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ORIGINAL PAPER

**Stefania Madonna · Rosanna Papa · Leila Birolo
Flavia Autore · Nunzianna Doti · Gennaro Marino
Eric Quemeneur · Giovanni Sannia · Maria L. Tutino
Angela Duilio**

The thiol-disulfide oxidoreductase system in the cold-adapted bacterium *Pseudoalteromonas haloplanktis* TAC 125: discovery of a novel disulfide oxidoreductase enzyme

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Abstract In prokaryotes, protein disulfide bond oxidation, reduction and isomerization are catalyzed by members of the thioredoxin superfamily, characterized by the conserved C–X–X–C motif in their active site. Thioredoxins and glutaredoxins contribute to the reducing power in the cytoplasm, while the Dsb system catalyzes disulfide bonds formation in the periplasmic space.This paper addresses the question of disulfide bonds formation in a cold-adapted micro-organism, *Pseudoalteromonas haloplanktis* TAC 125 (*Ph*TAC125) by characterizing the DsbA system. We found distinctive features respect mesophilic counterparts that highlighted for the first time the occurrence of two adjacent chromosomal *DsbA* genes organised in a functional operon. The sophisticated transcriptional regulation mechanism that controls the expression of these two genes was also defined. The two DsbA proteins, named *Ph*DsbA and *Ph*DsbA2, respectively, were expressed in *Escherichia coli* and characterized.Results reported in this paper provide some insights into disulfide bonds formation in a micro organism isolated in the Antarctic sea water.

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S. Madonna · R. Papa · L. Birolo · F. Autore · N. Doti
G. Marino · G. Sannia · M. L. Tutino · A. Duilio (✉)
Dipartimento di Chimica Organica e Biochimica,
Università di Napoli "Federico II"—Complesso Universitario
Monte Sant'Angelo, Via Cynthia, 80126 Napoli, Italy
E-mail: anduilio@unina.it
Tel.: +39-081-674314
Fax: +39-081-674313

L. Birolo · G. Marino · M. L. Tutino
Facoltà di Scienze Biotecnologiche,
Università di Napoli "Federico II", Napoli, Italy

E. Quemeneur
CEA, Direction des Sciences du Vivant, DIEP/SBTN,
Marcoule, Bagnols-sur-Ceze, France

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Introduction

A key step in oxidative protein folding is the formation of S–S bonds between correct cysteine pairs. In prokaryotes, oxidation of cysteine residues is a catalyzed process depending on the protein subcellular localization. Generally, cytoplasmic proteins do not contain structural disulfide bonds, although some enzymes like ribonucleotide reductase, thioredoxin peroxidase and methionine sulfoxide reductase form S–S bridges as part of their catalytic cycles (Bardwell et al. 1991; Schallreuter et al. 1991).

In prokaryotes, disulfide bond formation is achieved in the periplasmic space by the Dsb system, comprising a family of disulfide oxidoreductases belonging to the thioredoxin superfamily. This system can be divided into two pathways: an oxidation pathway, consisting of the DsbA and DsbB proteins, and an isomerization pathway that includes DsbC, DsbD and DsbG proteins (Hiniker et al. 2004). The initial oxidative event is catalyzed by DsbA, which interacts with reduced protein substrates and catalyses oxidation of their cysteine residues to disulfide bonds. Homologues of DsbA, characterized by the conserved –C–P–H–C– motif of the catalytic site, have been found in a wide range of bacteria (Hiniker et al. 2004), suggesting the conservation of the mechanisms involved in disulfide bond formation within the Gram negative bacteria.

Recently, besides the chromosomal DsbA, a second disulfide oxidoreductase homologue was identified in *Salmonella enterica* serovar *Typhimurium*, located in a virulence plasmid (Bouwman et al. 2003). This DsbA-like protein, named SrgA, is characterized by a –C–P–P–

C– motif and shows 37% identity with the canonical DsbA. Other SrgA homologues were also identified in *S. enterica* serovar Typhi and *S. enterica* serovar Enteritidis strains (Rodriguez-Penap et al. 1997). It is extremely unusual for organisms to contain more than one chromosomal DsbA; to the best our knowledge the only case reported so far is that of *Neisseria meningitidis* with three DsbAs suggested to have different activities in folding specific target proteins (Sinha et al. 2004; Tinsley et al. 2004).

This paper addresses the question of disulfide bonds formation in a cold-adapted micro-organism, *Pseudoalteromonas haloplanktis* TAC 125 (*Ph*TAC125) (Birolo et al. 2000) by characterizing the DsbA system. Results reported in this paper describe key enzymes of thiol-disulfide oxidoreductase system in a cold adapted micro-organism and demonstrate for the first time the existence of an uncommon DsbA gene organization.

Materials and methods

Bacterial strains, DNA constructs and media

Plasmids are all reported in Table 1. *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) was collected in 1992 from seawater near the French Antarctic Station Dumont d'Urville (60°40′; 40°01′E) and grown in aerobic conditions at 15°C in TYP broth, pH 7.5 (Birolo et al. 2000).

Escherichia coli strains TOPF'10 and HB1O1 were used as hosts for gene cloning. BL21(DE3) *E. coli* strain (Sambrook et al. 2001) was used as host for heterologous expression.

E. coli cells were routinely grown in LB (Sambrook et al. 2001) containing 100 μg/ml of ampicillin, when transformed. *E. coli* JCB570 and JCB571 were used for motility assays (Bardwell et al. 1991). *E. coli* JCB816 and JCB817 were used for Lac$^-$ phenotype assays (Grauschopf et al. 1995). All these strains were a kind gift from Prof. J.C. Bardwell. pTRC99A expression plasmid (Amersham Biosciences) was used for complementation assay. pUC18 plasmid (Roche, Penzberg, Germany) was used for the construction of the *Ph*TAC125 DNA genomic library. pPLB vector was used for transcriptional analysis (Duilio et al. 2004). pET22b(+) (Novagen) was used for the expression of recombinant proteins (*Ph*DsbA and *Ph*DsbA2).

Table 1 Plasmids constructed in this study

Plasmid	Description[a]
pT(*PhdsbA*)	pTRC99A Δ (*Nco*I- *Eco*RI) Ω (*PhdsbA* gene)
pT(*PhdsbA2*)	pTRC99A Δ (*Nco*I- *Eco*RI) Ω (*PhdsbA2* gene)
pT(*EcdsbA*)	pTRC99A Δ (*Nco*I- *Eco*RI) Ω (*EcdsbA* gene)
pE(*dsbA*)	pET22b Δ (*Nde*I- *Eco*RI) Ω (*PhdsbA* gene)
pE(*dsbA2*)	pET22b Δ (*Nde*I- *Eco*RI) Ω (*PhdsbA2* gene)
P(*yihE*)	pPLB Δ (*BamH*I- *Eco*RV) Ω (*Phdsb* 71–355)
P(*dsbA2*)	pPLB Δ (*BamH*I- *Eco*RV) Ω (*Phdsb* 1640–1990)
P(*dsbA*)	pPLB Δ (*BamH*I- *Eco*RV) Ω (*Phdsb* 1190–1341)
P(*dsbA*^{a})	pPLB Δ (*BamH*I- *Eco*RV) Ω (*Phdsb* 1190–1279)
P(*dsbA*^{b})	pPLB Δ (*BamH*I- *Eco*RV) Ω (*Phdsb* 1279–1341)

[a] Nucleotide coordinates of *Phdsb* locus as deposited in EMBL Databank (AJ634705). Ω, insertion; Δ, deletion.

Cloning and sequencing of *PhdsbA* locus

Genomic DNA preparation from *Ph*TAC125 was carried out as previously described (Tosco et al. 2003). The *PhdsbA* locus was isolated from a *Ph*TAC125 *Hind*III genomic library by dot blotting screening, by using degenerated oligonucleotides as probes, designed on the *E. coli* amino acidic sequences and from F_{29} to P_{41} *Ec*DsbA for *Ph*DsbA.

The screening was carried out as reported by Georlette et al. (2003), and allowed to identify positive clones for *PhdsbA*. The positive clones were sequenced by the TaqFS dye terminator kit (Perkin Elmer), Norwalk, CT, USA, using the Applied Biosystems Automatic Sequencer model 373A (Perkin Elmer).

In order to complete the sequence of the *PhdsbA* locus, sequencing reactions were performed directly on the genomic DNA by using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech., Freiburg, Germany), according to the procedure reported by Krin et al. (2001). In the case of DNA sequences with dyad symmetries containing dG and dC residues, a master mix containing dITP was used for the sequencing. Use of dITP required longer extension times (20 min) at 60°C.

The EMBL Databank accession number for *PhdsbA* locus is AJ634705.

Constructions of the expression plasmids

For the complementation assays, the *PhdsbA*, *PhdsbA2* and *EcdsbA* genes were amplified (Tosco et al. 2003) and cloned into the commercial expression vector pTRC99A, generating the pT(*PhdsbA*), pT(*PhdsbA2*) and pT(*EcdsbA*) plasmids, respectively (Table 1).

For the purification of the recombinant proteins, *PhdsbA* and *PhDsbA2* genes were PCR amplified and cloned into the commercial expression vector pET22b(+). The resulting constructs were indicated as pE(*dsbA*) and pE(*dsbA2*), respectively (Table 1).

For the transcriptional fusion experiments, the promoter regions upstream of *PhyihE* and *PhdsbA2* genes were amplified and cloned into the reporter vector pPLB (Duilio et al. 2001), generating P(*yihE*) and P(*dsbA2*) plasmids (Table 1). The two putative promoters upstream of *PhdsbA* gene were amplified and cloned together and separately, generating P(*dsbA*), P(*dsbA*^{a}), P(*dsbA*^{b}) plasmids, respectively (Table 1).

All the resulting plasmids were used to transform *Ph*TAC125 cells by interspecific conjugation (Duilio et al. 2001) and to perform the transcriptional assays.

Complementation of *E. coli* dsbA mutant strains

Two different phenotypes—dependent upon the presence of active DsbA homologous protein—were used for the complementation assays: motility and Lac^- phenotype of the MalF-β-galactosidase 102 fusion protein (Bardwell et al. 1991).

pT(*PhdsbA*), pT(*PhdsbA2*) and pT(*EcdsbA*) constructs were used for both types of assays. pTRC99A was used for negative control.

For motility assays, the constructs described were used to transform the non-motile *E. coli* strain JCB571 ($dsbA^-$) following standard procedure (Sambrook et al. 2001). *Ec*JCB570 ($dsbA^+$) and *Ec*JCB571(pT *EcdsbA*) strains were used as positive controls, while JCB571(pTRC99A) strain was used as negative control.

The complementation assays with MalF-β-galactosidase 102 fusion protein were performed in according to the procedure reported by Grauschopf et al. (1995), by using *Ec*JCB817 ($dsbA^-$) strain, *Ec*JCB816 and *Ec*JCB817 transformed with pT(*EcdsbA*) construct were used as positive controls.

Transcriptional assays

RNA isolation from *Ph*TAC125 cells (grown at 15°C up to 3 OD_{600}) and Northern blotting analysis were performed as described by Tosco et al. (2003). The P1, P2 and P3 probes used for these experiments were fragments internal to the *PhyihE*, *PhdsbA* and *PhDsbA2* genes, respectively, obtained by PCR amplification.

Primer extension experiments were performed as described by Tosco et al. (2003), by using 18-21 bp specific oligonucleotides as primers.

Reporter assays of transcriptional fusions measured activity of a cold-adapted β-galactosidase as described by Duilio et al. (2004).

Overexpression and purification of *Ph*DsbA and *PhDsbA2*

The recombinant *PhdsbA* and *PhdsbA2* genes were separately expressed in *Ec*BL21(DE3) cells, as follows: fresh cultures (2 ml) were inoculated into 200 ml LB medium containing 100 μg/ml of ampicillin. The recombinant cells were grown at 18°C for 20 h without any induction until the OD_{600} reached 4.5. The bacterial pellets were resuspended in 10 ml of buffer A (30 mM Tris–HCl pH 8, 20% sucrose, 1 mM EDTA pH 8) and incubated at room temperature for 20 min. The shocked cells were collected by centrifugation at 13,000 rpm at 4°C and resuspended in 10 ml ice-cold 5 mM $MgSO_4$. After incubation at 4°C for 20 min and centrifugation at 13,000 rpm, the supernatants (periplasmic fractions) containing the recombinant *Ph*DsbA and *Ph*DsbA2 proteins were collected and extensively dialyzed against 10 mM MOPS pH 7.2 and 10 mM MOPS pH 7.6, respectively.

For *Ph*DsbA protein purification, the sample was loaded on a DEAE-Sepharose *Fast Flow* column (Pharmacia Biotech. Inc., NJ, USA) equilibrated in 10 mM MOPS pH 7.2. Proteins were eluted with a linear NaCl gradient (0–0.3 M in equilibration buffer) and fractions were analysed for reducing activity with insulin as substrate (Holmgren 1979). The fractions containing the active protein were pooled, concentrated, dialyzed against 20 mM Tris–HCl pH 8, 1 M NH_4SO_4 and loaded on a *Phenyl Superose* column (Pharmacia Biotech. Inc.) equilibrated in the same buffer. The proteins were eluted with a linear gradient (20 mM Tris–HCl pH 8, 1 M NH_4SO_4—20 mM Tris–HCl pH 8).

For *Ph*DsbA2 protein purification, the sample was loaded on a SP-Sepharose *Fast Flow* column (Pharmacia Biotech Inc.) equilibrated in 10 mM MOPS pH 7.6 and proteins were eluted with a linear NaCl gradient (0–0.5 M in equilibration buffer). The active fractions were pooled, and loaded on a *Superdex 75* PC 3.2730 column (Pharmacia Biotech. Inc.) equilibrated in 50 mM Na-phosphate pH 7.5, 0.15 M NaCl, buffer.

Protein concentration was determined with the Bio-Rad protein assay (Bradford 1976), using bovine serum albumine as standard. The recombinant *Ph*DsbA and *Ph*DsbA2 proteins were stored at –20°C.

*Ph*DsbA and *Ph*DsbA2 antibodies production and Western blotting analyses

The anti- *E. coli* DsbA rabbit serum already described in Charbonnier et al. (1999) was shown to cross-react with *Ph*DsbA but not with *Ph*DsbA2. Thus, it could be used in Western blotting experiments. An antiserum against *Ph*DsbA2 was raised in rat, using inclusion bodies of the recombinant protein as antigen. The immunisation protocol involved three injections of 330 μg protein each; the first one in complete Freund adjuvant and the two further ones in incomplete Freund adjuvant at days 30 and 60. The optimal titer of antibodies were reached at day 75 where animals were bleeded (Charbonnier et al. 1999).

For Western blotting, protein samples were resolved by using 15% SDS-PAGE gel. Electrophoresis was done under reducing conditions using standard procedure (Sambrook et al. 2001). The proteins were transferred to a PVDF membrane using an electroblotting transfer apparatus (Trans-Blot Semi-Dry Transfer Cell, Bio-Rad, USA). *Ph*DsbA and *Ph*Dsba2 were detected by using the anti-rat polyclonal antibodies with ratio 1:1,000 and 1:3,000, respectively, and peroxidase-conjugated anti-rabbit secondary antisera (1:20,000) (A9169, Sigma, MO, USA). The membranes were developed by using SuperSignal West Femto Maximum Sensitivity Substrate detection kit (Pierce).

Mass spectrometric analysis

The molecular mass of the *Ph*DsbA and *Ph*DsbA2 proteins was determined by electrospray mass spectrometry (ESMS) on a ZQ single quadrupole mass spectrometer (Waters), by injecting protein solutions (10 pmol/μl) into the ion source at a flow of 5 μl/min. Data were elaborated using the Mass Lynx program (Waters).

CD spectroscopy

Spectroscopic characterization

Far-UV CD spectra were recorded on a Jasco J715 spectropolarimeter equipped with a Peltier thermostatic cell holder (Jasco model PTC-348), in a quartz cell of 0.1 cm light path at a protein concentration of 1.0 μM. Temperature was measured directly in the quartz cell, the solutions were filtered just before use on 0.22 μm pore size PVDF membrane (Millipore, Bedford, MA, USA), and data corrected by subtracting a control from which the protein was omitted. Spectra were recorded at 25°C from 280 to 184 nm at 0.2 nm resolution, 16 s response, at a scan rate of 20 nm/min. All data are the averages of three measures, and the results are expressed as mean residue ellipticity (θ), which is defined as $(\theta) = 100\ \theta_{obs}/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters.

Thermal denaturation of the protein was followed by recording temperature-induced changes in secondary structure. Ellipticity at 220 nm was measured as the temperature was varied from 20 to 100°C at a rate of 1°C min^{-1}. Enzyme concentration was 1 μM in 10 mM HEPES, 1 mM DTT and 0.15 M NaCl, pH 7.5.

Linear baselines were fitted above and below the transition zone and the apparent fraction of molecules in the unfolded state (F_u) has been derived from the experimental mean residue ellipticity according to Eq. 1:

$$F_u = \frac{(y_n - y_{obs})}{(y_n - y_u)} \quad (1)$$

where y_n and y_u are the pre-transitional and post-transitional baselines, that are assumed to depend linearly on temperature.

Thermal unfolding transitions were analyzed with the two-state $N \rightleftharpoons D$ model whose equilibrium constant is given by (2):

$$K_d(T) = \exp\{(-\Delta_d H(T_d)/R)(1/T - 1/T_d)\} \quad (2)$$

where T_d is the denaturation temperature at which $K_d = 1$ and $\Delta_d H(T_d)$ is the denaturation enthalpy change. The denaturation heat capacity change, $\Delta_d C_p$, is considered to equal zero because it cannot reliably be determined from CD measurements.

Correspondingly, the observed molar ellipticity is (3):

$$(\theta) = ((\theta)_N + (\theta)_D K_d)/(1 + K_d) \quad (3)$$

where $(\theta)_N$ and $(\theta)_D$ are the molar ellipticities of the native and denatured states, respectively, which are assumed to depend linearly on temperature. A non-linear least-squares regression was carried out to estimate the unknown parameters associated with the unfolding transition, using Micromath Scientist for Windows.

Results

Cloning and genetic organisation analysis of *PhdsbA* locus

Six thousand clones from a *Ph*TAC125 genomic library were screened using a degenerate primer as probe, designed on the basis of a multiple alignment of several amino-acid sequences from bacterial DsbA proteins available in protein databases. Two identical positive clones were identified, both containing a 1.9 kb insert, whose complete sequencing revealed the presence of three ORFs, two of which (ORF1 and ORF3) were incomplete. The complete ORF2, consisting of 621 bp, encodes a predicted protein of 207 amino acids, with theoretical *M*w of 22991 Da, exhibiting 33% identity with the *E. coli* DsbA protein (*Ec*DsbA) (Bardwell et al. 1991) and 40% identity with a DsbA homologue from *Vibrio parahaemolyticus* (entry Q87GP4).

With the aim of obtaining the complete sequence of the other two genes, the flanking regions of the ORF2 were sequenced by using the genomic DNA direct sequencing method (Krin et al. 2001). As a control, the genomic regions sequenced were amplified by PCR reactions using the *Ph*TAC125 genomic DNA as template, cloned in the pUC18 vector, and sequenced on both strands. Sequence data revealed that ORF1 is located 34 bp upstream ORF2 and consists of 972 bp, encoding a putative protein sharing 53% identity with the *E. coli* YihE protein (Belin et al. 1994), whose biological function is still unknown. ORF3, located 20 bp downstream ORF2 and consisting of 636 bp, encodes a protein of 212 amino acid residues with a theoretical *M*w of 23709 Da, displaying 39% identity with the DsbA homologue from *Vibrio vulnificus* (entry Q8DDF4).

Due to the canonical CPHC sequence of its active site, the protein encoded by the ORF2 was named *Ph*DsbA. The protein product of ORF3 showed a novel sequence at the active site (–C–P–A–C–), and, on the basis of homology considerations, it was tentatively identified as a DsbA-like protein. This protein shares 37% identity and 55% similarity with *Ph*DsbA and thereafter was named *Ph*DsbA2. In Fig. 1, the alignment of *Ph*DsbA and *Ph*DsbA2 to the *Ec*DsbA sequence is shown. Both *Ph*DsbA and *Ph*DsbA2, according to Von Heijne's rules (Von Heijne 1985), contain at the N-terminus a putative translocational signal, typical of the periplasmic proteins.

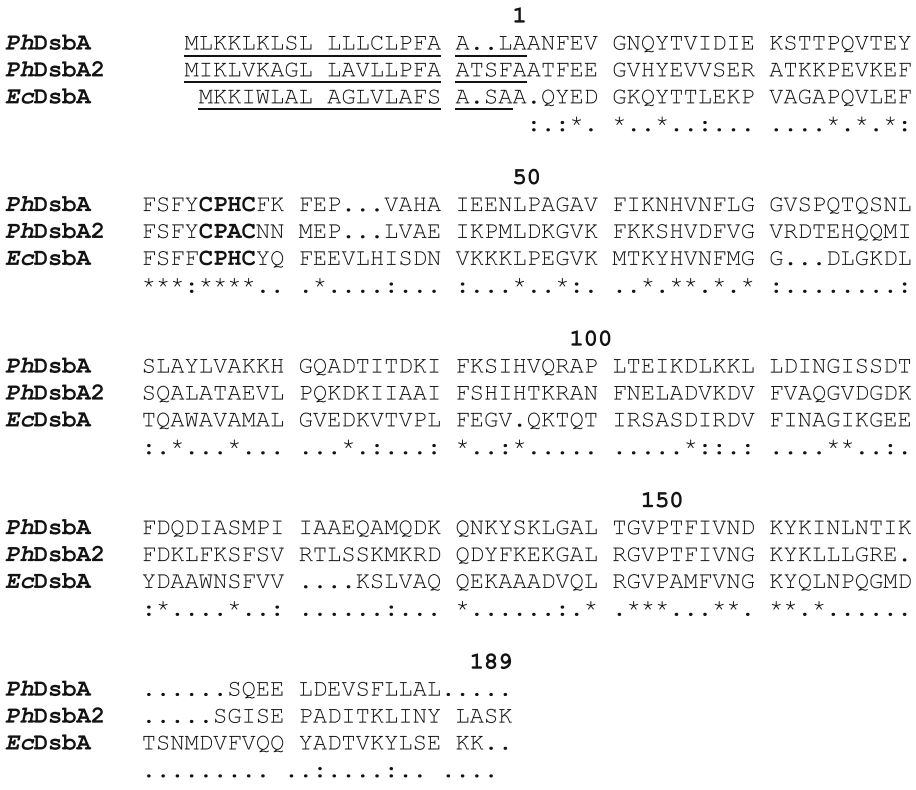

Fig. 1 Sequence alignment of DsbA homologues (*Ph*DsbA and *Ph*DsbA2) proteins from *Pseudoaltermonas haloplanktis* TAC125 with the corresponding counterparts from *E. coli* (*Ec*). Identical residues are labelled with (*), conservative substitutions with (:), and semi-conservative substitutions with (.), conserved translocation signal (_). The numeration of the amino acid residues refers to *Ec*DsbA

The nature of the flanking regions of the *PhdsbA* locus was further investigated by direct sequencing reactions, carried out on *Ph*TAC125 genomic DNA. Divergent oligonucleotides designed on the *PhdsbA* locus sequence were used as primers. Partial sequencing of the left side adjacent region revealed the presence of an ORF encoding a putative protein showing a high degree of similarity with ferredoxin from *Vibrio cholerae* (40%). Partial sequencing on the right side of *PhdsbA* locus revealed the presence of an ORF (ORFb), coding for a putative protein, which showed a significant similarity with the fimbriae-associated adhesion protein Fap1 from *Streptococcus parasanguis* (47%).

Transcriptional analysis of *PhdsbA* locus

Northern analyses were performed to investigate the transcriptional organization of the *PhdsbA* locus. [32] P-labeled DNA fragments internal to *PhyihE, PhdsbA* and *PhdsbA2* genes were used as probes. As shown in Fig. 2A, P1 probe recognized a 1.5 kb transcript corresponding to the co-transcription of the *PhyihE* and *PhdsbA* genes. P2 probe hybridises with two different transcripts: (1) a 1.5 kb transcript corresponding in size to *PhyihE* and *PhdsbA* genes together; (2) a 1.2 kb transcript that was attributed to the co-transcription of the *PhdsbA* and *PhdsbA2* genes. Finally, P3 probe detects two transcripts: (1) a 1.2 kb transcript corresponding in size to the co-transcription of *PhdsbA* and *PhdsbA2* genes; (2) a 0.65 kb transcript corresponding to the *PhdsbA2* monocistronic message. All probes recognize a 2.1 kb transcript whose size is consistent with the length of the mRNA corresponding to the three ORFs together.

Taken together (Fig. 2B), these results suggest the existence of: (1) an active promoter element located upstream of the *PhyihE* gene that is responsible for the full-length locus transcription and *PhyihE–PhdsbA* co-expression; (2) a promoter element upstream of the *PhdsbA* gene, responsible for the *PhdsbA* and *PhdsbA2* co-transcription; (3) a promoter sequence located upstream of the *PhdsbA2* gene, responsible for the synthesis of the *PhdsbA2* monocistronic message. Moreover, these data demonstrate the functionality of the putative transcriptional Rho-independent terminator, located 6 bp downstream of the ORF3 stop codon and indicate the occurrence of a transcriptional terminator, likely Rho-dependent, downstream of the *PhdsbA* gene.

Figure 3 shows primer extension experiments that explained the occurrence of the *PhdsbA– PhdsbA2* messenger. These analyses revealed two different transcriptional start sites for *PhdsbA* gene (Fig. 3A). A multiple distal start site (T/T) was identified 108 bp upstream of the translational start site, while a proximal start site, corresponding to a single thymine base, was identified 29 bp upstream of the *PhdsbA* start codon. The putative −10 and putative −35 boxes identified upstream of the two transcriptional start sites are shown in Fig. 3B. No transcriptional start site was experimentally detected for *PhyihE* and *PhdsbA2* genes although Northern analyses suggested their presence and putative promoter elements had been predicted by computational analysis.

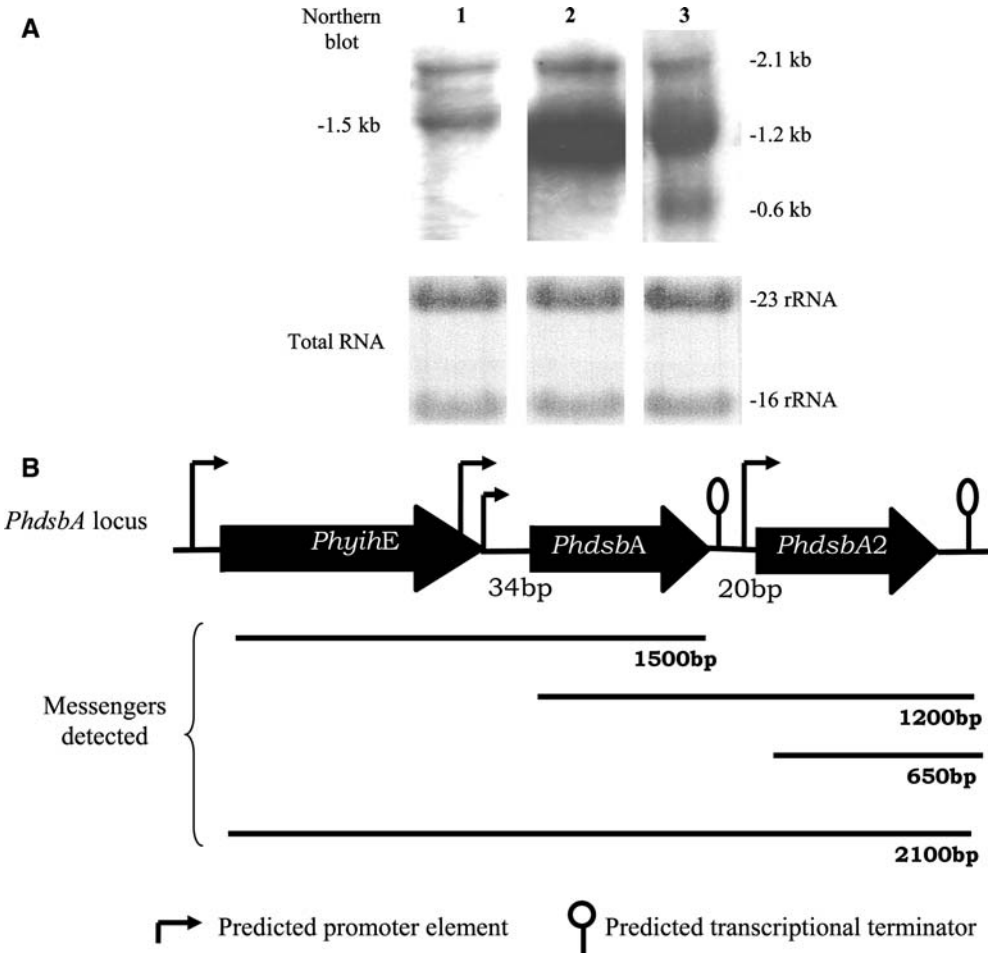

Fig. 2 **A** Northern blot analysis (*top panel*) and visualization of the same samples in an agarose-formaldehyde gel (*bottom panel*) of PhTAC125 total RNA. Total RNA samples (30 μg), isolated from mid-logarithmic-grown cells, were separated onto the 1.2% agarose gel, blotted to nitrocellulose membrane and hybridized with probes P1 (internal to *Ph*yihE, *lane 1*), P2 (internal to *Ph*dsbA, *lane 2*), and P3 (internal to *Ph*dsbA2, *lane 3*). **B** A schematic representation of the *Ph*dsbA locus and its transcriptional organization

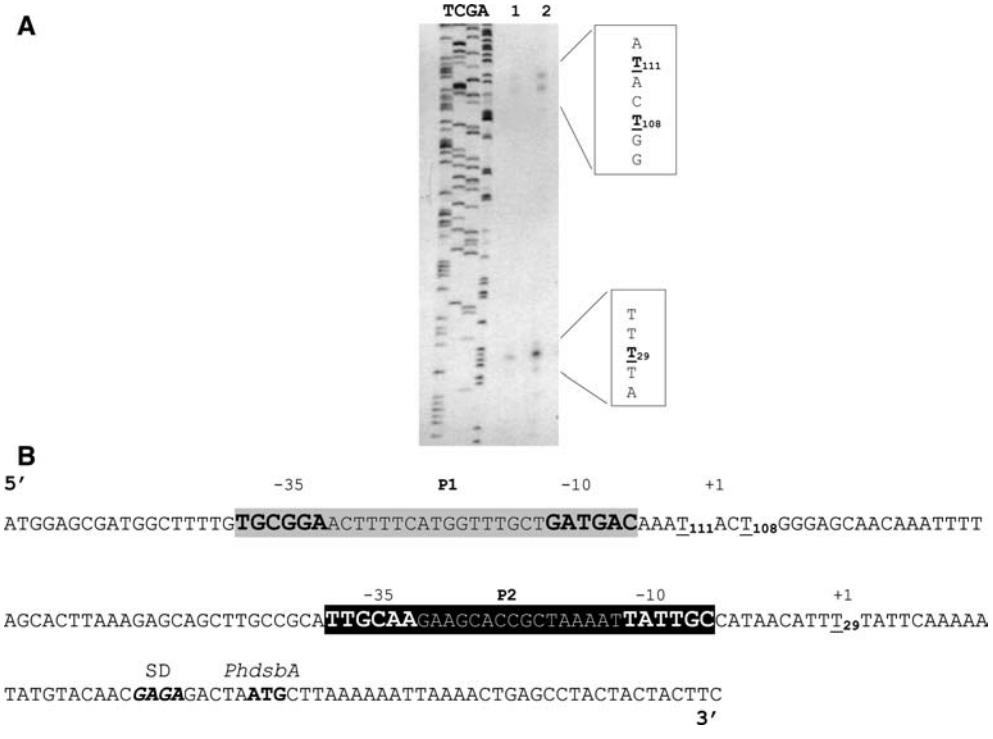

Fig. 3 **A** Primer extension analysis of the *Ph*dsbA transcript. A 18 bp oligonucleotide was annealed to 10 μg (*lane 1*) and 30 μg (*lane 2*) of *Ph*TAC125 total RNA and extended using AMV reverse transcriptase. The nucleotide sequence of the upstream region was determined using the same oligonucleotide as a primer (*lanes T, C, G* and *A*). **B** The putative P1 and P2 promoters are evidenced in *grey* and *black*, respectively. The corresponding −10 and −35 regions are in *bold*. The transcriptional start sites are *underlined*. The ribosome binding site (*SD*) is indicated as *italics*, while the *Ph*dsbA start codon is indicated as *bold*

The transcriptional mechanism of the *PhdsbA* locus was further investigated by transcriptional fusion experiments. DNA fragments immediately upstream of *PhyihE* and *PhdsbA2* coding regions (150 and 300 bp, respectively) were individually fused to a promoter-less *lacZ* gene contained in a pPLB plasmid (Duilio et al. 2004), generating the P(*yihE*) and P(*dsbA2*) vectors (Table 1). Both fusion vectors displayed significant β-galactosidase activity (Fig. 4A), compared to the control vector (pPLB), thus confirming the presence of promoter elements within the regions upstream of *PhyihE* and *PhdsbA2* genes.

The transcriptional activity of the *PhyihE* and *PhdsbA2* promoters was further investigated by monitoring β-galactosidase activity during the growth of recombinant *Ph*TAC125 cells. Figure 4A shows that P(*yihE*) transformed cells exhibited a poor promoter activity in the early growth phase, while, during the exponential phase (20–40 h), a rapid increase in β-galactosidase accumulation was observed, with maximum levels at the late exponential phase. As for P(*dsbA2*) transformed cells, β-galactosidase levels reached the maximum value during the stationary phase (Fig. 4A); in both cases, the enzyme levels were constant for at least 12 h. These experiments clearly showed that the regions upstream *PhyihE* and *PhdsbA2* are differently regulated, the former being much more active in the late exponential phase and the latter in the stationary phase.

Similar analyses were performed to investigate the activity of the two promoters (P1 and P2) located upstream of the *PhdsbA* gene. P1 and P2 promoter elements were cloned both together and individually into the pPLB plasmid, generating the P(*dsbA*), P($dsbA^a$) and P($dsbA^b$) plasmids, respectively (Table 1). The corresponding β-galactosidase activity was then measured in *Ph*TAC125 transformed cells during the growth phases, as shown in Fig. 4B. The promoter activity of the P1 region steadily increases up to a maximum value in the stationary phase, while the P2 region provided an essentially constant β-galactosidase activity during the cellular growth. The P(*dsbA*) plasmid, containing the whole promoter region, showed a transcriptional activity strongly growth-phase dependent: in the exponential phase, *Ph*TAC125 cells transformed with P(*dsbA*) exhibited a large increase in β-galactosidase activity reaching the maximum level in the late exponential phase.

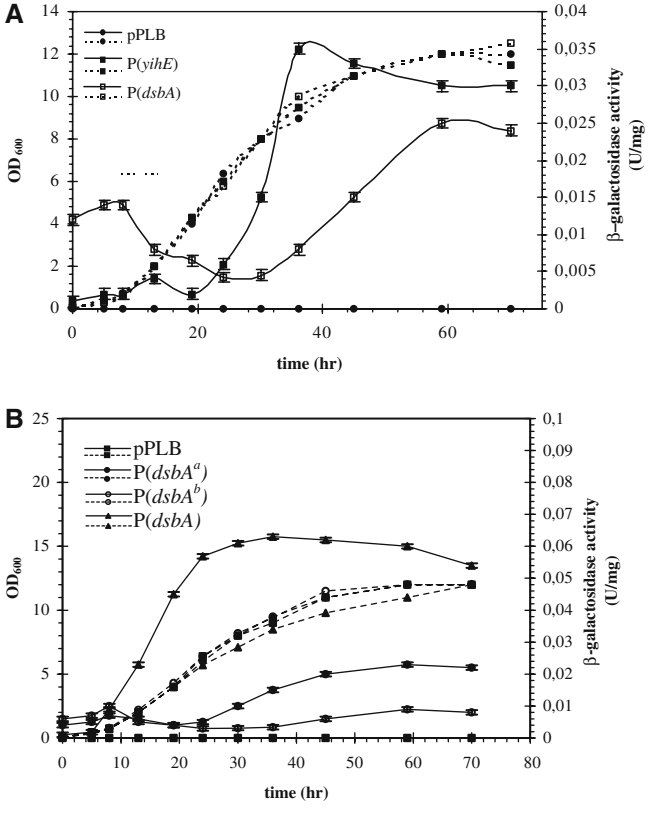

Fig. 4 Profiles of growth in aerated Typ cultures at 15°C and expression of *Ph*dsb::lacZ transcriptional fusion constructs in *Ph*TAC125. *Broken lines* indicate the growth curves, *solid lines* indicate the promoter activity. Each data point represents an average value from two samples. **A** pPLB (negative control), P(yihE) and P(dsbA2) cells. **B** pPLB (negative control), P(*dsbA*), P($dsbA^a$), P($dsbA^b$) cells.

*Ph*DsbA and *Ph*DsbA2 thermal stability

The *PhdsbA* and *PhdsbA2* genes were expressed in *E. coli* cells and the corresponding recombinant proteins purified from the periplasmic fractions by using the insulin reductase assay (Holmgren 1979), as described in the Materials and methods section. In both cases, ESMS analysis of the purified proteins showed the presence of a single component with a molecular mass of 20,808.8 ± 0.6 Da, and 21,450.4 ± 0.5 Da, for *Ph*DsbA and *Ph*DsbA2, respectively. These values were per se confirming the correct sequence of the recombinant proteins in agreement with the expected molecular mass of the mature proteins.

The thermal unfolding of *Ph*DsbA and *Ph*DsbA2 was investigated by means of CD measurements in comparison to *Ec*DsbA. The far-UV spectra are qualitatively similar (data not shown), thus suggesting a conserved secondary structure composition. Thermal unfolding of the "active" forms of DsbA was monitored by recording the molar ellipticity at 220 nm as a function of temperature (Fig. 5). Results are presented in Table 2, showing that *Ph*DsbA is less stable than *Ph*DsbA2 which is, in turn, less stable than *Ec*DsbA.

Preliminary analyses by differential scanning calorimetry on *Ph*DsbA and *Ph*DsbA2 revealed that the oxidized forms have denaturation points about 12–16°C lower than their reduced forms (data not shown). This is coherent with the thermodynamical mechanism of oxidative transfer of their disulfide bridge to the substrate protein in both cases, in agreement with the mechanism of *Ec*DsbA (Zapun et al. 1993; Moutiez et al. 1999).

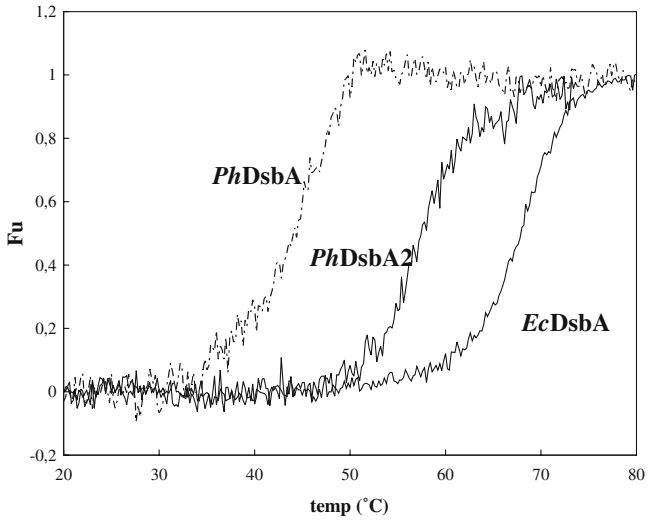

Fig. 5 The melting of secondary structure of DsbAs. Enzyme concentration was 10 μM in 50 mM Tris–HCl pH 7.0. Thermal transitions were followed at 220 nm as temperature was varied at a rate of 1°C min^{-1}. The fraction of unfolded protein was calculated from experimental data as described in the Materials and methods section

Table 2 Thermodynamic parameters of the thermal unfolding of oxidized DsbAs (*Ph*DsbA, *Ph*DsbA2, *Ec*DsbA), obtained by recording the molar ellipticity at 220 nm as a function of temperature

	T_d (°C)	$\Delta_d H(T_d)$ (kJ/mol)$^{-1}$
*Ph*DsbA (ox)	43.5 ± 0.4	4.4 ± 0.4
*Ph*DsbA2 (ox)	57.5 ± 0.5	3.3 ± 0.3
*Ec*DsbA (ox)	67.5 ± 0.3	4.5 ± 0.5

Cellular localization and functional characterization of *Ph*DsbA and *Ph*DsbA2 in *Ph*TAC125

The purified *Ph*DsbA and *Ph*DsbA2 proteins were used to produce-specific polyclonal antibodies in rat as described in the Materials and methods section. Western blotting analyses with anti- *Ph*DsbA and anti- *Ph*DsbA2 antibodies (Fig. 6) clearly showed that both proteins are actually produced by *Ph*TAC125, and totally translocated into the periplasm. Indeed, immunoreaction was only observed in the periplasmic fraction and not in the cytoplasmic portion.

With the aim of investigating the in vivo role of *Ph*DsbA and *Ph*DsbA2 proteins, we set up a complementation test taking advantage of the observation that *E. coli* cells harbouring null mutations in the *dsbA* gene have a pleiotropic phenotype as the correct folding of many proteins is affected. In particular, these mutants lack motility because of the improper assembly of the flagellar motor, due to incorrect disulfide bond formation in the P-ring protein (Dailey et al. 1993). We tested *Ph*DsbA and *Ph*DsbA2 proteins for their ability to restore the cellular motility of *E. coli* mutants.

The cold-adapted genes were PCR amplified, and separately cloned into pTRC99A expression plasmid. The resulting constructs, named pT(*PhdsbA*) and pT(*PhdsbA2*), were used for complementation assay of *E. coli dsbA*$^-$ strain JCB571. A construct containing the *EcdsbA* gene, named pT(*EcdsbA*), was used as positive control, and a non recombinant pTRC99A vector as negative control. As shown in Fig. 7, pT(*PhdsbA*) and pT(*PhdsbA2*) JCB571 recombinant cells (panels 3 and 4) exhibited a restored cellular motility on soft agar plates; panels 1 and 2 show the negative and positive controls, respectively.

A further complementation assay was performed in the *E. coli* strain JCB817, harbouring the *dsbA*:: *Km* null mutation, and encoding the MalF-LacZ102 fusion protein that confers a blue colour to bacterial colonies on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) (Bardwell et al. 1991). In this strain, due to the *dsbA* mutation the β-galactosidase fused protein is able to assemble into an active enzyme, yielding a Lac+ phenotype (blue colonies). In the presence of DsbA-like activity, β-galactosidase is enzymatically inactive (white colonies), since disulfide bond formation causes the fused protein to be entrapped in the cytoplasmic membrane. pT(*PhdsbA*) and pT(*PhdsbA2*) JCB817 transformed cells did not develop

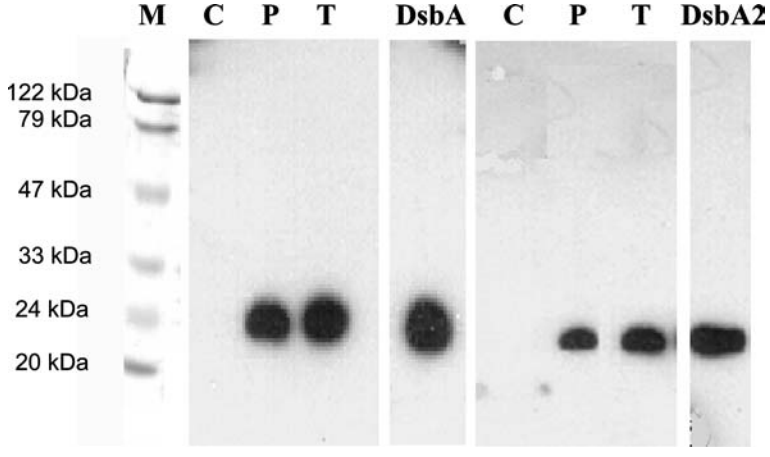

Fig. 6 Western immunoblot showing the cellular localisation of *Ph*DsbA and *Ph*DsbA2 proteins. *C* soluble cytoplasm fraction, *P* periplasmic fraction, *T* whole-cell lysate. DsbA purified protein, DsbA2 purified protein

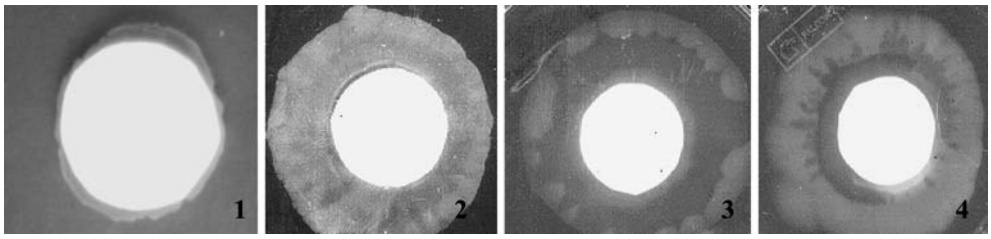

Fig. 7 Complementation assay of *E. coli* dsbA$^-$ strain JCB571. **1** non recombinant pTRC99A vector (negative control). **2** pT(EcdsbA) JCB571 recombinant cells (positive control). **3** pT(*Ph*dsbA) and pT(*Ph*dsbA2) JCB571 recombinant cells. **4** pT(*Ph*dsbA2) JCB571 recombinant cells

any blue colour, suggesting that both proteins were able to substitute for *Ec*DsbA. JCB817 transformed with the empty vector, used as negative control, readily developed a blue colour (data not shown).

Discussion

The present study on disulfide isomerases from the cold-adapted Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 provides some insights into disulfide bonds formation in a micro-organism isolated in the Antarctic sea water.

*Ph*TAC125 carries two adjacent chromosomal genes (*PhdsbA – PhdsbA2*), encoding two disulfide oxidoreductases belonging to the DsbA family, named *Ph*DsbA and *Ph*DsbA2. The expression of these two genes is controlled by a sophisticated transcriptional regulation mechanism. To the best of our knowledge, this is the first report of two genes coding for two DsbA-like proteins organised in a functional operon.

A comparison of the amino acid sequences of *Ph*DsbA and *Ph*DsbA2, showing an 33 and 36% identity with *Ec*DsbA, respectively, immediately gives evidence that they are characterized by different motifs of the catalytic site. The *Ph*DsbA contains the canonical –C–P–H–C– motif, highly conserved in other DsbA homologues proteins, while in *Ph*DsbA2 the His in the active site is substituted by an Ala (–C–P–A–C–). We infer *Ph*DsbA2 as a proper DsbA isoenzyme for several reasons: (i) a BLAST run gives DsbA with the highest score, (ii) since the protein is located within the periplasm, it has to be involved at some stage in the oxidative folding pathway, and (iii) finally, *Ph*DsbA2 is not a membrane protein, thus a DsbB or DsbD-like recycling role could be ruled out. Moreover, the protein is monomeric with a molecular mass of 21 kDa: therefore, it is not a DsbC or a DsbG-like protein, since both of them are dimeric (Zapun et al. 1993; Andersen et al. 1997). It is also worth mentioning that a proper homologue of DsbC is present in *Ph*TAC 125 (unpublished results).

Bardwell research group elegantly demonstrated, by in vitro experiments carried out on *Ec*DsbA (Guddat et al. 1997), the importance of the electrostatic contribution of the histidine residue, since mutants in the third position of the catalytic site are less efficient as oxidant than wild type enzyme. Therefore, we would suggest a lower oxidizing power for *Ph*DsbA2. It is worth mentioning that an easy spectroscopic determination of the redox potential of *Ph*DsbA2 is impaired by the absence of Trp125 residue (Sillen et al. 1999). Experiments aimed at determining this parameter by chromatographic analysis (Siedler et al. 1993) are currently in progress.

The occurrence of additional *dsbA* genes in bacteria is rare but well documented, such as in the case of *Shewanella oneidensis* (Entry names: Q8EB18 and Q8EAM7) and *Salmonella enterica* (Bouwman et al. 2003). However, the genes encoding these DsbA-like proteins are either scattered on the chromosome or located on extrachromosomal elements. Here we report the first direct evidence in a Gram-negative bacterium of a functional operon comprising the genes encoding two DsbA proteins.

We observed that the flanking regions of the *dsbA* genes are well conserved and have a high similarity with those of *Salmonella* strains and *E. coli*. Indeed, the presence of *yihE* immediately upstream of the *dsbA* gene is quite widespread among the Gram-negative bacteria (Suntharalingam et al. 2003), as revealed by a computational comparison of bacterial genomes.

Partial sequence data concerning the region downstream of the *PhdsbA2* gene revealed the presence of an ORF (ORFb), encoding a protein homologous to the fimbriae-associated adhesin proteins, generally involved in biofilm formation and in fimbriae assembly. A similar genetic organisation was observed in several *Salmonella* strains, where these DsbA-like proteins are involved in oxidation of specific components of the fimbrial system (Bouwman et al. 2003; Rodriguez-Penap et al. 1997).

Although any involvement of the ORFb in the adhesion of *Ph*TAC125 is still under investigation, this observation suggests a possible function for *Ph*DsbA2. This hypothesis is supported by the ability of *Ph*TAC125 to form biofilm at 4°C (data not shown).

A complex mechanism of transcriptional regulation in *Ph*TAC125 for this operon was highlighted. Northern blotting analysis demonstrated that these genes are transcribed as a tricistronic messenger including the *PhyihE* gene, probably under the control of a promoter region located upstream of it. Additionally, the *PhdsbA* gene can also be transcribed as two different bicistronic

messengers: the first including the *PhyihE* gene, and the second one including *PhdsbA2*. Moreover, the *PhdsbA2* gene is also transcribed as a monocistronic transcript. Two adjacent transcription start points upstream of *PhdsbA*, identifying two distinct promoters (P1 and P2), were located within the 3′-terminal region of *PhyihE*, as also observed in *Salmonella Typhimurium* (Goecke et al. 2002).

The P2 promoter can be classified as a constitutive σ^{70} -dependent promoter, resembling the σ^{70} consensus sequence of *Ph*TAC125 (Duilio et al. 2004). The P1 promoter shows significant differences with constitutive promoters, suggesting the possibility that it is controlled by alternative σ factors, possibly responsible for the transcription under specific conditions. These hypotheses are supported by transcriptional fusion analyses that revealed a fairly constant activity of the P2 promoter region during cellular growth, typical of the σ^{70} -dependent promoters. On the contrary, the P1 promoter region is growth-phase regulated, exhibiting maximum activity during the *Ph*TAC125 stationary phase. The whole promoter region exhibits a marked dependence on the cellular growth, with a maximum activity during the late exponential phase, suggesting a synergic effect of the two promoters.

Although no transcription start site was detected upstream of *PhyihE* and *PhdsbA2* genes, transcriptional fusion analyses clearly demonstrate the presence of two active promoters responsive to growth phase. Their activity increases during the late exponential and the stationary phases, suggesting their dependence on alternative σ factors.

This analysis revealed that *PhdsbA* and *PhdsbA2* expression clearly increases during the late exponential phase, in which oxidoreductase proteins are presumably required for folding of components involved in the physiological changes that occur during this cellular phase. It is possible to suppose that *Ph*TAC125 cells devise a fine-tuning of transcriptional control for each gene, according to different growth conditions and/or different extra-cytoplasmic stimuli.

Both *Ph*DsbA and *Ph*DsbA2 showed the ability to substitute for *Ec*DsbA in *E. coli* mutants lacking this enzymatic activity. These data indicated that both proteins display similar oxidoreductase abilities in vivo. When multiple genes are discovered for proteins with apparently identical functions, the genes are often described as redundant. Taking advantage of the *Pseudoalteromonas haloplanktis* TAC125 genome annotation (to be published), the identification of *Ph*DsbAs protein substrates may, in the near future, be approached by a combination of proteomic tools and site-directed random mutagenesis experiments (Kadokura et al. 2004).

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