTCA-3′	

E. coli strain DH5α (see Table 1) was used as host for the gene cloning, while *E. coli* strain S17-1(*λpir*) (Tascon et al. 1993) was used as donor in intergeneric conjugation experiments. *E. coli* cells were routinely grown in Terrific broth (Sambrook et al. 1989) containing 100 μg/ml of ampicillin if transformed.

Construction of the promoter-less vector pPLB

By using cloneQ (Tutino et al. 2001) as a starting plasmid, the pPLB promoter-less vector was constructed by inserting the cold-adapted strong *Phasp*C transcription terminator (T*asp*C) (Birolo et al. 2000) downstream of the promoter-less *Ph*TAE79 *lac*Z gene (Hoyoux et al. 2001). The transcription terminator was amplified by PCR reaction using a vector containing the whole *Phasp*C gene and its relative regions as template, and the oligonucleotide pair T*asp*1 and T*asp*2 as primers (see Table 1). The amplification was performed in a mixture containing 60 ng of template, 50 pmol of each oligonucleotide primer, 1.7 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl pH 8.3, 0.1% gelatine, 200 μM dNTP, and 1,25 units of *Taq* DNA polymerase, in a final volume of 50 μl. Twenty cycles of amplification, consisting of 1 min at 95°C, 1.5 min at 55°C and 1 min + 5 sec/cycle at 72°C, were carried out. The DNA fragment corresponding to T*asp*C was digested by *Pst*I and *Hind*III and inserted into cloneQ corresponding sites.

The *P. haloplanktis* TAE79 reporter β-galactosidase gene (*Ph*TAE79*lac*Z) (Hoyoux et al. 2001) was inserted using a two-step procedure, the first one consisting of the double *Xba*I/*Hsp*92I digestion of a 280-bp fragment (PCR amplified by using the primers BG1 and BG2 and the genomic subclone pSP79β-gal as template). In the second step, a 3.2 Kb fragment, containing the 3′ region of *Ph*TAE79*lac*Z gene, was recovered from pSP79 β-gal clone (Hoyoux et al. 2001) by a double *Hsp*92I/*Pst*I digestion. Finally, the two fragments were ligated into the cloneQ-T*aspC* shuttle vector, previously digested with *Xba*I and *Pst*I, generating the promoter-trap plasmid pPLB (Fig. 1). A DNA sequencing reaction was performed on the pPLB amplified region to rule out the occurrence of mutations introduced by the PCR synthesis.

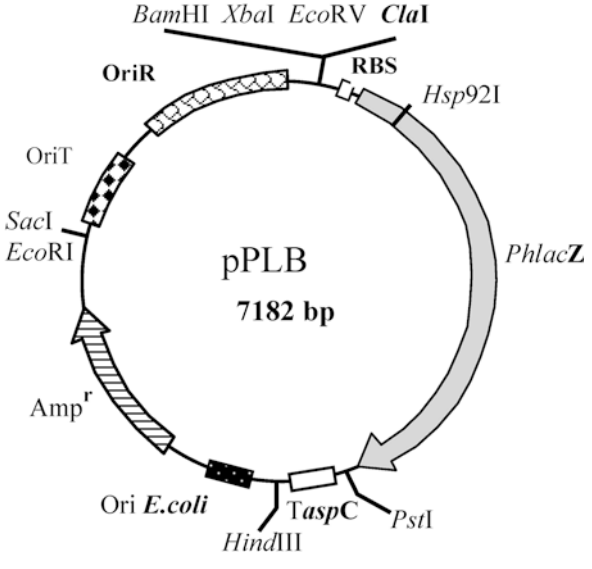


Fig. 1 Map of the shuttle promoter-less pPLB vector. This plasmid derives from the psychrophilic cloneQ shuttle vector (Tutino et al. 2001), in which a promoter-less *Ph*TAE79 *lac*Z gene (Hoyoux et al. 2001), the transcription terminator from the *Phasp*C gene (T*asp*C) (Birolo et al. 2000), and a small polylinker were inserted. *OriT* origin of conjugative transfer from the pJB3 plasmid (Blatny et al. 1997); *OriR* origin of replication of the pMtBL plasmid (Tutino et al. 2001); *RBS* natural *Phlac*Z ribosome binding sequence; *Ori E. coli*, origin of replication from pGEM4Z; *Amp*^{r}, β-lactamase coding gene from pGEM4Z

Construction and screening of a *Pseudoalteromonas haloplanktis* TAC125 promoter library

*Ph*TAC125 genomic DNA was extracted as previously described (Birolo et al. 2000), and partially digested with *Taq*I. The digestion mixture was ligated into the alkaline phosphatase-treated *Cla*I site of pPLB. The recombinant DNA was electroporated into *E. coli* S17-1(*λpir*) cells, and the overnight-grown recombinant cells were used as an intergeneric donor for the mobilization of recombinant plasmids into *Ph*TAC125 cells, as described by Tutino et al. (2001). Single transformed *Ph*TAC125 colonies were selected after 48–72 h of incubation at 4°C onto TYP plates containing ampicillin (100 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (320 μg/ml).

DNA sequencing and analysis

E. coli cells containing the appropriate promoter fragment plasmid (pP#, see Table 1) were grown for 16 h in LB/ampicillin medium and plasmid DNA was prepared using Quiagen spin columns. Automated DNA sequencing was performed with a "PRISM ready reaction dye deoxy terminator cycle sequencing kit" (Protocol part number 401388 Rev.A; Applied Biosystems) on a thermal Cycler 480 (Perkin-Elmer), using various deoxy-oligonucleotide primers. Reaction sequences were loaded on an ABI 373A fluorescent automatic DNA sequencer (Perkin-Elmer, Applied Biosystem division). The dideoxy chain termination method using a Sequenase 2.0 kit (US Biochemical) and [^{35}S]dATP (Amersham Pharmacia Biotech, Uppsala, Sweden) were performed when a sequence ladder was required, as in primer extension analysis. Nucleotide and amino acid sequences were compared with sequences in the GenBank and EMBL databases using the FASTA and BLAST programs (Altschul et al. 1990). Alignments of the promoter regions were performed with MultiAlin version 5.4.1 (Corpet 1988).

β−galactosidase assay

Duplicate cultures of *Ph*TAC125 containing the pP# plasmids were grown at 4° and 15°C in TYP/ampicillin medium up to a late exponential phase (about 10 OD_{600nm}); cell pellet recovered from 2.5 ml of each culture was resuspended in 1 ml of lysis buffer (sodium phosphate 0.1 M pH 7.8, EDTA 2 mM pH 8.0, Triton X100 2%, DTT 1 mM, lysozime 5 mg/ml, PMSF 250 μM) and incubated at 15°C for 15 min. After centrifugation for 20 min at 4°C, the supernatant was collected. Soluble active β-galactosidase was assayed as previously reported (Hoyoux et al. 2001).

RNA isolation and primer extension reaction

RNA isolation from *Ph*TAC125 cells (grown at 15°C up to 3 OD_{600}) and primer extension reactions were performed as described by (Tosco et al. 2003), using 10–30 μg of each total RNA and two oligonucleotides complementary to the *Phlac*Z gene as alternative primers (Pe1*lac*Z and Pe2*lac*Z), For the P3 promoter fragment, primer extension reactions were also performed using a specific P3 oligonucleotide (oligo PeP3). Total RNA isolation from *E. coli* recombinant cells was performed using the RNeasy Mini Kit (Quiagen) following the manufacturer's instructions. The length of the primer extension products was calculated by comparing the electrophoretical mobility in a 6.0% polyacrylamide gel with that

of the products of a sequencing reaction generated with the promoter plasmids as templates and the same primer as used for the reverse transcription reactions.

Construction of promoter deletion mutants

A promoter-less version of the pP3 plasmid was constructed by cloning the 1-kb-long fragment, deriving from the double *Eco*RI/*Bgl*II digestion of the pP3 plasmid, into the *Eco*RI/*Bam*HI digested pPLB plasmid, generating the pΔP3 vector.

Two mutants (pP2ΔUP and pP3ΔUP) were constructed from pP2 and pP3 vectors, respectively, applying a similar PCR-based strategy. Either the FW2Δ or FW3Δ primers were designed to introduce a *Bam*HI restriction site, thereby destroying the respective putative UP proximal region. The RV2 and RV3 primers were complementary to the 3′ end of each cloned fragment and introduced an *Eco*RV restriction site. The resulting PCR products (204 bp long for P2ΔUP, and 157 bp long for P3ΔUP) were double digested with *Bam*HI and *Eco*RV and ligated into pPLB corresponding sites, generating pP2ΔUP and pP3ΔUP plasmids.

Results and discussion

Construction and screening of a *Ph*TAC125 library in a promoter-trap vector

The cold-adapted cloneQ shuttle vector (Table 1) was modified by sequentially inserting (1) the strong *Phasp*C rho-independent transcription terminator, and (2) a promoter-less β-galactosidase gene from *P. haloplanktis* TAE79 (*Ph*TAE79*lac*Z) (Hoyoux et al. 2001), generating the promoter-trap plasmid pPLB (Fig. 1). The cloning procedure allowed the insertion of a unique *Cla*I restriction site upstream of the promoter-less reporter gene, which still retained its natural ribosome binding site sequence.

A promoter-trap library was constructed by cloning *Ph*TAC125 genomic DNA fragments, resulting from the *Taq*I partial digestion, into pPLB *Cla*I restriction site. 15,000 recombinant vectors were mobilized into *Ph*TAC125 cells by intergeneric conjugation following the procedure previously described by Tutino et al. (2001). The transformed psychrophiles were selected at 4°C on solid medium containing ampicillin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Amongst about 15,000 recombinant psychrophilic colonies screened, some 30% showed various intensities of blue color, suggesting the very likely presence of a cold-adapted promoter correctly located with respect to the promoter-less reporter gene. Eight relatively dark blue colonies, possibly containing strong promoters, were selected for further analysis.

The selected inserts were fully sequenced (sizes and EMBL accession numbers are shown in Table 2) and the direct nucleotide similarity search yielded a single positive result, leading to the confident identification of the P3 insert (173 bp long) as the promoter of a ribosomal RNA operon. This fragment showed 68% identity to the *E. coli rrn*DP1 promoter and its flanking regions (between positions 67 to 244 of the sequence stored under the accession number ECRGNDS1).

Table 2 PhTAC125 promoter fragments cloned in pPLB. The data shown are averages of 12 measurements. SD, standard deviation vector

Promoters	Size[a] (bp)	Accession no	β-galactosidase activity[b] (U/mg) ± SD		Ratio 4°/15°C
			4°C	15°C	
P2	218	AJ557251	1.31 ± 0.03	1.20 ± 0.04	1.1
P3	173	AJ557252	0.45 ± 0.03	0.16 ± 0.01	2.8
P4	422	AJ557253	5.40 ± 0.30	2.30 ± 0.30	2.3
P6	1768	AJ557246	0.20 ± 0.10	0.20 ± 0.04	1.0
P14	459	AJ557247	0.20 ± 0.05	0.20 ± 0.01	1.0
P15	188	AJ557248	0.10 ± 0.05	0.20 ± 0.01	0.5
P17	184	AJ557249	1.10 ± 0.40	0.30 ± 0.01	3.6
P25	146	AJ557250	0.02 ± 0.01	0.04 ± 0.01	0.5

[a] The precise lengths were determined by sequencing
[b] The relative strength of each promoter clone is given as β-galactosidase activity in U/mg

Four inserts (namely P2, P17, P15, and P25) proved to be less than 250 bp long, and no significant open reading frames (ORFs) were highlighted. The same result was recorded when the nucleotide sequence of P4 and P14 inserts was analyzed, although these fragments were 422 and 459 bp long, respectively. The cloned P6 fragment proved to be very long (1,768 bp) and did not contain any *Taq*I restriction site. Three ORFs were identified, all oriented in the same direction with respect to the reporter gene. The upstream ORF was incomplete and only its C-terminal 323 amino acid residues were encoded. This sequence showed significant homology with several poly(A) polymerases (EC 2.7.7.19) from other Gram-negative bacteria with the highest identity score (48%) being recorded for the PcnB from *Escherichia coli* (Cao and Sarkar 1992). Only 5 bp separated the *Phpcn*B stop codon from the translation start site of the following ORF, which consisted of 163 amino acids. The sequence of this ORF shared 52% identity with *E. coli hppK* gene product, the 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3). The *hppK* gene is located downstream of *pcnB* even in *E. coli*, thus suggesting an identical genome organization in both the mesophile and *Ph*TAC125, at least for this genetic locus. Finally, the third identified ORF, partially contained in the insert, starts 26 bp downstream from the *PhhppK* stop codon. Its N-terminal 90 amino acid long sequence was identified as the psychrophilic homologue of *E. coli panB* gene (56% identity), whose protein product, 3-methyl-2-oxobutanoate hydroxymethyltransferase (EC 2.1.2.11), is involved in the biosynthesis of pantothenate (Merkel and Nichols 1996).

Determination of relative promoter strength

*Ph*TAC125 recombinant clones were grown in duplicate in liquid culture at two different temperatures (4° and 15°C) until the late exponential phase. The recombinant

β-galactosidase activity was measured in the cell lysates as previously described by Hoyoux et al. (2001). Since *Ph*TAC125 strain has no detectable β-galactosidase activity, pPLB recombinant cell lysate was used as negative control. The recorded specific β-galactosidase activities are shown in Table 2, where the ratio between the average data obtained for each recombinant clone at the two tested temperatures is also reported. No temperature effect was observed on the reporter enzyme production in pP2, pP6, and pP14 recombinant cells. On the contrary, the amount of reporter enzyme produced by pP3, pP4, and pP17 at lower temperatures was 2.3–3.6 times higher than that found at 15°C. Only pP15 and pP25 recombinant clones seemed to produce a slightly higher amount of β-galactosidase at 15°C.

Identification of a cold-adapted consensus promoter sequence

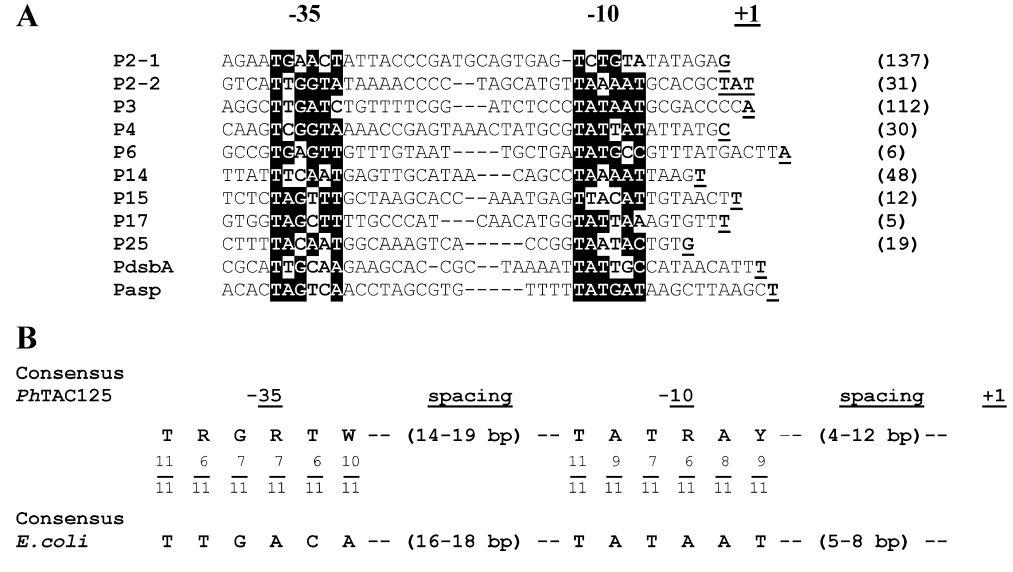
The m-RNA initiation sites of the cloned *Phlac*Z-fusion transcripts were determined by primer extension analysis using total recombinant *P. haloplanktis* RNA as template and two oligonucleotides complementary to the *Phlac*Z gene as alternative primers (Pe1*lac*Z and Pe2-*lac*Z). The determination of the putative transcription start site was also performed on two other *Ph*TAC125 genes, namely *Phasp*C (2) and *Phdsb*A (unpublished results from this laboratory), by using specific primers (Pe*asp* and Pe*dsb*). In all cases, at least one band was detected as a primer extension product, and the determined +1 sites and their respective upstream DNA regions are presented in Fig. 2A. In the case of the P2 promoter, the primer extension pattern indicated two distinct transcriptional start (TS) sites, located at a distance of 104 bp, thus indicating the likely occurrence of two tandem promoters (indicated as P2-1 and P2-2). Moreover, the putative P2-2 promoter showed the occurrence of three adjacent bands, likely representing multiple TS sites.

In Fig. 2A the distance between the determined +1 nucleotide and the downstream *Taq*I cloning site is also reported for each cloned promoter. Only in the case of the P3 fragment is the transcribed portion of the insert longer than 50 bp (112 bp). This ruled out the possibility of identifying the specific gene regulated by each mapped promoter.

The agreement of the TS sites mapped on the recombinant plasmids with those occurring in native *Ph*TAC125 was demonstrated by a retrotranscription reaction carried out in parallel on total RNA extracted from both *Ph*TAC125 wild-type or pP3 recombinant cells and using an oligonucleotide complementary to the P3-transcribed region as specific primer (PeP3). As shown in Fig. 3 (lanes 1 and 2), a unique product of identical size was detected in the reactions.

The sequences of the 11 putative promoters from *Ph*TAC125 were aligned using the MultiAlign 5.4.1 software (Corpet 1988). On the basis of the resulting alignment (Fig. 2A), two 6-nucleotide consensus sequences were identified, representing the putative −35 and −10 regions. As far the identity of the first transcribed nucleotide, in 6 cases out of 11 (54%), the first transcribed base was T; moreover, for the P2-2 promoter two of the three adjacent bands detected were thymine.

Fig. 2 Alignment of 11 promoter regions from *Ph*TAC125 (*panel A*) and identification of a cold-adapted consensus sequence (*panel B*). The promoter regions P2-1 and P2-2 were identified in the same genomic fragment (P2). The determined transcriptional start sites are *boldface and underlined*, while the distance between each +1 nucleotide and the downstream cloning site is reported *in brackets*. *Dashes* indicate gaps introduced to maximize the alignment. Nucleotides *highlighted in black* are those considered for the definition of the consensus sequence shown in panel B. This consists of nucleotides that are present in any given position in more than 51% of the sequences. R: A or G; Y: T or C; W: A or T. *E. coli* promoter consensus sequence was derived from Lisser and Margalit (1993)



This observation could indicate that *Ph*TAC125 prefers a T residue as the TS site, while a systematic analysis of the known *E. coli* promoters revealed a general lack of the +1 preference, the frequencies ranging from 28% for C to about 22% for G (Lisser and Margalit 1993; van der Vossen et al. 1987).

The −10 region is positioned 4–12 bp upstream of the transcription start site and the consensus sequence of this region was shown to be TATRAY, with the positions one, two, and five being the most conserved (100%, 81%, and 72%, respectively). The spacing between the −10 and −35 regions ranged between 14 and 19 bp. As far the consensus for the −35 hexamer, it was established as TRGRTW, although a generally lower extent of conservation was observed, since only the first position (T) proved to be fully invariant. The above consensus sequences identified were shown to be very similar to the *E. coli* σ^{70}-dependent promoter sequences. Indeed, as far as this small promoter sample is concerned, the cold-adapted promoters seem to diverge from the mesophilic ones mainly in the second and fifth positions of the −35 box, where the occurrences of T and C have been more frequently observed (Lisser and Margalit 1993). The high similarity between the *Ph*TAC125 and *E. coli* transcriptional signals was also demonstrated by mapping the TS site of P3, P14, and *dsb*A promoters when placed in an *E. coli* context. The results obtained with P3 are reported in Fig. 3, lane 3, as

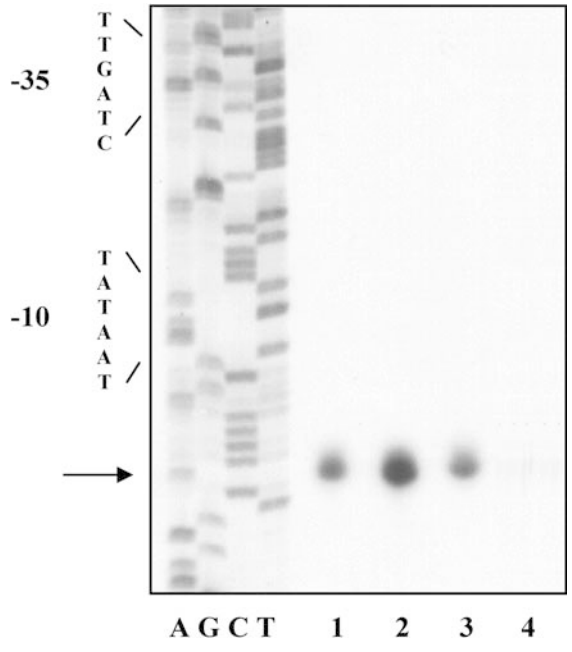


Fig. 3 Primer extension analysis. Comparison of transcriptional start sites mapped from m-RNA isolated from *Ph*TAC125 *wild-type* (*lane 1*), *Ph*TAC125 harboring pP3 plasmid (*lane 2*), *E. coli* harboring pP3 plasmid (*lane 3*), and *Ph*TAC125 harboring pΔP3 plasmid, which contains the promoter deletion (*lane 4*). The sequencing reaction and the first three retrotranscriptions were performed with the PeP3 primer, while lane 4 contains the reaction carried out with the Pe*lac*Z primer. The start site is highlighted with an *arrow*, and −10 and −35 regions are *bracketed*

an example. In all the tested promoters, the *E. coli* transcription machinery started the m-RNA synthesis at exactly the same nucleotide used by *Ph*TAC125.

To further confirm the cold-adapted promoter identification, a mutant of the P3 insert was constructed (ΔP3). A 1-kb-long fragment, deriving from the double *Eco*RI/*Bgl*II digestion of the pP3 plasmid, was ligated with the *Eco*RI/*Bam*HI-digested pPLB plasmid, generating a 156-bp-long deletion which encompasses the P3 −35/−10 sequences as well as the TS site and the downstream transcribed region. The deleted plasmid (pΔP3) was transferred into *Ph*TAC125 and a primer extension analysis was performed on its total RNA using Pe2*lac*Z as primer. As shown in Fig. 3, lane 4, no transcription activity was detected for the pΔP3 plasmid, thus confirming the absence of any recombinant β-galactosidase activity (data not shown).

Identification of putative cold-adapted transcription regulative sequences

Two promoters, P2-1 and P3, were characterized by a long AT-rich sequence located between −59 and −38 bp from the determined TS site. Several prokaryotic promoters possess a positive transcription regulative sequence in this region, the so-called UP element (Rao et al. 1994; Ross et al. 1993). Figure 4, panel A, shows the alignment of the nucleotide sequences of *E. coli rrn*D P1, *Ph*TAC125 P2-1 and P3 promoters with the *E. coli* UP consensus sequence as defined by Gourse et al. (2000). The bases matching the consensus, highlighted in black, are mainly located into the proximal subsite (between positions −46 and −38), which is considered the preferred binding site for the C-terminal domain of the RNAP α-subunit by virtue of its location within the RNAP-promoter complex (Estrem et al.1999). To investigate whether the presence of the putative cold-adapted UP sequence could influence the amount of recombinant β-galactosidase produced, two plasmid mutants (pP2ΔUP and pP3ΔUP) were constructed by PCR, characterized by the deletion of the whole AT-rich upstream region. Recombinant psychrophilic cells were grown at two temperatures (4° and 15°C) until a late exponential phase, and β-galactosidase-specific activities were compared with the corresponding values obtained with the wild-type promoters. In both cases, the deletion of the upstream sequence resulted in a decrease in β-galactosidase production. The decrease in enzymatic activity could not be accurately determined for the pP2 plasmid since it contains two adjacent promoters (P2-1 and P2-2), both of which might affect the production of recombinant β-galactosidase. In the case of the P3 promoter (Fig. 4B), the presence of the UP element resulted in a maximal activation (3.8-fold) at 4°C, while the value dropped 2.3-fold at 15°C.

Furthermore, a plasmid containing a mutated version of the P3 promoter was isolated by serendipity (pP3*). This clone differed from the wild-type only in the change

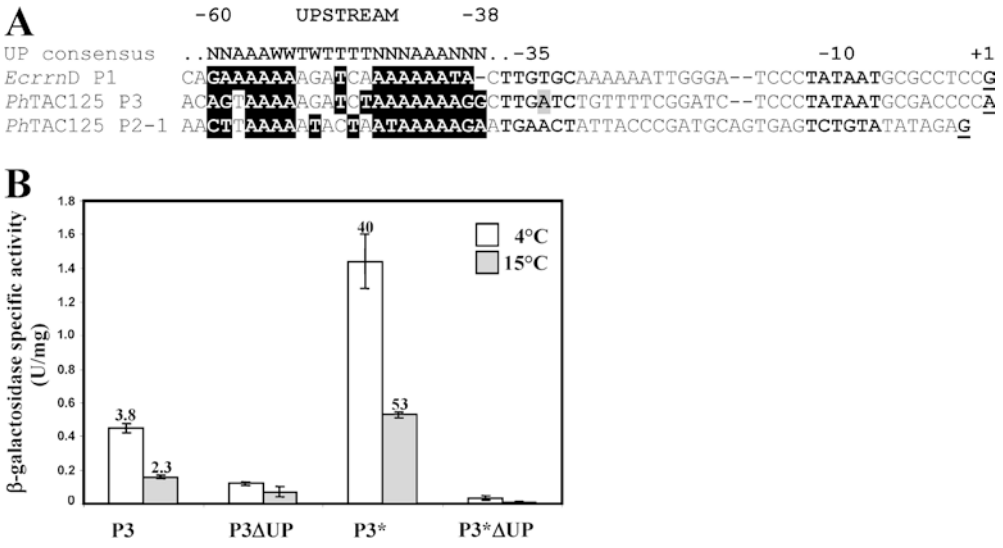


Fig. 4 Identification and functional characterization of two cold-adapted promoters containing putative upstream regulative sequences. *Panel A* alignment of *Ph*P2-1, *Ph*P3, and *E. coli rrnD*P1 promoters and their respective upstream regions with the *E. coli* UP consensus sequence as defined by Gourse et al. (2000). Nucleotides *highlighted in black* are those matching the UP consensus. Core promoter hexamers are *boldface*, while the transcription start sites are *underlined*. The nucleotide *highlighted in gray* is replaced by a T nucleotide in the P3* promoter. *Panel B* β-galactosidase-specific activities from *Ph*TAC125 cells harboring P3, P3ΔUP, P3*, and P3*ΔUP promoters–*lac*Z fusion. *Numbers above bars* refer to fold activation by the UP element (P3/P3ΔUP; P3*/P3*ΔUP). Means and standard deviations are derived from four independent assays

of the base located at the fourth position of the −35 box (from A to T; see Fig. 4A, highlighted in light gray). This base substitution makes the P3* −35 sequence more similar to the *E. coli rrnD* P1 region, while at the same being less faithful to the cold-adapted consensus. A pP3*ΔUP was constructed, and the specific β-galactosidase activities in pP3* and pP3*ΔUP recombinant cells were determined and compared with the corresponding values obtained with the wild-type promoter (Fig. 4B). The substitution in the −35 box made the P3* core promoter (P3*ΔUP) less efficient than the wild-type at both tested temperatures. Surprisingly, the P3* promoter was shown to function about three times better than the wild-type (P3) at both temperatures, and the activation ratio (P3*/P3*ΔUP) was calculated to be between 40- and 50-fold.

Conclusions

This paper represents the first systematic attempt to define the consensus of housekeeping promoters from a cold-adapted *Pseudoalteromonas haloplanktis* species (*Ph*TAC125), isolated from Antarctic seawater (Birolo et al. 2000). The data collected in this study highlight the strong similarity between the defined psychrophilic transcriptional signals with their *E. coli* counterparts. Furthermore, this resemblance is not limited to the −35/−10 sequences but is also supported by their functionality in the mesophilic context, since the *E. coli* transcriptional machinery initiates the m-RNA synthesis from exactly the same transcriptional start point used by *Ph*TAC125, in all the tested examples. On the basis of the latter observation, we could speculate on the likely structural conservation of the RNA polymerase subunits involved in the promoter recognition and transcription initiation.

Although the fine analysis of the cold-adapted promoter structure is outside the scope of the present paper, the fortuitous occurrence of a single-point mutation in the P3 promoter allowed us to investigate the effect of this change on the mutated promoter efficiency, expressed as specific reporter enzyme produced. In particular, the fact that the A to T mutation occurred at the fourth position of the P3 −35 hexamer makes the resulting sequence less faithful to the suggested cold-adapted consensus. In line with this observation, the mutated core promoter (P3*ΔUP) was proven to be less efficient than the wild-type (P3ΔUP), the efficiency ratio (P3ΔUP/P3*ΔUP) being about 3 (at 4°C) and 7 (at 15°C).

Furthermore, between the nine cold-adapted promoters picked out by applying the promoter-trap strategy, two of them (i.e., P2-1 and P3) were shown to be characterized by the presence of a putative UP element, an AT-rich sequence found immediately upstream of the −35 box of some σ^{70}-dependent eubacterial promoters. The deletion analysis described in this paper demonstrated that the identified cold-adapted UP sequences positively contribute to the efficiency of their respective downstream core promoter. The availability of the previously described P3 mutant (P3*) allowed us to evaluate the effect of a single nucleotide substitution in the −35 box on the resulting activity of the whole promoter (UP element + core hexamers). Interestingly, our results demonstrate that P3* is always more active than P3, and that the activation ratio for P3* (P3*/P3*ΔUP) is between 10 and 20 times higher than the corresponding

P3 values. Taken together, these functional data are in agreement with those reported for the *E. coli rrn* P1 promoters by Gourse and co-workers (Hirvonen et al. 2001), which demonstrated that the extent of activation by the same UP element greatly depends on the sequence of the following core promoter.

Although our data highlighted the effect of growth temperature on the efficiency of some cold-adapted promoters, no conclusions can be drawn about the specific molecular mechanism involved. A further investigation on promoters, which differently sense the growth temperature, and on their downstream genes, would help in clarifying the strategies evolved in this marine bacterium to cope with these environmental changes.

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