Regulation of gene expression in psychrophilic micro organisms: molecular aspects and biotechnological applications

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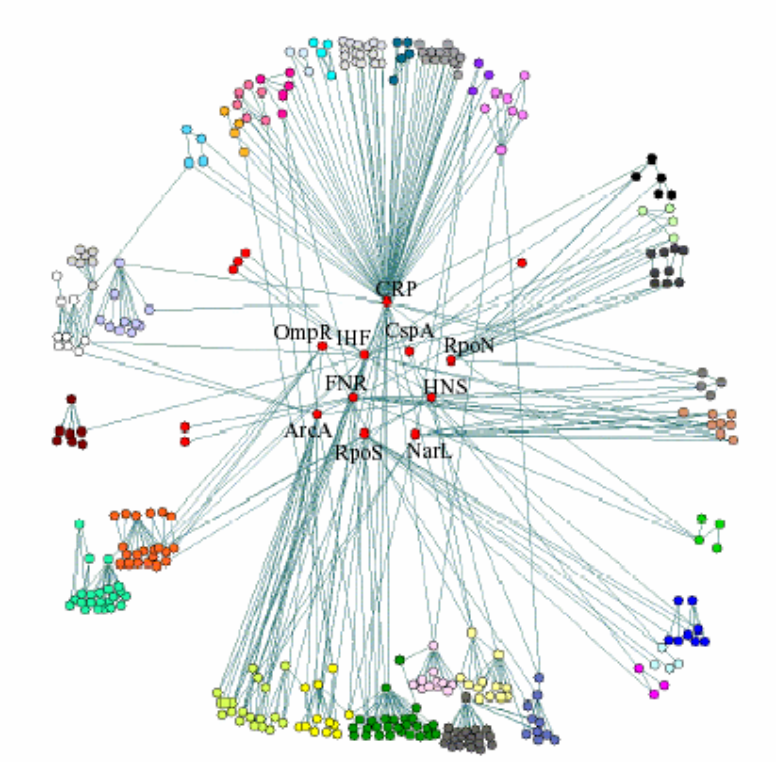
A mio marito

Genome sequencing and high-throughput technologies of functional genomics generate a huge amount of information about cellular components and their functions in an unprecedented pace. These advances make it possible to reconstruct large scale biological networks (metabolism, gene regulation, signal transduction, protein-protein interaction etc.) at a whole cell level. One of the key issues in the contemporary genomic biology is to understand the structure and function of these cellular networks at different molecular levels. Among them, the transcriptional regulatory network (TRN) plays a central role in cellular function because it regulates gene expression and metabolism and is often the final step of signal transduction.



Functional modules in the transcriptional regulatory network of *E. coli*. Operons in different modules are shown in different colors. The ten global regulators form the core part of the network. The periphery modules are connected mainly through the global regulators. Depending on the connectivity between the modules and their connectivity to the global regulators, these modules can be further grouped to larger modules at an higher level.

Hong-Wu Ma, Jan Buer and An-Ping Zeng (2004)
Hierarchical structure and modules in the *Escherichia coli* transcriptional regulatory network revealed by a new top-down approach
BMC Bioinformatics **5**:199

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Summary

An *in silico* analysis performed on the *Ph*TAC125 genome sequence (1) showed that this cold adapted microorganism does not possess the classical PTS (2), dedicated to the transport and modification steps needed for carbohydrates metabolism. Moreover the genes encoding key enzymes of the Embden-Meyerhoff pathway could not be identified. In contrast, it does contain the genes coding for the enzymes of the alternative metabolic Entner-Doudoroff pathway. In order to define the favourite carbon source for *Ph*TAC125 we analyzed its growth behaviour in the presence of four different carbon sources. Data obtained showed that L-malate is degraded from *Ph*TAC125 at a considerable rate during cellular growth. The analysis of the proteome of *Ph*TAC125 was performed and the differences in the protein expression profile of cells grown in minimal medium in the presence and in the absence of L-malate as carbon source were identified. A classification into functional groups of the proteins identified was carried out. Using this approach, a total of 31 proteins differently expressed in the presence of L-malate was identified. Our attention has been focused on the gene *PSHAb0363* coding for a putative outer membrane porin (named *Phdct*A). In fact, the upstream region of the gene coding for the putative outer membrane porin has a rather specific organization. Two CDSs encoding a putative C4-dicarboxylates sensor kinase and a putative C4 response regulator were respectively located upstream of the porin gene. Furthermore, a CDS coding for a periplasmic transporter of dicarboxylic acids (named *PhdctB*) was found downstream of the porin gene. This association strongly suggests the occurrence of a two-component sensor regulatory system. The transcriptional fusion experiments demonstrated that this regulatory element (named *Ph*MAlRS) is remarkably influenced by the presence of L-malate in the growth medium (3). The transcriptional regulation of the *PhMalRS* and *PhdctAB* loci was first investigated by RT-PCR experiments. The promoter element located upstream the *PhMalS* gene is only active in the presence of L-malate whereas the promoter located upstream the *PhdctA* gene is able to dictate transcription of *PhdctA* in both conditions but it is strongly induced by L-malate. On the contrary, the presence of L-malate appeared to be irrelevant on *PhdctB* transcription. The promoter region upstream *PhdctA* region was investigated by primer extension experiments. This analysis revealed two different transcriptional start sites: putative σ^{70} and a putative σ^{54} promoter element were also identified. Upstream the σ^{54} promoter element, a DNA region, that represents the *cis*-acting region requested for the binding of transcriptional regulatory proteins leading to the induction of the *PhdctAB*, was also identified.

In conclusion, we used the regulative region comprising the *Ph*MalRS system to construct an inducible expression vector, named pUCRP. We demonstrated that the inducible expression system was effective in the production of the psychrophilic β-galactosidase from *Ph*TAE79 and mesophilic α-glucosidase from *Saccharomyces cerevisiae*. The performances of the inducible and the constitutive system (4) for the

production of these proteins were also compared. Data presented demonstrated that when these proteins are produced in *Ph*TAC125, they are completely soluble and catalytically competent in both expression systems used.

In the second part of this PhD thesis, the attention has been focused on the utilization of microorganisms to reduce environmental contamination that occur in sea waters and in effluents of industrial processes which are characterised by low temperatures (5). With the aim to investigate the degradation of aromatic compounds at low temperatures the Antarctic psychrophilic bacterium *Ph*TAC125 was used as representative recipient for the biodegradative gene belonging to the upper pathway of *Pseudomonas stutzeri* OX1 coding for the toluene/*o*-xylene-monooxygenase (ToMO) (6). Moreover, a preliminary characterization of the recombinant strain is also reported (7). Recombinant TAC/*tou* cells were grown in a broad range of temperatures (4-25°C). An active rToMO, able to hydroxylate phenol to catechol, was detectable at all temperatures tested. Catalytic parameters of *Ph*-rToMO on phenol, *o*- and *p*-cresol were determined and compared with those determined for *Ec*-rToMO (8). Both *Ph*-rToMO and *Ec*-rToMO hydroxylate *p*-cresol more efficiently than *o*-cresol, which, on the other hand, is hydroxylated more efficiently than phenol. Catalytic parameters determined for *Ph*-rToMO are considerably lower than those determined for *Ec*-rToMO suggesting an effect of temperature on ToMO activity when the enzyme is produced in TAC/*tou* cells (9). TAC/*tou* cells grown at 15°C up to late exponential phase were used to assay rToMO activity on three different substrates as function of different incubation temperature: *Ph*-rToMO retains about half of its activity in a broad range of temperature (10-37°C). Data obtained support the possibility of developing specific degradative capabilities for the bioremediation of chemically contaminated marine environments and of industrial effluents characterised by low temperatures, using a naturally cold adapted bacterium. Further experiments will be performed with the aim of cloning the entire metabolic pathways necessary for the complete degradation of aromatic compounds.

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Riassunto

La regolazione dell'espressione genica in microrganismi adattati al freddo: aspetti molecolari ed applicazioni biotecnologiche.

1. Stato dell'arte

Uno dei principali obiettivi che le biotecnologie si propongono riguarda la produzione di proteine ricombinanti. La messa a punto di un sistema d'espressione alternativo, che sia in grado di assicurare un'efficiente produzione di proteine d'interesse biotecnologico la cui espressione risulta inefficiente nei sistemi convenzionali attualmente a disposizione potrebbe riscuotere notevole interesse. Da queste osservazioni nasce, dunque, l'idea di utilizzare i microrganismi adattati alle basse temperature come ospiti per l'espressione e la produzione ricombinante di proteine d'interesse biotecnologico (1,2). Tali organismi, inoltre, possono ricoprire un ruolo importante nell'ecologia degli ambienti freddi: infatti, potrebbero essere utilizzati, se opportunamente ingegnerizzati, in processi di biodegradazione di sostanze xenobiotiche come nella mineralizzazione di reflui contaminati da sostanze organiche (3).

2. Premesse scientifiche e metodologiche

Una delle tematiche di cui si occupa il gruppo di ricerca del prof. Marino e collaboratori riguarda lo studio di sistemi genetici in batteri antartici (4,5). Recentemente nel suddetto laboratorio sono stati isolati e caratterizzati alcuni promotori costitutivi, alcuni dei quali sono stati utilizzati per la costruzione di vettori di espressione alle basse temperature (6). Tuttavia per rendere il sistema più versatile è necessario avere a disposizione elementi in grado di modulare l'espressione del prodotto ricombinante d'interesse, come ad esempio promotori modulabili e proteine regolatrici.

Grazie alle informazioni derivanti dal sequenziamento del genoma di *Ph*TAC125 (7), è possibile oggi condurre uno studio sistematico volto sia alla comprensione dei meccanismi molecolari di adattamento alle basse temperature sia alla delucidazione degli elementi genici coinvolti nella regolazione dell'espressione genica.

3. Obiettivi e risultati attesi

Questo progetto si è proposto di studiare la regolazione dell'espressione genica nel batterio antartico *Ph*TAC125 mediante un approccio di tipo proteomico. L'identificazione e la caratterizzazione degli elementi di regolazione trascrizionale sono stati infine utilizzati per sviluppare nuovi strumenti genetici più versatili ed efficaci per l'espressione a basse temperature dei prodotti d'interesse.

Inoltre, essendo *Ph*TAC125 un batterio marino in grado di crescere e riprodursi in un ampio intervallo di temperatura (4-27°C), lo si è considerato l'ospite ideale per studiare e ampliare il campo applicativo di un enzima biodegradativo come la toluene *o*-xilene monoossigenasi, che rappresenta il primo enzima della via metabolica di degradazione degli idrocarburi aromatici in *Pseudomonas stutzeri* OX1 (8, 9).

4. Risultati conseguiti

Nel corso di questo progetto di dottorato l'attenzione è stata rivolta all'identificazione e alla caratterizzazione di alcuni sistemi di regolazione del catabolismo di *Ph*TAC125, al fine di isolare promotori regolati.

La ricerca di promotori regolati nell'organismo oggetto di studio è stata condotta mediante un approccio di tipo proteomico avvalendosi delle informazioni derivanti dal progetto di sequenziamento del genoma di *Ph*TAC125 (7). La strategia utilizzata ha previsto le seguenti tappe sperimentali:

- Formulazione di opportuni mezzi di coltura;
- Analisi dell'andamento di crescita del batterio nelle differenti condizioni;
- Analisi dei *pattern* proteici di espressione nelle differenti condizioni utilizzate;
- Identificazione delle proteine differenzialmente espresse;
- Isolamento degli elementi di regolazione trascrizionale (sequenze promotrici, siti di regolazione);
- Caratterizzazione strutturale e funzionale dei elementi di regolazione identificati;
- Costruzione di vettori di espressione inducibili.

Una volta definito un mezzo di coltura opportuno per l'organismo in esame si è proceduto ad analizzare l'andamento di crescita del batterio in presenza di quattro differenti fonti di carbonio: D-glucosio, D-fruttosio, D-xilosio e L-malato; in particolare la condizione prescelta per la successiva analisi dei *pattern* proteici è stata L-malato, poiché questo substrato era l'unico ad essere metabolizzato con una velocità apprezzabile da *Ph*TAC125. Successivamente, i *pattern* proteici estratti a differenti fasi della crescita batterica sono stati separati mediante elettroforesi bidimensionale e gli spot proteici visualizzati sono stati analizzati mediante spettrometria di massa MALDI. Per facilitare il confronto dei *pattern* proteici di *Ph*TAC125 in presenza e in assenza di malato è stato utilizzato il software DECODON basato sulla tecnica Dual Channel Imaging (10), la quale permette una rapida comparazione dei *pattern* proteici ottenuti in condizioni differenti mediante un'analisi colorimetrica.

Successivamente è stata condotta un'analisi mediante spettrometria di massa MALDI che ha permesso di identificare circa 300 proteine. Confrontando i *pattern* proteici ottenuti estraendo le proteine da crescite in presenza ed in assenza di L-malato sono state identificate circa 30 proteine differenzialmente espresse nelle due condizioni suddette. Successivamente sono state analizzate le regioni di DNA poste a monte dei geni codificanti alcune di queste proteine, regioni potenzialmente coinvolte nella trascrizione di queste ultime, al fine di identificare eventuali elementi di regolazione trascrizionale (sequenze promotrici, siti di regolazione, proteine regolatrici).

L'analisi funzionale delle potenziali regioni coinvolte nella regolazione trascrizionale dei geni differenzialmente espressi è stata condotta mediante esperimenti di fusione trascrizionale (6). In particolare, le regioni di DNA poste a monte dei geni codificanti alcune di queste proteine, potenzialmente coinvolte nella trascrizione di queste ultime sono state isolate e clonate in un vettore *promoter-less* a monte di un gene *reporter* codificante l'enzima β-galattosidasi (6). Gli esperimenti condotti hanno permesso di identificare una sequenza promotrice fortemente regolata

dalla presenza di L-malato nel mezzo di coltura. Tale regione è caratterizzata dalla presenza di un tipico sistema di regolazione a due componenti (11), la cui attivazione è strettamente correlata alla presenza nell'ambiente extracellulare di acidi C4-dicarbossilici (12).
Successivamente sono stati definiti, mediante caratterizzazione strutturale e funzionale, i determinanti molecolari coinvolti nella regolazione della regione genomica identificata. Infine, la regione identificata è stata utilizzata per sviluppare un sistema di espressione inducibile alle basse temperature e ne è stata verificata la versatilità nella produzione di due proteine ricombinanti d'interesse biotecnologico: la proteina psicrofila β-galattosidasi da *Ph*TAE79 e la proteina mesofila α-glucosidasi da *Saccharomyces cerevisiae*.
La seconda parte del progetto di dottorato è stata invece rivolta alla realizzazione di un microrganismo ricombinante capace di esprimere l'intera via degradativa di sostanze aromatiche ai fini di sviluppare un sistema applicabile nella decontaminazione di ambienti marini o comunque caratterizzati da basse temperature.
*Ph*TAC125, essendo un batterio capace di vivere in un ampio intervallo di temperatura, è stato considerato l'ospite ideale per l'espressione ricombinante del gene codificante la toluene *o*-xilene monoossigenasi (ToMO), il primo enzima della via degradativa dei composti aromatici nel batterio mesofilo *Pseudomonas stutzeri* OX1. Esperimenti condotti in precedenza avevano già dimostrato che l'enzima ToMO era efficientemente prodotto in *Ph*TAC125, ed in particolare ne era stata verificata l'attività su cellule ricombinanti (TAC/*tou*) cresciute a diverse temperature (4-15-25°C) in terreno ricco, utilizzando il fenolo come substrato (13, 14).
Nel corso di questo progetto di dottorato è stata verificata l'attività dell'enzima ToMO in cellule TAC/*tou* (*Ph*-rToMO) cresciute a differenti temperature (4°C, 15°C and 25°C) in terreno minimo usando il fenolo come substrato; è stata riscontrata attività a tutte le temperature analizzate a partire dalla media fase esponenziale fino alla tarda fase stazionaria.
Inoltre sono stati determinati i parametri catalitici dell'enzima ricombinante *Ph*-rToMO su differenti substrati (fenolo, *p*-cresolo e *o*-cresolo) i quali sono stati confrontati con quelli calcolati per l'enzima prodotto in *Escherichia coli* (*Ec*-rToMO). I dati ottenuti hanno messo in luce che *Ph*-rToMO conserva sostanzialmente le stesse caratteristiche dell'enzima prodotto in *E.coli*; tuttavia i valori numerici ottenuti per *Ph*-rToMO sono più bassi di quelli ottenuti per *Ec*-rToMO, probabilmente per un effetto dovuto alla differente permeabilità delle membrane dei due batteri ai composti aromatici.
Infine è stata determinata l'attività di ToMO su tre differenti substrati in funzione della temperatura di incubazione. I dati ottenuti hanno dimostrato che ToMO conserva circa il 40% della sua attività su *p*-cresolo e *o*-cresolo su un ampio intervallo di temperatura (10-37°C), mentre, per quanto riguarda le prestazioni dell'enzima calcolate utilizzando il fenolo

come substrato, esso conserva circa il 50% della sua attività tra 20 e 37°C e solamente il 20% di essa tra 5 e 20°C.

5. Prospettive

I risultati ottenuti nel corso di questo progetto di dottorato potranno essere utilizzati non solo per chiarire i meccanismi molecolari di adattamento della vita alle basse temperature, ma soprattutto per implementare le potenzialità dei sistemi genetici preesistenti nel batterio antartico *Pseudoalteromonas haloplanktis* TAC125.

Inoltre, la realizzazione di un microrganismo ricombinante in grado di esprimere una capacità monoossigenasica, supporta la possibilità di sviluppare organismi normalmente adattati a vivere in un ampio intervallo di temperature, in grado di degradare completamente i composti aromatici. Questi organismi così ingegnerizzati potrebbero essere utilizzati per la decontaminazione di siti inquinati caratterizzati da temperature relativamente basse come reflui industriali, fiumi, etc.

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PART 1

Regulation of gene expression in *Pseudoalteromonas haloplanktis* TAC125

1.1 Introduction

1.1.1 Regulation and environmental complexity

The complexity of an organism can be defined as regulatory circuitry: a reaction network that can involve transcription factors, promoters, enzymes, structural genes, functional RNAs and metabolites. Regulatory networks control activation of genes in development, in the cell cycle and in the activation of metabolic pathways (fig. 1) (McAdams, H.H. *et al.*, 2004).

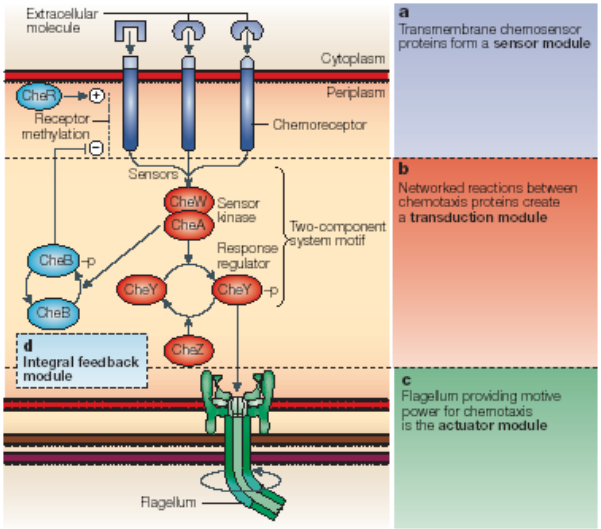

Fig. 1
Example of a bacterial regulatory circuit.
The figure illustrates the modular nature of the *Escherichia coli* chemotaxis network.
The sensor module includes several different chemoreceptors that are sensitive to different extracellular molecules. The transduction module comprises biochemical reactions between different chemotaxis molecules that create a pathway that communicates a signal to the distant flagella.
Because of its strong modular organization, the chemotaxis system is evolutionarily flexible, as shown by the diversity of chemical signals and response regulator functions that are found among motile bacteria.

Environmental changes can be regarded as random fluctuations over some frequency range, frequently overlaid on a trend (such as global warming) or on top of periodic patterns (for example, seasons or day/night cycles). Enteric bacteria, soil bacteria and other free-living bacteria live in complex environments and have correspondingly complex sensor–response–control subsystems (Cases, I. *et al.*, 2005). Surface-dwelling bacteria experience a broad distribution of timescales in the fluctuations in environmental parameters, such as broad annual temperature swings and the rapid changes in osmolarity that can occur within minutes following a sudden rainstorm. These bacteria have many environmental sensors and corresponding control circuits to invoke a wide variety of contingent responses. By contrast, obligate symbiotic bacteria live in a more constant host environment, and generally have both fewer genes and a simplified regulatory structure (Wilcox, J. L. *et al.*, 2003; van Ham, R.C. *et al.*, 2003).

Pre-existing pathways in the genome that can be quickly activated by sensor–response control systems provide contingent responses for these emergencies. The heat shock response and various metabolic-pathway activating responses are examples.

The bacterial kingdom provides many examples of the remarkable adaptability that results from evolutionary selection. These remarkable organisms have a wonderful diversity of behaviours, morphologies and

natural habitats, and their small size and rapid growth rates allow direct observation of evolutionary processes in the laboratory. The availability of many bacterial sequences and new genomic analysis techniques produce new and surprising discoveries every year that deepen our understanding of evolutionary mechanisms (McAdams, H. H. *et al.*, 2004).

1.1.2 Promoters in the environment: transcriptional regulation in its natural context

Transcriptional activation of many bacterial promoters in their natural environment is not a simple on/off decision. The expression of cognate genes is integrated in layers of iterative regulatory networks that ensure the performance not only of the whole cell, but also of the bacterial population, and even the microbial community, in a changing environment. Unlike *in vitro* systems, where transcription initiation can be recreated with a handful of essential components, *in vivo*, promoters must process various physicochemical and metabolic signals to determine their output. This helps to achieve optimal bacterial fitness in extremely competitive niches. Promoters therefore merge specific responses to distinct signals with inclusive reactions to more general environmental changes.

The growing availability of complete bacterial genome sequences has substantiated the idea that regulation of gene expression is the evolutionary response to the challenge of surviving in a changing environment (McAdams, H. H. *et al.*, 2004).

A gross but still useful simplification of this problem is to assume that transcription is the main component in the control of gene expression. An environmental signal can upregulate or downregulate the activity of many promoters. All the genes and gene clusters (operons) that respond to the same transcriptional regulator, which becomes competent for activation/repression of cognate promoters in response to a given environmental signal constitute a regulon. From a mechanistic point of view, regulons involve regulatory proteins that allow different promoters to respond to the same signal. All the genes and gene clusters that are expressed in response to a distinct physicochemical input, that typically involve the action of more than one transcription factor (more regulons) delineate a stimulon (fig. 2).

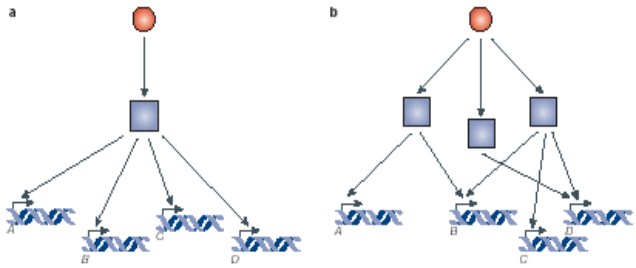

Fig. 2
Regulons and stimulons.
a. A single given environmental signal can act on many promoters to drive the expression of related gene clusters (*A*, *B*, *C*, *D* and so on) such that they are coordinately expressed, often through the same regulatory factor (or factors). This arrangement is known as a regulon.
b. In other cases, a given environmental signal triggers a response that is mediated by various regulatory elements that are sometimes not connected mechanistically. In these instances, the term 'stimulon' is preferred, as it refers predominantly to the phenomenon, not to the underlying mechanisms.

The extent to which nearly every gene in every bacterial genome is co-regulated by general

environmental changes is becoming increasingly apparent with the popularization of DNA-chip technologies, which frequently makes the comprehension of complex regulatory circuits a considerable challenge. The regulon concept is typically used in the laboratory, where many growth conditions can be kept constant, and nutritional and numerous environmental variables can be controlled. However, in their natural niches, microorganisms experience many different stresses and can be exposed to several nutrients that vary in availability with time. This is the second problematic aspect of signal integration in bacterial promoters. As shown in fig.2, specific promoters can then be influenced by many different inputs that, ideally, must be processed to ensure the optimal level of transcription.

1.1.3 The regulation of bacterial transcription initiation

The central component in transcriptional regulation in bacteria is the multi-domain DNA-dependent RNA polymerase, which is responsible for all transcription (Ebright, R.H. 2000). The main step in initiation is promoter recognition by RNA polymerase, and the different DNA sequence elements that are responsible for this have been extensively studied (Gross, C.A. *et al.*, 2000; Busby, S. *et al.*, 1994).

Distinct molecular mechanisms seem to ensure the distribution of RNA polymerase between competing promoters. These involve promoter DNA sequences, transcription factors, small ligands, σ factors and the folded bacterial chromosome structure. The different mechanisms are addressed, in turn, below, including how they are exploited to alter profiles of gene expression in response to environmental change. Each mechanism allows variation in the level of expression of genes.

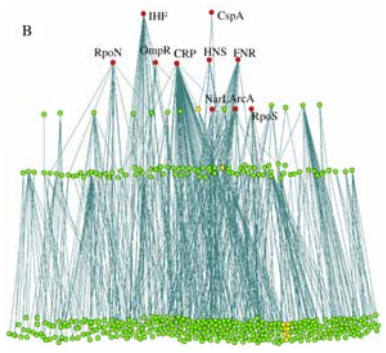

Fig. 3
Hierarchical structure of *E. coli* transcriptional regulatory network. The hierarchical regulation structure in which all the regulatory links are downward.
Nodes in the graph are operons.
Links show the transcriptional regulatory relationships. The global regulators found in this work are shown in red. The yellow marked nodes are operons in the longest regulatory pathway related with flagella motility.

Promoter sequences play a key role as powerful drivers in the unequal distribution of RNA polymerase between different transcription units. We know that promoters with near-consensus sequence elements function more efficiently. Many of the strongest bacterial promoters have effective upstream region (UP), that seem to function simply by binding to the carboxy-terminal domain of RNA polymerase α subunit (α CTD)(Gourse, R.L. *et al.*, 2000). Although differences in promoter sequence elements provide a useful way to control a wide range of promoter activities, these differences provide only static regulation that cannot normally be modulated according to environmental conditions. So, most adaptive regulation is due to modulation by *trans*-acting factors.

The *E.coli* genome contains more than

300 genes that encodes proteins that are predicted to bind to promoters, and to either up- or downregulate transcription (Perez-Rueda, E. *et al.*, 2000, Babu, M. M. *et al.*, 2003). Most of these proteins are sequence-specific DNA binding proteins, and this ensures that their actions are targeted to specific promoters. Some of these proteins control large numbers of genes, whereas other control just one or two genes (fig. 3).
It was estimated that seven transcription factors (CRP, FNR, IHF; Fis, ArcA, NarL and Lrp) control 50% of all regulated genes, whereas approximately 60 transcription factors control only a single promoter (Martinez-Antonio, A. *et al.*, 2003).
Transcription factors couple the expression of genes to environmental signals, and they must be regulated either by controlling their activity or by controlling their expression. Different mechanism are used to achieve this. First, the DNA binding affinity of transcription factors can be modulated by small ligands, the concentration of which fluctuate in response to nutrient availability or stress. Second the activity of some transcription factors is modulated by covalent modification. For example, some response regulator bind their target DNA only when phosphorylated by their cognate sensor kinase. The sensor kinases are located in the inner membrane, and are regulated by extracellular signals (Stock, A. M. *et al.*, 2000). Third, the concentration of some transcription factors in the cell controls their activity. In these cases, cellular concentration is determined either by regulation of expression of the transcription factor or by proteolysis.
Then, small molecules can look like ligands and provide an alternative mechanism by which RNA polymerase can respond quickly and efficiently to the environment. The best example is guanosine 3',5' biphosphate (ppGpp), which is synthesized when aminoacid availability is restricted to the extent that translation is also limited (Chatterji, D. *et al.*, 2001). ppGpp works by destabilizing open complexes at promoters that control synthesis of the machinery for translation (Barker, M.M. *et al.*, 2001; Barker, M.M. *et al.*, 2001).
The global pattern of gene transcription is believed to be determined through competition between available σ subunits and replacement of one RNA polymerase core enzyme-associated σ subunit by another should be the major determinant in switching of the global transcription pattern (Maeda, H. *et al.*, 2000). In *Escherichia coli*, seven different species of σ subunit are known to exist, each directing transcription of a specific set of genes with different binding affinity.
In a way, σ factors are at the top of the regulatory hierarchy because they determine the ability of the RNAP to recognize and bind certain promoters, which can then, in turn, be subject to further regulation by transcription factors. Therefore, they define 'transcription programmes' or sets of genes that have to be transcribed under certain conditions in an efficient way. Generalist bacteria are enriched in σ factors, especially in a particular class of extra-cytoplasmic function (ECF) factors, which respond to extra-

cytoplasmic stimuli, and which allow them to respond to a wide range of environmental inputs (Martinez-Bueno, M. A. *et al.*, 2002).

Regulation of alternative σ factor activity can be very complicated, involving transcriptional, translational and post-translational control. In many cases, the activity of σ factor is controlled by anti-sigma factor, which sequesters it away from RNA polymerase (Hughes, K.T. *et al.*, 1998). The anti-sigma factor is a negative transcriptional regulator that acts by binding to a sigma factor and preventing its activity.

All these system act together to control the sophisticated transcriptional machinery of a bacterial cell and to make the right protein products in the correct amount at the appropriate time.

1.1.4 The evolution of genetic regulatory systems in bacteria

The genomes of bacterial species show enormous plasticity in the function of individual genes, in genome organization and in regulatory organization. The field of evolutionary genomics has recently devoted considerable research effort towards understanding the evolution of coding region sequences and of genome organization — research that has been boosted by the availability of a large number of complete genome sequences. Less attention, however, has been devoted to explaining the evolution of the overall genetic regulatory circuitry that controls cellular functions.

Recent functional genomics (the use of genome-wide or system-wide experimental approaches to assess gene function) studies are beginning to address this topic (Cases, I. *et al.*, 2003).

The regulatory circuitry includes environmental sensors, sensors that reflect the internal state of the cell and a wide variety of signalling pathways. These signalling pathways comprise a network of protein-level reactions and genetic regulatory mechanisms that determines how the cell responds to the sensed conditions. It is interesting to know how the organization and mechanisms of this control system have evolved alongside genomic evolution. Key questions include the degree of plasticity of the regulatory network structure and how the complex, highly organized and biochemically based regulatory systems in cells emerged. For example, in *E. coli*, 22% of all regulatory interactions that involve paralogous genes are regulated by the same transcription factor (Babu, M. M. *et al.*, 2004; Teichmann, S. A. *et al.*, 2004).

1.1.5 Regulatory descriptors of bacterial lifestyles

As regulatory mechanisms and signal integration are key for the adaptation of bacterial species, there must be a relationship between the ecological niche that the bacterium occupies and the functional contents of its genome, in particular the proportion of genes that are involved in the transcription-regulation machinery. That larger genomes possess more transcription factors per gene than smaller genomes (Stover, C. K. *et al.*, 2000). As free-living bacteria that inhabit soil or aquatic environments usually have large genomes, it was suggested that microorganisms that face more variable niches devote more of their genes to control mechanisms. Similarly, it was also observed that intracellular pathogens and symbionts, which have undergone severe genome reduction - for

example, *Rickettsia* spp. or the aphid symbiont *Buchnera* (Andersson, J. O. *et al.*, 2001) - are deficient in transcription-related proteins. This strengthens the idea that microorganisms that have adapted to the stable conditions provided by the host have no use for regulatory mechanisms and, consequently, they are lost during evolution. As more genomes of bacteria from diverse phylogenies and ecological niches have become available (Ranea, J. A. *et al.*, 2004; van Nimwegen, E. 2003; Konstantinidis, K. T. *et al.*, 2004; Cases, I. *et al.*, 2003), more detailed analyses of this point have followed.

Species such as *Vibrio cholerae* or *Pseudomonas aeruginosa*, which were included in the pathogen group, are also mostly inhabitants of soil and water, and only rarely produce disease. Such versatility is reflected in their genome in the form of many regulators. On the other side, the genome of *Helicobacter pylori*, a pathogen that normally resides in the human stomach and has specialized in surviving the extremely acidic conditions that are present there, has a limited number of transcription factors.

Interestingly, the combined analysis of the metabolic content and the regulatory content of bacterial genomes provides a descriptor of the degree of specialization of the lifestyle of the bacterium. Bacteria that are highly specialized to a particular niche have a proportionally higher content of genes encoding enzymes, versus a lower number of genes encoding regulators. By contrast, generalist bacteria have a large number of genes coding for control-associated proteins and a proportionally lower number of metabolic genes (Konstantinidis, K. T. *et al.*, 2004; Cases, I. *et al.*, 2003). Another feature of genome comparisons is that the enzyme versus regulator lifestyle descriptor is particularly evident for some classes of transcription associated proteins, namely σ factors and two-component regulators.

In particular, two-component systems participate in a wide range of signal-processing mechanisms, from chemotaxis and flagellar movement to internal nitrogen availability or pathogenicity, and constitute an efficient way of transmitting a signal from the extracellular environment to the transcription machinery (Galperin, M. Y. 2004).

Nearly all bacteria (mycoplasmas are exceptions) encode multiple systems of this type for diverse signaling processes. There are also analogous signalling systems in cells of lower eukaryotes, including fungi, amoebae, and plants (Maeda, T. *et al.*, 1994; Wang, N. *et al.*, 1996; Wurgler-Murphy, S. M. and H. Saito. 1997).

A typical two-component regulatory system is comprised of a signaling histidine kinase (HK) (also called a sensor kinase) that is usually membrane associated and a cytoplasmic response regulator (RR) that is usually a transcription factor (an activator or repressor) (fig. 4). Similar systems control the expression of genes for nutrient acquisition, virulence, antibiotic resistance, and numerous other pathways in diverse bacteria.

Due to the involvement of these two-component systems in so many cellular processes, several reviews of them have now been published (for

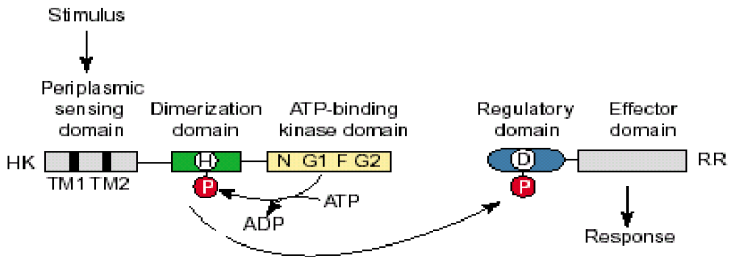

Fig. 4
Schematic organization of typical two-component regulatory system.

example review published by West AH and Stock AM, 2001). *E. coli* is thought to encode 31 different two-component regulatory systems, based on experimental evidence and protein sequence similarities (Zhou, L. *et al.*, 2003). The functions of many of these systems remain undefined. In addition, some cross-talk reactions have been described *in vitro* between several two-component systems (Arora, S. K. *et al.*, 1997). The biological meaning of such cross reactions *in vivo* is still debatable. It is tempting to speculate on the capability of these systems to integrate disparate signals by means of such cross talk. This would benefit generalist bacteria, as they would be able to sense a large number of different signals with a relatively compact set of genes (fig. 5).

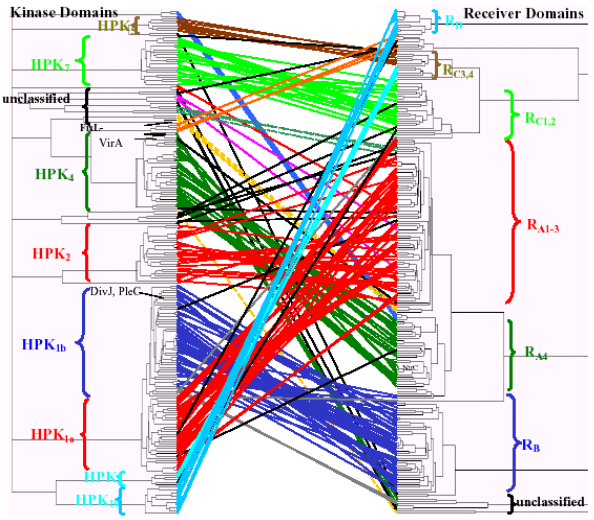

Fig. 5
Schematic representation of cross-talk reactions between several two-component systems.

Two-component regulators are composed of two elements, a signal-receptor protein, which undergoes auto-phosphorylation in response to a specific signal, and a regulator component, the activity of which is modified when phosphorylated using the signal-sensing component (fig. 4). The sensor component is normally structured in two domains: the phospho-transfer domain and the input domain, this last (often a trans-membrane domain) being the one that actually senses the signal. The regulator component also typically has two regions, the one that receives the phospho-group, and the output module, which can be a DNA-binding domain (Galperin, M. Y. *et al.*, 2001). While the domains that are involved in the phosphotransfer reaction are highly conserved, the input domain (the one that is involved in signal sensing) and the output domain (the activity that is modified) are highly variable.

This is another example of a useful regulatory element (a two-protein mechanism for fast point to- point signalling) that has maintained its internal interfaces while showing much plasticity in its interconnections to the different regulatory networks of its host.

Understanding how global and specific transcriptional regulation operates at the level of single cells or homogeneous bacterial populations is still a

challenge, but is becoming increasingly tractable with the rise of 'omics' technologies and network theory. In fact, new experimental methods allow the generation of an enormous volume of data on the control of gene expression, and bioinformatics and network theory allow the results to be presented in a comprehensible format.

1.1.6 Life at low temperatures

A considerable fraction of life develops in the sea at temperatures lower than 15°C. In 1887, Forster was the first to call attention to the growth and reproduction of bacteria at low temperatures by reporting that microorganisms isolated from fish could grow well at 0°C (Forster J., 1887). Since then, numerous organisms, prokaryotic but also eukaryotic, have been found to have successfully colonized low-temperatures habitats (for review, see Margesin, R. *et al.*, 2002). Evolution has allowed these cold-adapted organisms, called psychrophiles, not to merely survive, but to breed and grow successfully in the restrictive conditions of cold habitats. Psychrophiles display metabolic fluxes at low temperatures that are more or less comparable to those exhibited by closely related mesophiles living at moderate temperatures (Morita R.Y., 1975; Mohr P.W. *et al.*, 1980; Clarke, A. 1983; Feller G. *et al.*, 1994; Russell, N.J 2000; Deming, J.W., 2002), clearly showing that mechanisms of temperature adaptations are involved. Such mechanisms include a vast array of structural and physiological adjustments in order to cope with the reduction of chemical reaction rates induced by low temperatures.

Although low-temperature tolerant microorganisms were discovered long ago, limited information on the transcription machinery in cold adapted bacteria is still available (Cavicchioli, R. *et al.*, 2002). This knowledge represents a necessary background for the investigation of the adaptation of gene-expression mechanisms at low temperatures.

1.1.7 The marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

Pseudoalteromonas haloplanktis TAC125 (*Ph*TAC125) (Birolo, L. *et al.*, 2000) is a Gram-negative bacterium isolated from an Antarctic costal sea water in the vicinity of the French Antarctic station Dumont d'Urville, Terre Adélie (66° 40' S; 140° 01' E) during the European expedition IFREPOL in 1992. Although it was isolated from a permanently cold environment, *Ph*TAC125 is able to grow in a wide temperature range (4-25°C), and its lowest observed doubling time was detected at 20°C (31 min). This reason, together with many others, justify the interest on this psychrophilic bacterium. In fact, a) *Ph*TAC125 is the cold adapted bacterium so far characterized with the highest specific growth rate at temperature as low as 4°C; b) is able to reach very high cell density even in uncontrolled laboratory conditions; c) recently, a reliable gene transfer technology has been successfully established (Duilio, A. *et al.*, 2004). Due to the above properties, *Ph*TAC125 is considered a promising novel host system for the recombinant protein production at low temperatures. Indeed, the lowering of expression temperature is a common strategy applied when the recombinant products have the tendency to form insoluble aggregates (Baneyx, F. 1999), since low temperature minimize

the so called hydrophobic effect. The use of a cold adapted bacterium allows to perform the production at very low temperature, thus overcoming the physiologic limit observed for the growth of mesophilic hosts. *Ph*TAC125 contains a medium copy number plasmid, named pMtBL that was instrumental for the construction of first cold expression system (Tutino, M.L. *et al.*, 2001). Over the last years, *Ph*TAC125 was used for the production in soluble and active form of many proteins, which are not successfully produced in conventional mesophilic hosts.

Because *Ph*TAC125 was considered a likely candidate to study the molecular basis of physiological adaptation with the added value of being an interesting biotechnological tools, an European consortium recently has sequenced its genome (Medigue, C. *et al.*, 2005).

1.1.7.1 Genome organisation

Using genome sequencing, corroborated by in silico and in vivo analyses we have uncovered exceptional genomic and metabolic features of the γ-proteobacterium *Pseudoalteromonas haloplanktis* TAC125 as compared to other bacteria from aqueous environment (Table 1).

Table1: list of marine bacteria for which complete genomes are available– The *Psychrobacter* sp. genome has been found at the JGI web site (http://img.jgi.doe.gov/v1.0/main.cgi).

Species	Size (Mb)	Nb chr.	Division	Ecosystem	Temperature
Aquifex aeolicus	1.55	1	Aquificae	Near volcanoes or hot springs	Hyperthermophilic (growth at 95°C)
Thermotoga maritima	1.86	1	Thermotogae	Marine sediments	Hyperthermophilic (growth at 80°C)
Thermoanaerobacter tengcongensis	2.69	1	Firmicutes	Hot springs	Thermophilic (Range: 50°C – 80°C)
Vibrio vulnificus	5.21	2	γ-Proteobacteria	Surface sea water	Mesophilic (Range: 20°C - 40°C)
Shewanella oneidensis	4.75	1	γ -Proteobacteria	Lake or sea water	Mesophilic (Range : 4°C - 46°C) (Opt growth: 30°C)
Oceanobacillus iheyensis	3,63	1	Firmicutes	Deep sea	Mesophilic (Opt growth: 30°C)
Prochlorococcus marinus MED4	1.66	1	Cyanobacteria	Sea water / high-light-adapted ecotype (surface waters)	Mesophilic (Opt growth: 15°-20°C)
Photobacterium profundum	6.40	2	γ -Proteobacteria	Deep sea / high pressures	Psychrophilic (Range: 2°C - 20°C) (Opt growth : 15°C)
Desulfotalea psychrophila	3.66	1	δ- Proteobacteria	Cold marine sediments	Psychrophilic (growth at < 0°C)
Idiomarina loihiensis	2.84	1	γ -Proteobacteria	Cold water at the periphery of the vent waters	Psychrophilic (Range: 4°C - 46°C)
Silicibacter pomeroyi	4.11	1	α-Proteobacteria	See water / oceanic mixed-layer bacterioplankton	Mesophilic (Opt growth: 28°C)
*Psychrobacter sp. 253-4**	2.65	1	γ -Proteobacteria	Soil sea-ice/siberian permafrost core	Psychrophilic (Opt growth: unknown)
Pseudohalteromonas haloplanktis TAC125 (this study)	3.85	2	γ -Proteobacteria	Antarctic costal sea water	Psychrophilic (Range: < 0°C - 25°C) (Opt growth: ca 15°C)

As in many marine γ-proteobacteria, the *Ph*TAC125 genome is made of two chromosomes (fig. 6). The replication origin of chromosome I maps near *dnaA* (Lobry, J.R. *et al.* 2003; McLean, M.J. *et al.*, 1998) in a region that is highly conserved in γ-proteobacteria (fig. 6). However, in remarkable contrast with the genomes of the vibrios (Okada, K. *et al.*, 2005), the second chromosome does not display a standard GC-skew (fig.6). The pattern observed is likely to be caused by unidirectional replication. To the best of our knowledge, this is the first time that such a system would be uncovered in an authentic bacterial chromosome.

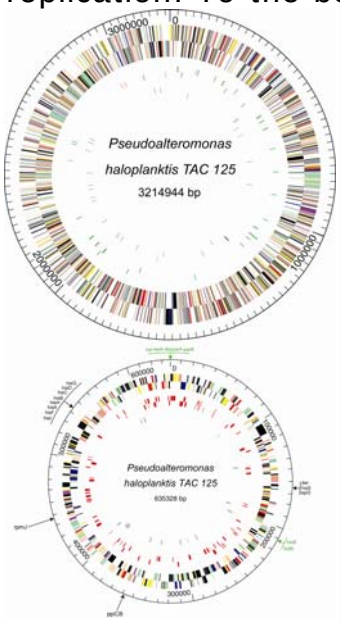

Fig. 6
Circular representation of the *Pseudoalteromonas haloplanktis* genome.
Circles display (from the outside): (1) predicted coding regions transcribed in the clockwise direction. (2) predicted coding regions transcribed in the counterclockwise direction. Genes displayed in (1) and (2) are color-coded according to different functional categories: salmon, amino acid biosynthesis; orange, purines, pyrimidines, nucleosides, nucleotides; purple, fatty acid and phospholipid metabolism; light blue, biosynthesis of cofactors, prosthetic groups and carriers; light green, cell envelope; red, cellular processes; brown, central intermediary metabolism; yellow, DNA metabolism; green, energy metabolism; pink, protein fate/synthesis; blue, regulatory functions; grey, transcription; teal, transport and binding proteins; black, hypothetical and conserved hypothetical proteins. (3) tRNAs (green) and rRNA (pink) on chromosome I / genes similar to phage proteins (red) on chromosome II, (4) and *tonB* and *tonB-like* genes in grey. Chromosome II gene names similar to that of the R1 plasmid replication apparatus (unidirectional) are colored in green.

The G+C content and gene density of chromosome II match that of chromosome I (Table 2). It contains the essential genes *hisS* and *gcpE*, in addition to a series of genes ubiquitous in γ–proteobacteria. A third of chrII genes have orthologs in *E.coli*. Remarkably, the whole metabolism of histidine is coded in chrII, in a highly conserved gene cluster (fig. 6). 19% of the *P.haloplanktis* chrII genes show high similarities with plasmid-encoded genes, further suggesting that this replicon was a plasmid recruited to become a chromosome encoding essential genes (fig. 6 and Table 2).

Table 2: General features of the *Pseudomonas haloplanktis* genome

	Chromosome 1	**Chromosome 2**
Size (bp)	3,214,944	635,328
G+C percentage	41.0	39.3
Number of predicted CDSs	2,942	546
Average size of CDSs (bp)	950	1013
Percentage coding	88.6	87.3
Number of rRNA operons (16S-23S-5S)	9	0
5S rRNA (extra copies)	1	0
Number of tRNAs	106	0
CDSs similar to known proteins	1123	157
Putative functions (limited homology/structural features)	759	251
Conserved hypothetical proteins	694	75
Orphan proteins	325	61
Doubful CDS and gene remnant	41	2
Similarities with genes from plasmid origin	10%	18.7%

Chromosome I codes for 9 rDNA clusters (23S, 5S, and 16S RNAs, one operon has two copies of the 5S RNA gene), a large number as compared to that found in most sequenced γ-proteobacteria (Ussery, D.W. *et al*., 2004). In the same way, the number of tRNA genes is quite high (106 genes), a feature in common with that in vibrios and in *Photobacterium profundum*. Because the speed of transcription/translation must be limited at low temperature, the large number of rRNA and tRNA genes may participate in the adaptation allowing fast growth of the organism in the cold.

In *Ph*TAC125 several genes relevant to adaptation to cold conditions are clustered together: genes coding for cold-shock proteins, nine paralogues of *cspA*, as in *E.coli* (four in chrII, three of them clustered together), as well as genes coding for a class of putative short secreted proteins that could bind calcium, next to a divalent metal exporter system, most likely used in calcium export. Calcium is known to be involved in cold adapation and formation of exopolysaccharides (EPS) in bacteria (Dominguez, D.C. 2004; Kierek, K. *et al.*, 2003).

1.1.7.2 Metabolic features.

Marine bacteria are facing a medium generally unbalanced in terms of carbon, nitrogen, and phosphorous supply (Moran, M.A. *et al.*, 2004) but not depleted in sulfur sources. Strain TAC125 is adapted to fast growth, suggesting that it regularly encounters a fairly rich medium (this is probably due to its propensity to make a water/air biofilm (see below) allowing it to live in region full of plankton debris). Excess of several easily metabolized carbon sources present simultaneously is unlikely, making catabolite repression the exception rather than the rule. Indeed, *P.haloplanktis* TAC125 is lacking the cAMP-CAP complex that regulates carbon availability in related organisms such as vibrios and *Shewanella*. Furthermore, in contrast to many γ-proteobacteria (including vibrios), it does not possess a phospho*enol*pyruvate-dependent phosphotransferase (PTS) system for the transport and first metabolic step of carbohydrate degradation (fig. 7).

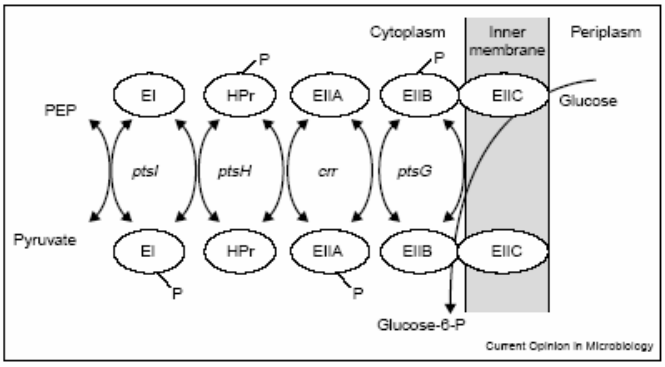

Fig. 7
The PTS phosphorylation cascade the five conserved PTS domains, as exemplified by the glucose PTS in *E.coli*, are shown. Enzyme I (EI) and HPr are common to most sugars and are encoded by the *ptsI* and *ptsH* genes, respectively. The Enzyme II components are sugar-specific transporters with three domains, EIIA, EIIB, and EIIC, EIIA and EIIB are soluble proteins, whereas EIIC is an integral membrane protein. In the process called vectorial phosphorylation, the phosphate, supplied by phospho*enol*pyruvate (PEP), passed through the chain of proteins and is eventually transferred to the incoming sugar from the EIIB domain as it passes through the pore created by the EIIC domain.

The PTS permits either the translocation with concomitant phosphorylation of a variety of sugars or amino-sugars while regulating carbon metabolism as a whole in response to their availability (fig. 8).

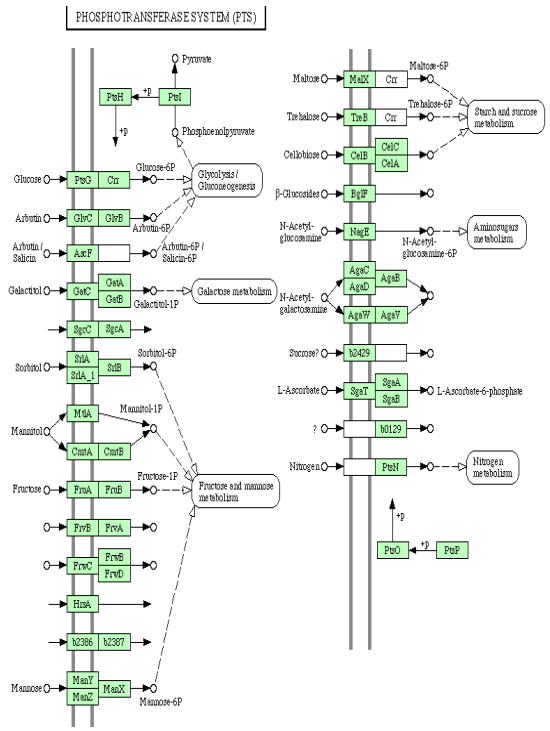

Fig. 8
Schematic representation of sugars and amino-sugars that enter in the bacterial cell through the PTS system.

An essential step for biomass construction is formation of pyruvate which, because of the absence of the PTS, must go through an alternative pathway starting with pyruvate kinase. In contrast to *E. coli*, with two such enzymes, and vibrios, with three, TAC125 possesses only one pyruvate kinase. Interestingly, it is homologous to the cold-adapted PykA enzyme of *E. coli*. This is further in line with gluconate as a preferred carbon source, providing pyruvate directly through the Entner-Doudoroff pathway (Edd, Eda) (fig. 9).

The metabolism of nitrogen appears to be highly similar to that in phylogenetically related organisms, and be controlled by a phosphorylation cascade involving PtsP (an homologue of the PTS enzyme PtsI) that phosphorylates PtsO and the regulator PtsN, controlling all sigma54 dependent operons (a typical two component regulatory system). Arginine catabolism could provide a direct source of ammonia under nitrogen-limiting conditions while providing metabolites for adapation to cold (Schneider, B.L. *et al.*, 1998). The organism can metabolize N-acetyl-glucosamine, a carbon and nitrogen source ubiquitously present in marine environment (Riemann, L. *et al.*, 2002). In the same way, phosphate input in metabolism is controlled by the counterparts of PhoB, PhoR and PhoU, with several putative transport systems, including one of high affinity.

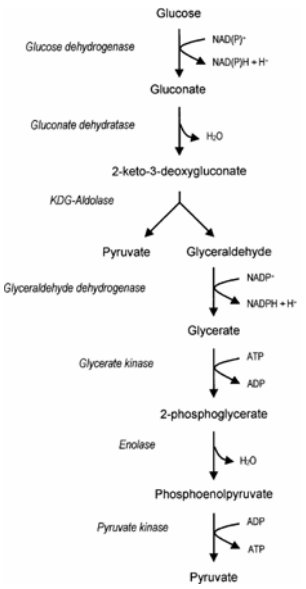

Fig. 9 The Entner-Doudoroff pathway.

1.1.7.3 Growth, yield and adaptation to salt.

A remarkable feature of TAC125 is that, when provided with sufficient nutrients and aeration, it grows to very high density under laboratory settings, even at 0°C. The very high growth yield indicates that respiration must be particularly efficient in this bacterium. The cells are well adapted

to salt, and, although they can grow in low osmolarity media, optimal growth is between 1.5 and 3.5 % NaCl.
Genes for the synthesis of polar flagellum are present, and these appendages are indeed visible under the microscope. In salty water the cells are highly motile. However, in contrast to the situation with several vibrios, pseudomonads and related bacteria, the cells have a reduced motility in low salt media, while they still grow well under such conditions. In minimal medium, the strain grew in a large range of NaCl concentration (0% to up 11% NaCl). On rich media however, the growth of the strain is remarkably sensitive to the presence of salt. In fact, at 20°C no growth occurs in the absence of NaCl. In remarkable contrast, slow but significant growth is observed at 4°C in the absence of salt, suggesting some adaptation to ice or melting ice water. Interestingly the shape of the cells under various salt conditions is extremely variable, with cells with a small volume under high salt and a larger volume (both in diameter and length) under low salt conditions. Taken together these observations indicate fine tuning of growth and form by availability of salt, with concomitant adaptation to cold.

1.1.7.4 Regulation of gene expression in *Ph*TAC125

Limited information about the transcription machinery of *Ph*TAC125 is still available. This knowledge rapresents a necessary background for the investigation of the adaptation of gene-expression mechanisms at low temperature. The development of a shuttle genetic system (Tutino, M.L. *et al.*, 2001) for the transformation of the cold-adapted Gram-negative bacterium *Ph*TAC125 has made possible the isolation of the psychrophilic promoters and the definition of housekeeping promoters consensus sequence (Duilio, A. *et al.*, 2004).
Nevertheless no information about regulated promoters and the specific mechanisms involved in modulating their activity has obtained so far.
An in silico analysis performed on *Ph*TAC125 genome sequence revealed the presence of a large number of CDSs coding for typical two-component regulatory systems (Table 3), suggesting, also for *Ph*TAC125, a fine regulation depending by external environmental conditions.
This ascertainment seems to be in contrast with the fact that complex regulative mechanisms are commonly characteristic of free-living bacteria rather than of bacteria

Table 3: Putative two-component regulatory systems in *Ph*TAC125 identified on the basis of similarities with other bacterial species.

CDS	*System*
PSHAb 0012 PSHAb 0013	cusRS
PSHAb 0161 PSHAb 0162	Metal resistance
PSHAb 0361 PSHAb 0362	C4-dicarboxylates regulation
PSHAa 0163 PSHAa 0164	phoBR
PSHAa 0597 PSHAa 0598	ntrCB
PSHAa 1282 PSHAa 1283	pho QP
PSHAa 1591 PSHAa 1592	uhp AB
PSHAa 2195 PSHAa 2196	bae SR
PSHAa 2409 PSHAa 2408	yea NM
PSHAa 2849 PSHAa 2850	envZ, ompBR
PSHAa 0551 PSHAa 0375	cpx AR, cpx CR
PSHAa 1404 PSHAa 1405	Unknown
PSHAa 0737 PSHAa 1916	sir AS, bar A, uvr Y
PSHAa 0853 PSHAa 0854	rst AB urpT uspT
PSHAa 0860 PSHAa 0861	rst AB
PSHAb 0275 PSHAa 2380	Chemiotaxis specific methylesterase
PSHAa 2275	Histidine kinase
PSHAa 0810	Histidine kinase
PSHAa 0913	Histidine kinase
PSHAa 1150	Histidine kinase
PSHAa 2259	Histidine kinase

that inhabit stable niches (as marine and Antarctic environments). *Pseudoalteromonas haloplanktis* TAC125 has found much unexpected solutions to cope with cold. In fact, it grow fast under such conditions and this makes this bacterium not only a model for the study of adaptation to cold marine conditions, but also a promising tool for biotechnology production of proteins.

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1.2 Results and Discussion

Proteomic identification of a two-component regulatory system in *Pseudoalteromonas haloplanktis* TAC125

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Running title: Identification of a two-component system in *Ph*TAC125

Keywords: Psychrophiles, Carbon source response, Two components regulatory system

Summary
The capability of microorganisms to utilize carbohydrates reflects the availability of these substrates in their habitat. To support growth and cell survival, the induction of specific metabolic enzymes, carbohydrates transporters, and uptake systems is required. We analyzed the growth behaviour and the protein patterns of a marine Antarctic bacterium, *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125), grown in a minimal medium both in the presence and in the absence of four different carbon sources. Expression profiling of the proteome showed the occurrence of several differences in the protein expression profile of *Ph*TAC125 in the presence and in the absence of L-malate. Our results demonstrate, for the first time, the presence of a functionally active two-component regulatory system for sensing the availability of C4-dicarboxylic acids in the Antarctic bacterium *Ph*TAC125.

1. Introduction
The ability of microorganisms to utilize carbohydrates likely reflects the availability of these substrates in their habitat. Different sugars, provided to bacteria as single source of carbon and energy, require the induction of different uptake systems and metabolic enzymes to support growth and cell survival (Cases, I. *et al.*, 2005). Exploration of the repertoire of carbohydrate-related proteins, in parallel with analysis of the regulation of their expression is likely to reveal much about relevant metabolic features of the organism while providing information about the interactions within its ecosystem.
Bacteria utilize different transport mechanisms for the uptake of solutes: facilitated diffusion, active transport driven by ATP or ion-gradients and group translocation. Group translocation of carbohydrates, such as D-glucose and D-fructose, is mediated by the bacterial phospho*enol*pyruvate-dependent phosphotransferase system (PTS) (Kornberg, H.L. 2001; Tchieu, J.H. *et al.*, 2001).The PTS permits either the translocation with concomitant phosphorylation of a variety of sugars or amino-sugars while regulating carbon metabolism as a whole in response to their availability.

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We analyzed the growth behaviour and the proteome of the marine Antarctic bacterium, *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) (Birolo, L. *et al.*, 2000), when grown in a minimal medium both in the presence and in the absence of four different carbon sources: D-glucose, D-fructose, D-xylose and L-malate.

Data presented in this article show that *Ph*TAC125 i) is unable to grow on fructose and xylose; ii) glucose is metabolized very slowly; whereas iii) L-malate is degraded at a considerable rate during cellular growth. Expression profiling of the proteome has been exploited to identify differences in the protein expression profile of *Ph*TAC125 cells grown in minimal medium in the presence and in the absence of L-malate as carbon source. Bacterial proteins differently expressed in the two conditions were identified on the basis of the genome sequence (Medigue, C. *et al*., 2005). These results demonstrate that *Ph*TAC125 possesses a functionally active two-component regulatory system for sensing C4-dicarboxylic acids in the environment. To the best of our knowledge this is the first report on the occurrence of such a system in Pseudoalteromonads.

2. Results and discussion

2.1 Carbon source response of *Ph* TAC125

An *in silico* analysis performed on the *Ph*TAC125 genome sequence (Medigue, C. *et al*., 2005) showed that, in contrast to many γ-proteobacteria (including vibrios), this cold adapted microorganism does not possess any member of the classical PTS (Kornberg, H.L., 2001; Tchieu, J.H. *et al.*, 2001; Plumbridge, J. 2002; Siebold, C. *et al.*, 2001), dedicated to the transport and modification steps needed for carbohydrates metabolism. Moreover the genes encoding key enzymes of the Embden-Meyerhoff pathway could not be identified. In contrast, the *Ph*TAC125 genome does contain the genes coding for the enzymes of Entner-Doudoroff pathway, an alternative metabolic pathway that accounted for the marked preference of the bacteria for gluconate as carbon source instead of glucose.

This observation led us to investigate the *Ph*TAC125 response to different carbon sources. Bacteria were grown at 16°C in minimal marine salt medium supplemented with a complex amino acids mix in the presence and the absence of D-glucose, D-fructose, D-xylose and L-malate respectively. The concentration of supplemented amino acids was carefully selected to support a cellular growth able to produce the amount of protein extract needed for further proteomic analysis, even in the absence of other carbon source. The growth behaviour of *Ph*TAC125 is shown in Fig 1, together with the consumption of individual carbon sources. *Ph*TAC125 is unable to degrade D-fructose and D-xylose, the concentration of both carbon sources in the medium being virtually unchanged after 50 hours. Interestingly, D-glucose was very slowly metabolized by *Ph*TAC125; after 50 hours only 40% of this substrate was actually used. In contrast, L-malate was metabolized very efficiently by *Ph*TAC125 cells. (Fig. 1).

The data are fairly in agreement with the *in silico* observations in that the absence of a classical PTS accounted for the inability of *Ph*TAC125 to grow on fructose and for the poor growth in the presence of glucose. Preliminary experiments have shown that addition of tyrosine very much improves the growth of *Ph*TAC125 on glucose. Therefore it might well be that the observed glucose uptake is due to tyrosine present in the supplemented amino acids mix. The latter aspect requires further investigation.

As a case in point, the *Ph*TAC125 genome does not seem to possess the *xyl* genes responsible for the expression of proteins specifically involved in the

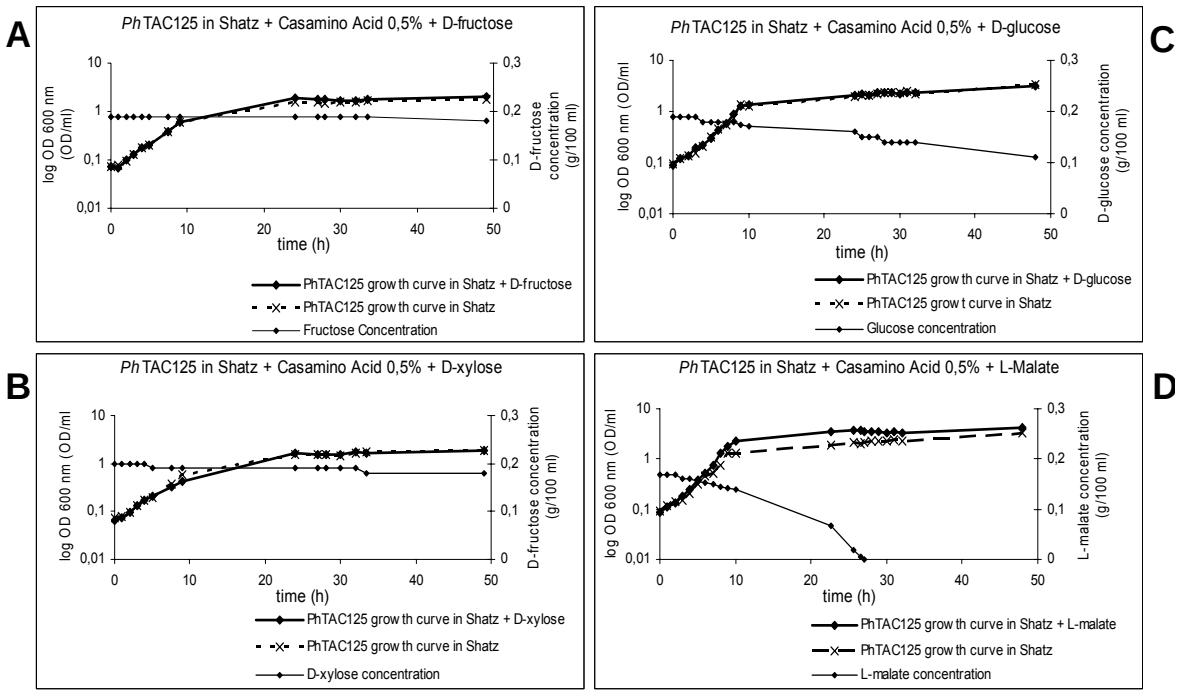

Figure 1
*Ph*TAC125 growth behaviour at 15°C in minimal medium supplemented with casamino acids.
A: Growth curve in the presence or in the absence of D-fructose and the consumption of D-fructose in the medium during cellular growth.
B: Growth curve in the presence or in the absence of D-xylose and the consumption of D-xylose in the medium during cellular growth.
C: Growth curve in the presence or in the absence of D-glucose and the consumption of D-glucose in the medium during cellular growth.
D: Growth curve in the presence or in the absence of L-malate and the consumption of L-malate in the medium during cellular growth.

uptake and metabolism of D-xylose (Song, S. *et al.*, 1997).

In contrast, two genes presumably coding for malic enzymes were identified in the *Ph*TAC125 genome. These enzymes play a key role in allowing cellular growth on L-malate, a key intermediate of Krebs' cycle (Iwakura M. *et al.*, 1979). These results match the *in silico* analysis of the genome sequence (Medigue, C. *et al.*, 2005).

2.2 Intracellular protein map of *Ph* TAC125

Proteomic investigations allow analysis of the protein pattern of a cell, while comparing the proteins expressed in different conditions. The protein profile of *Ph*TAC125 grown in minimal medium was constructed using two-dimensional gel (2-D) electrophoresis (Fig. 2A). A pH 4-7 IPG gradient was chosen for isoelectric focusing followed by a second dimension on SDS-PAGE. All protein spots stained by Colloidal Coomassie blue G-250, were excised from the gel and digested with trypsin. Proteins were identified by Matrix-Assisted Laser Desorption Ionization time-of-flight (MALDI-TOF) mass spectrometry of the corresponding peptide digests using an in-house database developed from the genome sequence of *Ph*TAC125 (Medigue, C. *et al.*, 2005).

Approximately 270 protein spots were identified in the gels. A classification into functional groups of the proteins identified so far on the basis of the genome annotation (Medigue, C. *et al.*, 2005) is shown in Table 1 (Appendix 1, supplementary materials cap. 2.1). Among these, approximately 40 enzymes involved in glycolysis and the TCA cycle, 36 enzymes involved in amino acid metabolism and 10

aminoacyl-tRNA synthetases were identified. Moreover, approximately 60 proteins involved in cell envelope and membrane-associated cellular processes were detected, some of them associated to the cell wall and others involved in membrane bioenergetics and motility. Only 10% of those were reported as unknown in the genome annotation.

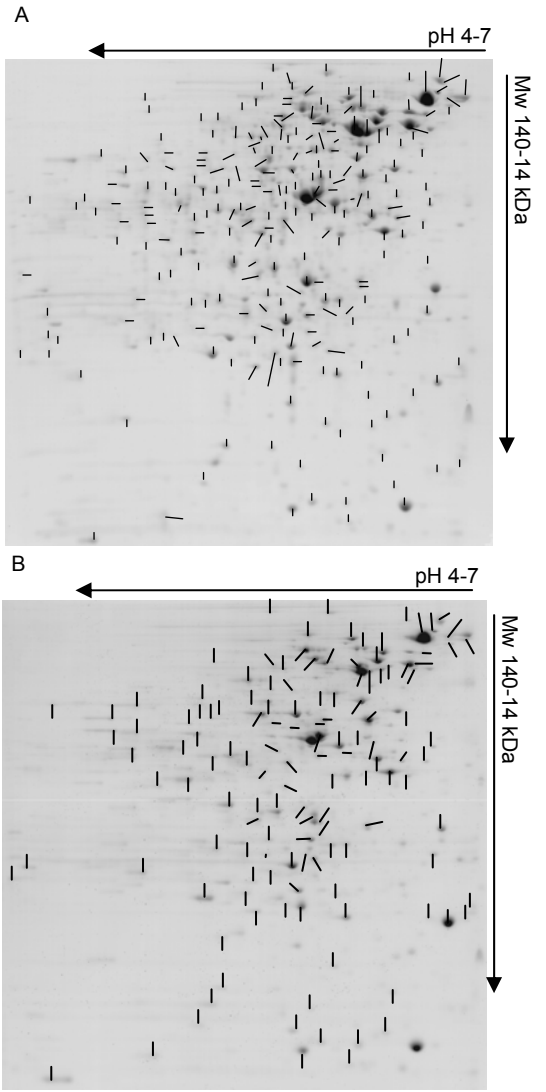

Figure 2
2D-PAGE of the intracellular protein fraction of *Ph*TAC125 during growth on minimal medium (A) in the absence and (B) in the presence of L-malate. A pH 4-7 IPG gradient was chosen for isoelectric focusing. Protein spots were stained with colloidal Coomassie blue G-250.

Using this approach, a total of 31 proteins differently expressed in the presence of L-malate was identified (Fig. 3, Table 2). Approximately 30% of them were proteins involved in membrane associated processes such as transport and binding of proteins and lipoproteins or chemotaxis and motility events. Approximately 45% of all proteins were metabolic enzymes but only 16% of them are directly involved in the metabolism of carbohydrates and related molecules.

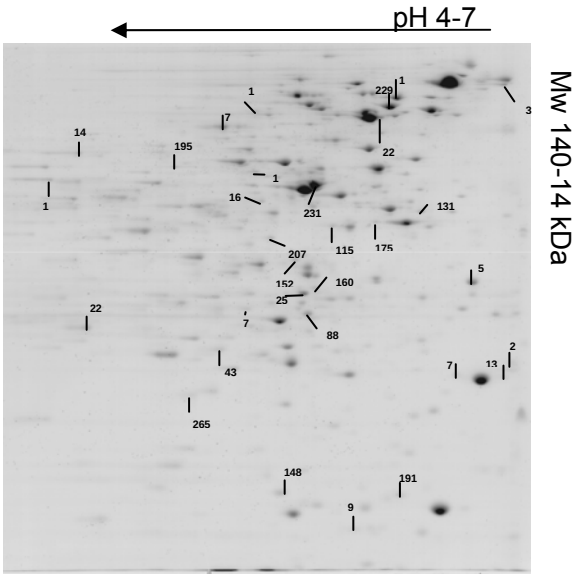

Figure 3
Two-dimensional electrophoresis map of soluble proteins expressed by *Ph*TAC125 during growth on minimal medium supplemented with casamino acids in the presence of L-malate. Differentially expressed proteins are numbered according to Table 1(supplementary materials).

Among all the proteins identified, six proteins were selected for further transcriptional analysis as these proteins were expressed in particularly high amounts in the presence of L-malate.

2.3 The transcriptional analysis of the genes coding for the differentially expressed proteins of *Ph*TAC125.

Table 3 shows the features of the six proteins that, considering their relative abundance in the gel, were chosen for the transcriptional analysis. Three of these are key enzymes of the metabolism of carbohydrates and related molecules, and the remaining are involved in transport/binding of proteins and lipoproteins and motility processes. According to the literature, the genes coding for these proteins are all transcriptionally regulated (Cai, J. *et al.,* 1997; Cunningham, L. *et al.*, 1997;

Davies, S.J. *et al.*, 1999; Hayashi, M. *et al.*, 2002; Kromer, J.O. *et al.*, 2004; Lai, E.M. *et al.*, 2004; Shen, L. *et al.*, 2004; Thompson, D.K. *et al.*, 2002; Tang, Y. *et al.*, 2004); therefore their differential expression might be ascribed to the activation of transcriptional stimuli induced by the presence of L-malate.

Table 2

Pseudoalteromonas haloplanktis proteins expressed only from cells grown in the presence of L-malate. Proteins were clustered into functional groups according to the genome annotation [5].

Spot	Function	pI	MW (kDa)	Accession number
	1. Cell envelope and membrane-associated cellular processes			
14	putative enzyme with a phosphatase-like domain	6,52	10,2	PSHAa2478
15	Na(+)-translocating NADH-quinone reductase subunit alpha	5,52	48,6	PSHAa2241
33	putative TonB-dependent receptor	4,38	92	PSHAa1840
13	putative outer membrane porin	4,55	34,4	PSHAa2567
22	Iron-regulated outer membrane virulence protein homolog	4,81	73,3	PSHAb0251
26	outer membrane protein W (OmpW) family	4,98	23,5	PSHAa2155
25	putative outer membrane porin	4,98	38,4	PSHAb0363
52	putative flagellin (FliC-like)	4,64	40,2	PSHAa0782
43	Ubiquinol-cytochrome c reductase, iron-sulfur subunit	7,7	20,9	PSHAa2530
	2. Intermediary metabolism			
62	bifunctional protein [Includes aconitate hydratase]	4,96	93,9	PSHAa0184
73	Succinate dehydrogenase flavoprotein subunit	5,84	64,6	PSHAa1649
78	succinyl-CoA transferase, subunit A	5,44	25,1	PSHAa1447
71	citrate synthase	5,74	48,1	PSHAa1653
88	transaldolase B	5,24	35	PSHAa2559
93	putative fructose-bisphosphate aldolase	5,56	33,1	PSHAb0548
115	probable isopropylmalate dehydrogenase	6,14	35,7	PSHAb0465
131	4-hydroxyphenylpyruvate dioxygenase	4,64	38,8	PSHAa2168
148	nucleoside diphosphate kinase	5,5	15,5	PSHAb0142
143	IMP dehydrogeanse	8,12	52	PSHAa0648
152	Alpha keto acid dehydrogenase complex, beta subunit	5,13	35,4	PSHAa1631
153	Alpha keto acid dehydrogenase complex, E2 component	5,32	57,3	PSHAa1630
160	malonyl-CoA-[acyl-carrier-protein] transacylase	4,95	32,3	PSHAa1809
162	3-oxoacyl-[acyl-carrier-protein] synthase I	5,2	42,6	PSHAa2080
	3 Information transfer pathways			
175	nucleoid-associated protein	5,46	34,5	PSHAa2381
187	adenylosuccinate synthetase	5,38	47,6	PSHAa0275
191	DNA-directed RNA polymerase omega chain	4,87	10,2	PSHAa2791
195	putative ATP-dependent RNA helicase	9,32	48	PSHAb0411
207	phenylalanine tRNA synthetase, alpha-subunit	5,38	37,3	PSHAa1904
224	50S ribosomal subunit protein L4	9,76	21,9	PSHAa0145
226	30S ribosomal subunit protein S4	10,12	23,4	PSHAa2807
229	Chaperone protein DnaK	4,59	68,7	PSHAb0357
	5. Unknown proteins			
265	conserved hypothetical protein	8,88	27,8	PSHAa0394
7	putative membrane-associated protein with TPR-like domain	4,87	22,2	PSHAb0136

The regulation of the six genes was investigated by transcriptional fusion experiments. DNA fragments immediately upstream of the genes were individually fused to a promoter-less *lacZ* gene contained in a pPLB plasmid (Duilio, A. *et al.*, 2004), generating the P(PSHA*X*) vectors (Table 4).

The upstream region of the gene coding for the putative outer membrane porin in *Ph*TAC125 has a rather specific organization. Two CDSs encoding a putative C4-dicarboxylates sensor kinase and a putative C4 response regulator were respectively located upstream of the porin gene. Furthermore, a CDS coding for a periplasmic transporter of dicarboxylic acids was found downstream of the porin gene. This association strongly suggests the occurrence of a two-component sensor regulatory system. The complete genome region upstream of the porin gene (i.e. the genes coding for sensor kinase and C4 response regulator) was subsequently used in the transcriptional fusion experiments.

T**able 3:**

List of the six most abundant proteins expressed from *Pseudoalteromonas haloplanktis* cells in the presence of L-malate.

Spot	Protein	pI	MW (KDa)	Accession number
26	outer membrane protein W (OmpW) family	4,98	23,5	PSHAa2155
71	citrate synthase	5,74	48,1	PSHAa1653
52	putative flagellin (FliC-like)	4,64	40,2	PSHAa0782
62	bifunctional protein [aconitate hydratase]	4,96	93,9	PSHAa0184
88	transaldolase B	5,24	35	PSHAa2559
25	putative outer membrane porin	4,98	38,4	PSHAb0363

Recombinant *Ph*TAC125 cells harbouring the β-galactosidase gene individually fused to the six promoters were grown in minimal medium either in the absence or in the presence of L-malate. Figure 4 shows the β-galactosidase activity measured in the mid-stationary phase. All recombinant *Ph*TAC125 cells transformed with the constructs displayed notably higher β-galactosidase activity when compared to the control vector (pPLB). However, in most cases, the β-galactosidase activity remained unchanged following L-malate supply. In contrast, *Ph*TAC125 cells harbouring the porin promoter region showed a 13fold activity increase when L-malate was added to the medium.

The transcriptional activity of the six genomic regions was further investigated by monitoring the β-galactosidase activity during the exponential and the late-stationary growth phase. These experiments demonstrated that the transcriptional activity of the six promoters was not growth-phase dependent (data not shown). As a whole, these data showed that the regulatory

element, located upstream of the porin gene, is remarkably influenced by the presence of L-malate in the growth medium. This finding indicated that *Ph*TAC125 contains a functional two-component system, consisting of a membrane sensor kinase and a cytoplasmic response regulator (Janausch, I.G. *et al*., 2002; Stock, A.M. *et al.*, 2000), for sensing the availability of C4-dicarboxylic acids.

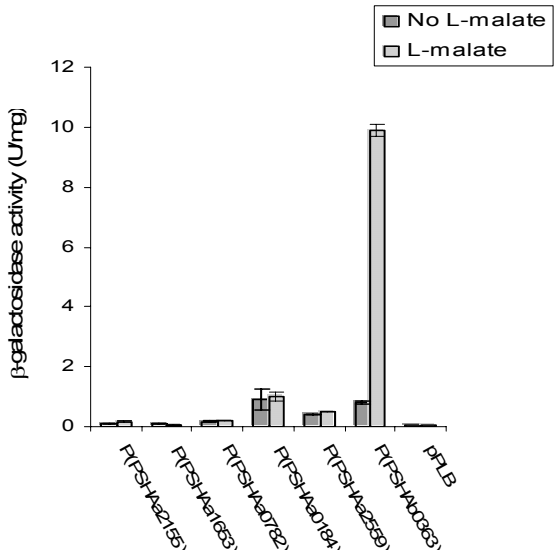

Figure 4
Profiles of the β-galactosidase activity of the *P*(PSHA*X*)*::lacZ* transcriptional fusion constructs in *Ph*TAC125 grown in minimal medium in the presence or in the absence of L-malate.
pPLB (negative control)

It is also worth to note that such an arrangement, to the best of our knowledge, has never been reported before to occur in members of Pseudoalteromonads. The identification of a regulatory element induced by L-malate might also constitute a key step in the development of an efficient and inducible cold expression system (Duilio, A. *et al.*, 2004).

3. Experimental procedures

3.1 Strains and growth conditions

Pseudoalteromonas haloplanktis TAC125 (*Ph*TAC125) (Birolo, L., *et al.*, 2000), a member of the gamma-proteobacteria was grown in aerobic conditions at 16°C in modified DSMZ medium 79 (a minimal synthetic sea water medium containing 1 g/l KH_2PO_4, 1 g/l NH_4NO_3,10 g/l NaCl, 0.2 g/l $MgSO_4$ x $7H_20$, 10 mg/l $FeSO_4$, 10 mg/l $CaCl_2$ x $2H_2O$), supplemented with casamino acid 0.5% w/v and 0.2% w/v carbon source. Care was taken to use in all experiments the same casamino acid batch (Difco Laboratories GmbH, Augsburg, Germany: batch 0231-01).

3.2 Preparation of protein extracts

Bacteria were harvested at the time points indicated, washed in 10 mM Tris, pH 7.5, 10 mM EDTA, and resuspended in the same buffer containing 1.4 μM phenyl methyl sulfonyl fluoride. Cells were disrupted by passage through a French Press. After centrifugation (20 000 x *g*, 4°C, 45 min) the protein concentration of the extract was determined with the RotiNanoquant Kit (Roth, Karlsruhe, Germany).

3.3 2D PAGE

For isoelectric focusing, protein extracts (300 μg proteins) were loaded onto commercially available IPG-strips (pH 4–7; Amersham Biosciences) according to Bernhardt, J. *et al.* (Bernhardt, J. *et al.*, 1999). In the second dimension polyacrylamide gels of 12.5% acrylamide and 2.6% bisacrylamide were used. Gels were stained with Colloidal Coomassie Brilliant Blue. For the Coomassie staining, a 5% w/v stock solution of Coomassie Brilliant Blue G-250 was prepared. This stock solution was then used to make up the staining solution containing 10% w/v $(NH_4)_2SO_4$, 1% v/v phosphoric acid, and 2% v/v (final concentrations) of the Coomassie stock solution. After fixation in a 40% ethanol/10% acetic acid v/v solution, the gels were stained for about 20 h in the staining solution diluted with ethanol (20% v/v, final concentration). Excess Coomassie stain was removed by washing the gels in distilled water.

3.4 Protein identification

Proteins were identified by MS. All the protein spots detectable by Coomassie stain were excised from gels with the Proteome Works Spot Cutter System (Bio-Rad). In-gel trypsin digestion of the proteins and extraction of the peptides were mostly done in the Ettan Spot Handling Workstation (Amersham Biosciences). Peptide masses were measured either in a Voyager-DE STR or in a Proteomics Analyzer 4700 (both Applied Biosystems). Peptide mass fingerprints were analyzed using an in-house database developed from the genome sequence of *Ph*TAC125. The resulting MS data were analyzed with the Bioanalyst Software (Applied Biosystems) and the integrated Mascot (Matrix Science) script.

Table 4: Plasmids and oligonucleotides

Plasmid	Description	Ref.
pPLB	Promoter-trap cold-adapted vector containing the promoter-less *Ph*TAE79 *lacZ* gene	6
P(PSHAa2155)	pPLB containing the PSHAa2155 promoter region (231 bp)	
P(PSHAa0782)	pPLB containing the PSHAa0782 promoter region (307 bp)	
P(PSHAa1653)	pPLB containing the PSHAa1653 promoter region (407 bp)	
P(PSHAa0184)	pPLB containing the PSHAa0184 promoter region (234 bp)	
P(PSHAa2559)	pPLB containing the PSHAa2559 promoter region (232 bp)	
P(PSHAb0363)	pPLB containing the PSHAb0363 promoter region (3600 bp)	
Oligonucleotide		
PSHAa2155 Fw	5' GCTTTAGAAATCTAGACTGAAGAAACAGC	
PSHAa2155 Rev	5' GTTCTCCAAGATATCTTTGTGATTGCC	
PSHAa1653 Fw	5' GAGATAGTCTAGATTATTTGAAGCACAACG	
PSHAa1653 Rev	5' CCATCGATATCTCTCCTATTAAGATAAC	
PSHAa0782 Fw	5' GAGATAGTCTAGATTATTTGAAGCACAAC	
PSHAa0782 Rev	5' GCCATGATATCACTCCTGATTACTTTCG	
PSHAa0184 Fw	5' CACTAAAGCAGCTCTAGAAGAAAACCG	
PSHAa0184 Rev	5' GTAGCACAAGATATCCCTCTTGGTGACG	
PSHAa2559 Fw	5' CAGGTGATCTAGAAGTGATCATAGTTAGC	
PSHAa2559 Rev	5' CGTTAGATATCTGCTTTCCTCATTCC	
PSHAb0363A Fw	5' CCAAAGCTAGGATCCGCTTAATTATAC	
PSHAb0363A Rev	5' CCTGGATCCAATATCGATAGTTTTACG	
PSHAb0363B Fw	5' GATGGACGTCTAGAACTATCGATATTAG	
PSHAb0363B Rev	5' CCTTCAATCTAGATATCTGCAGGAGTATC	
PSHAb0363C Fw	5'GATACTTCCTGCAGATATATTAATTG	
PSHAb0363C Rev	5' CCTGTGTCCCGGGTATCATCATGTGTCC	

3.5 Denomination of the proteins

Proteins are numbered according to the genome annotation (Medigue, C. *et al.*, 2005).

3.6 Transcriptional fusion assays

Reporter assays of transcriptional fusions were performed by measuring β–galactosidase activity as described by Duilio, A. *et al*. (Duilio, A. *et al.*, 2004).

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A two-component system regulates the expression of genes involved in uptake of L-malate in the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125.

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Abstract

Two-component systems are the most common mechanism of transmembrane signal transduction in bacteria and are involved in many cellular processes. They control the expression of genes for nutrient acquisition, virulence, antibiotic resistance, and numerous other processes in diverse bacteria. These systems are often used by soil bacteria that have to adapt to frequent changes in nutritional availability and in environmental conditions to regulate their gene expression. By contrast, bacteria that live in stable niches (symbionts of aphids, extremophiles or some marine hydrocarbon-degrading bacteria, etc.) seem to have less regulatory systems. Recently, a functionally active two-component system (named *Ph*MAlRS) probably involved in the of expression regulation of a C4-dicarboxylate transporter system was identified in the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125.

Our data demonstrated that the expression of the C4-dicarboxylate transporter system (consisting of genes coding for an outer membrane porin and a periplasmic transporter, renamed *Phdct A* and *B*, respectively) is induced by the presence of L-malate under the control of the *Ph*MalRS two-component regulatory system. The DNA region located upstream the *PhdctA* porin gene was further investigated; two different transcriptional start sites and two different promoter sequences were defined, one of which is a typical σ^{54} core promoter. A 53 bp inverted repeat sequence located 77 bp upstream the distal transcriptional start site was also identified. Functional analysis of this element showed that the inverted repeat sequence was specifically recognised by protein extracts of *Ph*TAC125 only when the cells were grown in the presence of L-malate, suggesting a putative regulatory role for this DNA region.

We can speculate that this DNA sequence represents the *cis*-acting region requested for the binding of transcriptional regulatory proteins leading to the induction of the *PhdctAB* operon expression.

These results demonstrated the occurrence of a fine transcriptional mechanism that regulates the expression of a C4-dicarboxylate transporter operon in *Pseudoalteromonas haloplanktis* TAC125. In the presence of L-malate, the *Ph*MalRS regulatory system controls the expression of the *PhdctA* and *B* genes very likely through binding of a regulatory protein to the *cis*-acting region located upstream the outer membrane porin gene. The interaction of the regulatory protein with this DNA sequence probably strengthens the binding of RNA polymerase on the σ^{54} core promoter sequence and activates the transcription of downstream genes responsible for L-malate uptake.

Keywords: Psychrophile, two-component regulatory system, L-malate, σ^{54} core promoter.

1. Introduction

Two-component regulatory systems are the most common mechanism of transmembrane signal transduction in bacteria. These regulatory systems are

widespread in nature and not only exist in nearly all prokaryotes and many Archaea but also in eukaryotes such as plants, fungi and yeasts [1]. A typical two-component regulatory system consists of a signaling histidine kinase (HK) (also called the sensor kinase) usually associated to the membrane and a cytoplasmic response regulator (RR), generally a transcription factor (an activator or repressor) (Fig. 1). Binding of the environmental signal ligand to the sensory domain located within the C-terminal region of the histidine kinase induces autophosphorylation of the transmitter domain. Once phosphorylated, the sensor kinase is able to phosphorylate the cognate response regulator, thus activating the regulatory protein, generally leading to transcription activation.

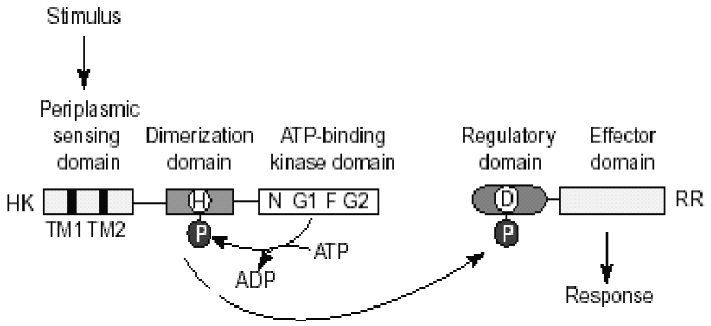


Fig. 1: Schematic organization of typical two-component regulatory system as reported in West AH and Stock AM (2001) [1].

The two-component systems are involved in many cellular processes and they control the expression of genes for nutrient acquisition, virulence, antibiotic resistance, and numerous other pathways in diverse bacteria (for a comprehensive review see 1).

Moreover, many examples suggest that a cross-regulation among different two-component systems can also take place [2]. This interactive function may be important for the integration of cellular processes involving multiple two-component systems [3]. In fact, several two-components sensor kinases (HK) were shown to be able to replace a mutated, non functional HK in phosphorylating a non-partner RR belonging to a different regulatory system, thereby complementing defects in the corresponding mutated system.

Two component regulatory systems are often used by soil bacteria that have to adapt to frequent changes in nutritional availability and, more generally, in environmental conditions to regulate their gene expression. By contrast, bacteria that live in stable niches (for example, symbionts of aphids, rickettsias, extremophiles or some marine hydrocarbon-degrading bacteria) seem to have less regulated promoters and less regulators [4].

Recently, the genome sequencing of the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 was completed [5]. Annotation of the genome highlighted that TAC125 possesses a large number of regulatory mechanisms including some typical two-component systems, although this microorganism usually lives in stable conditions. Studies on Gram-negative bacteria have revealed that two-component regulatory systems are usually employed to regulate the expression of C4-dicarboxylate transporters, which are only induced in the presence of the external substrate [6].

Recently, we analyzed the protein profile of the Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 when L-malate was added in minimal medium using a proteomic approach [7]. A functionally active two-component system consisting of CDSs PSHAb0361 and PSHAb0362 (hereby indicated as *PhMalRS)* was identified. 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 periplasmic transporter of dicarboxylic acids, respectively.
The two component regulatory system was then investigated both at the structural and functional level. A typical σ^{54} core promoter was identified upstream the gene coding for the outer membrane porin together with a canonical σ^{70} sequence. Moreover, an inverted and repeat DNA sequence was located upstream σ^{54} promoter and demonstrated to act as a *cis*-activating element in the presence of L-malate. The results reported here showed that the *Ph*MalRS two-component system is involved in the transcriptional operon following induction of L-malate.

2. Results and Discussion

2.1. Genetic organisation analysis of C4-dependent two-component system locus in *Ph*TAC125

The arrangement of the genes likely involved in the uptake of C4-dicarboxylic acids in *Pseudoalteromonas haloplanktis* TAC125 chromosome II is schematically shown in Fig. 2.

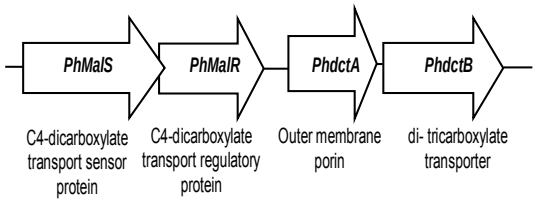


Fig. 2: Schematic organization of *PhmalRS* two-component regulatory system and *PhdctAB* loci.

The PSHAb0361 and PSHAb0362 CDSs, hereby indicated as *PhMalS* and *PhMalR* respectively, encoded two putative proteins of 576 and 454 amino acids with theoretical *Mw* of 64880 Da and 50340 Da, respectively. These proteins exhibited 31,7% and 58,3% identity with a C4-dicarboxylate transport sensor protein from *Vibrio parahaemolyticus* and a C4-dicarboxylate transport transcriptional regulatory protein from *Shewanella oneidensis*, respectively.
Computational analysis revealed that *Ph*TAC125 *Ph*MalS and *Ph*MalR exhibited further sequence similarities with several other regulatory proteins. Extensive similarities were observed for the C-terminal domain of the *Ph*MalS protein and the C-terminal domains of the sensor proteins NtrB, CpxA, EnvZ and PhoR. The N-terminal domain of the *Ph*MalR protein shared sequence similarities with the N-terminal domains of several well defined regulatory proteins, such as NtrC, ArcA (then called SfrA and thought to interact with CpxA), OmpR, PhoB, CheB, and CheY. It should be reminded that signal transduction in a two-component system occurs through binding of the environmental ligand to the C-terminal domain of a sensor kinase protein and it is then transferred to the N-terminal region of the partner regulatory protein [8, 9].
The CDS *PSHAb0363* located downstream of the *PhMalR* gene and consisting of 921 bp encoded a putative protein sharing 33.1% identity with an outer membrane porin from *Shewanella oneidensis*. A second ORF (*PSHAb0364*) consisting of 1476 bp and coding for a putative protein sharing 46,3% identity with a di- and tricarboxylate transporter from *Corynebacterium glutamicum* was found 12 bp downstream the outer membrane porin gene, thus suggesting the involvement of these two ORFs in the transport and the uptake of C4-dicarboxylic acids. Accordingly, these ORFs were renamed *PhdctA* and *PhdctB* (C4-dicarboxylate transport) respectively. Data from computation analysis together with the genetic organization of the four ORFs depicted in Fig. 2 strongly suggest that the uptake of C4 dicarboxylic acids in TAC125 is under the control of a two-component regulatory system.

2.2. Functional analysis of malate-dependent two-component regulatory system

The transcriptional regulation of the *PhMalRS* and *PhdctAB* loci was first investigated by RT-PCR experiments. Total RNA extracted from *Ph*TAC125 coltured in minimal medium both in the presence and in the absence of L-malate was amplified using oligonucleotide pairs specifically designed for each gene (Table 1, Fig. 3A).

These experiments clearly demonstrated that *PhMalS* is transcribed only when L-malate was added to the medium while the expression of *PhMalR* gene is independent from the presence of the substrate. As the *PhdctAB* locus is concerned, a *PhdctA* transcript was detected in both conditions (Fig. 3A, lane XY) but it was strongly induced by the presence of L-malate in the medium, supporting the hypothesis of an involvement of the outer membrane porin in the uptake of C4-dicarboxylic acids. On the contrary, the presence of L-malate appeared to be irrelevant on *PhdctB* transcription.

We also analyzed the possible presence of bicistronic transcripts for both *PhMalRS* and *PhdctAB* loci using suitable oligonucleotide pairs designed on the 3' sequence of *PhMalS* and the 5' sequence of *PhMalR*, and the 3' sequence of *PhdctA* and the 5' sequence of *PhdctB,* respectively (Fig. 3B). A co-transcriptional regulation for the *PhMalS* and *PhMalR* genes could not be observed, although previous sequence data analysis had revealed that these two genes are partially overlapped (7 bp), suggesting a common transcriptional regulation. A very small amount of bicistronic transcript comprising the *PhdctAB* locus was indeed detected when L-malate was added to the minimal medium.

The results reported in Fig. 3C indicated that each gene in both the *Ph*MalRS and *Ph*DctAB loci is transcribed by its own promoter. However, functional differences exist among the different promoter. The promoter element located upstream the *PhMalS* gene is only active in the presence of L-malate whereas the promoter located upstream the *PhdctA* gene is able to dictate transcription of *PhdctA* in both conditions but it is strongly induced by L-malate.

Moreover, the occurrence of a small amount of the bicistronic transcripts for the *PhdctAB* locus suggested the presence of a further promoter element located upstream the *PhdctA* gene responsible for the co-transcription of *PhdctA* and *PhdctB* genes.

These data also demonstrated the functionality of three putative transcriptional *Rho*-independent terminators previously identified by computational analysis. These terminators are located downstream the *PhMalS, PhMalR and PhdctB* stop codons, respectively. The presence of the *PhdctA* monocistronic message indicates the occurrence of a further likely Rho-dependent transcriptional terminator downstream the *PhdctA* gene. In figure 3C a schematic view of the transcriptional regulation of the *PhMalRS* and *PhdctAB* loci is reported.

The occurrence of both the single *PhdctA* and the bicistronic *PhdctA-PhdctB* messengers was investigated by primer extension experiments. The results of these analyses shown in Fig. 4A revealed two different transcriptional start sites, a proximal start site, corresponding to an adenine base, was identified only 13 bp upstream the *PhdctA* start codon (P1), while a distal start site (adenine) was identified 33 bp upstream the translational start site (P2). A putative σ^{70} promoter element, according to the σ^{70}-dependent consensus sequence for *Ph*TAC125 [10], was identified upstream the proximal start site, while a putative σ^{54} promoter element was inferred upstream the distal transcriptional start site (Fig. 4B). To

define the σ^{54}-dependent core promoter in *Ph*TAC125, a consensus sequence deriving from a collection of 186 promoters from 47 different bacterial species was used as reported in Studholme and Dixon [11]. The σ^{54} core promoters are, in fact, remarkably conserved both in sequence and structure in contrast to the flexible consensus of σ^{70}-dependent promoters. This analogy possibly reflects the strict requirements for promoter recognition and function required for a highly controlled regulation [12].

In conclusion, *PhdctA* gene is very likely expressed from two promoters: the σ^{70} P1 promoter and the σ^{54} P2 promoter. On the basis of known features of bacterial promoters (for a recent review see 1), it is conceivable that the σ^{70} promoter element guarantees a basal level of *PhdctA* transcript also in the absence of L-malate. On the contrary, the σ^{54}-dependent promoter element efficiently recruits the RNA polymerase and induces transcription of *PhdctA* at high levels only in the presence of L-malate in the medium, suggesting a possible upregulation by the *Ph*MalRS two-component system.

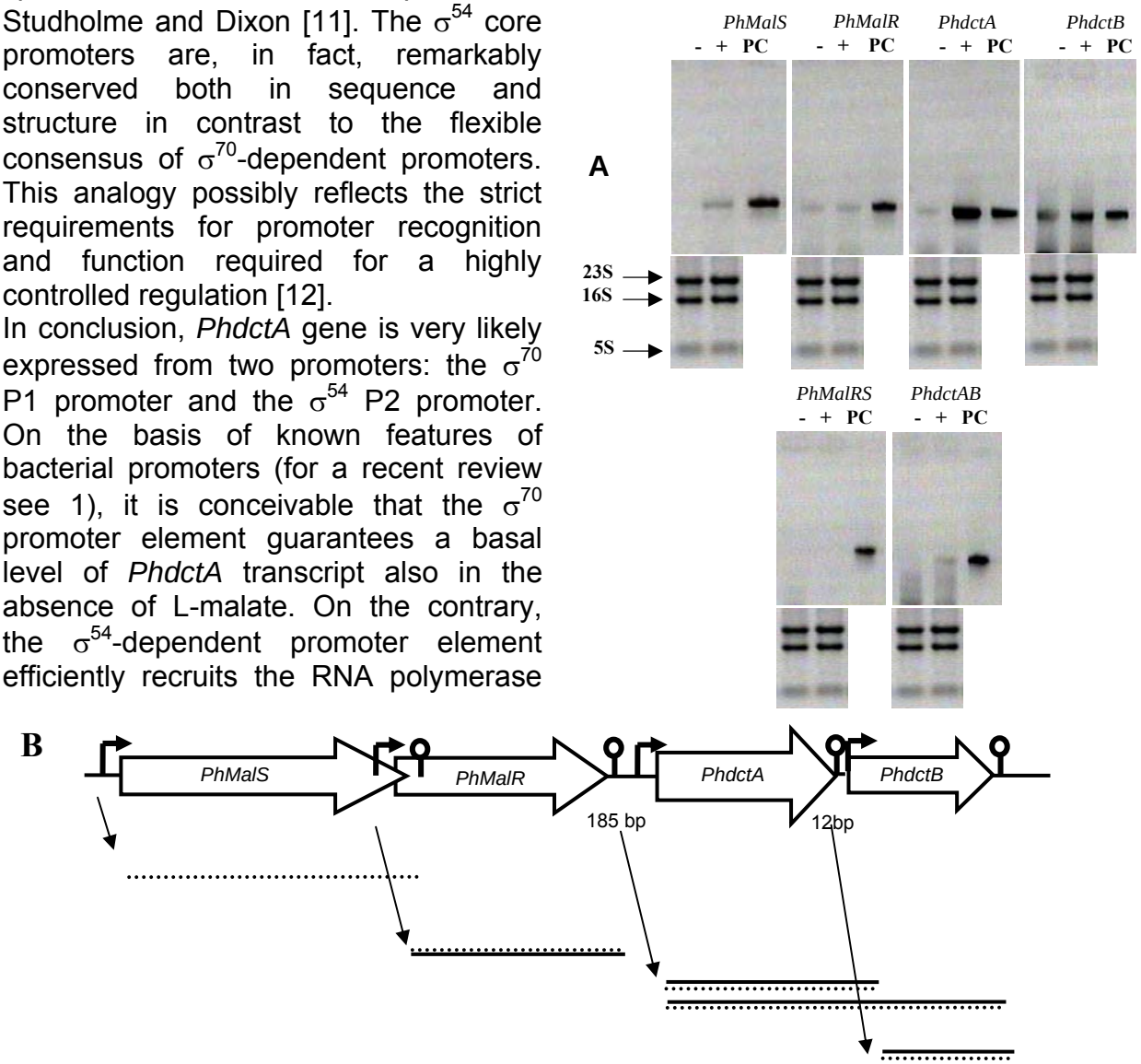


Fig. 3: Transcriptional analysis of malate-dependent two-component regulatory system using RT-PCR experiments.

A: Transcriptional induction malate dependent of the *malRS* and dct*AB* genes, monitored by RT-PCR (top panel) and visualization of the same samples in an agarose-formaldehyde gel (bottom panel) of *Ph*TAC125 total RNA. Total RNA samples were extracted from cells grown in minimal medium (Shatz) in the presence (+) and in the absence (-) of L-malate. *Ph*TAC125 genomic DNA was used as positive control (PC).

B: A schematic representation of the *malRS* and dct*AB* locus and their transcriptional organization.

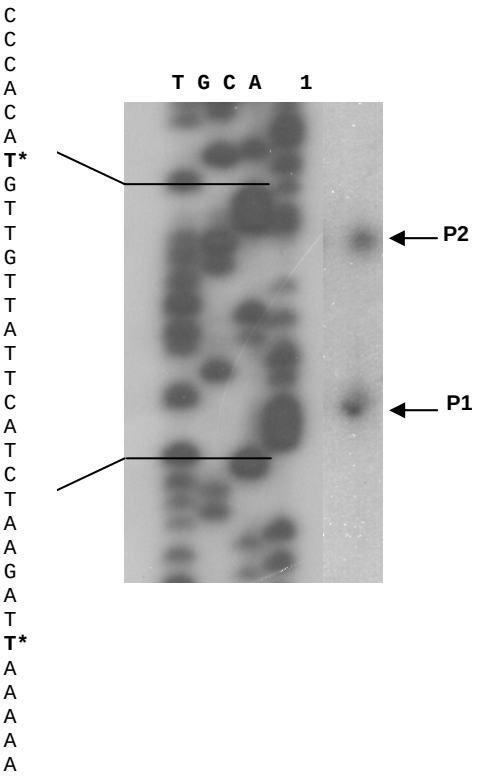
: putative promoter element

: putative terminator element

: transcript detected in the absence of L-malate

: transcript detected in the presence of L-malate.

A



B

GGCATGTAGTAGTGTGCGATAAAGGTGA**TGTTTTTAAATAAAAGTGTGCGGATATCCGCACA**

CTTTTTTGCTTTAAAAACAGTGTTAATCATTTAACTAGCTGAAATTTATGTTTATTTAGTAG

σ 54 -24 box **σ 54 -12 box** **+1P2** **+1P1** **SD**

T**TGGCCAT**CATT**TTGCTT**AATGGGTGT**A**CAACAA**TAAGTA**GATTCTA**A**TTTTTT*AGGA*CAC**ATG**

σ70 -35 box **σ70 -10 box**

σ54 CONSENSUS SITE (——) Y T G G C W Y W N N N N T T G C W

σ70 CONSENSUS SITE (·····) T R G R T W –- (14-19 bp) -- T A T R A Y

Y: C,T
W: A,G
R: A,G

Fig. 4: Transcriptional analysis of *PhdctAB* operon by primer extension analysis.
A: Primer extension analysis of the *PhdctA* transcript. A 19 bp oligonucleotide was annealed to 40μg (lane 1) of recombinant P(PSHAb0363) *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 (lane T, G, C and A).
B: The nucleotide sequence upstream PhdctA gene.
The putative P1 and P2 promoters are underlined and evidenced in bold. The corresponding –10 and –35 regions are in bold (σ^{70} core promoter is evidenced with broken lines). The transcriptional start sites are underlined and evidenced in grey (σ^{54} start site is evidenced with broken lines). The ribosome binding site (SD) is indicated as italics, while the PhdctA start codon is indicated as bold.
The putative binding site recognized by *PhMalR* is evidenced in black.

2.3. Identification of a putative DNA binding region involved in the transcriptional activation of malate-dependent two-component regulatory system

The regulation of expression of *PhdctA* was further investigated by analysing the genomic region located upstream the putative σ^{54}–dependent core promoter. A 53 bp length sequence identifying a perfect inverted repeat was recognized 71 bp upstream of transcriptional start site P2, as shown in Fig. 4B. Functional analysis of this element was carried out by electrophoretic mobility assays (EMSA) to investigate possible interactions with TAC125 proteins.

A 120 bp DNA fragment containing the inverted repeat sequence was amplified and incubated with protein extracts from *Ph*TAC125 cells grown either in the absence or in the presence of L-malate. Figure 5 shows the results of the EMSA analysis: a clear gel mobility shift was observed only when the inverted repeat sequence was incubated with protein extract of *Ph*TAC125 cells grown in the presence of L-malate (Fig. 5, lane 4), suggesting a putative regulatory role for this DNA region. We can speculate that the inverted repeat sequence represents the *cis*-acting region requested for the induction of the *PhdctAB* operon expression through the binding of transcriptional regulatory proteins. Binding of regulatory proteins on this DNA sequence probably strengthens the interaction of RNA polymerase on the σ^{54} core promoter sequence and activates the transcription of the downstream genes.

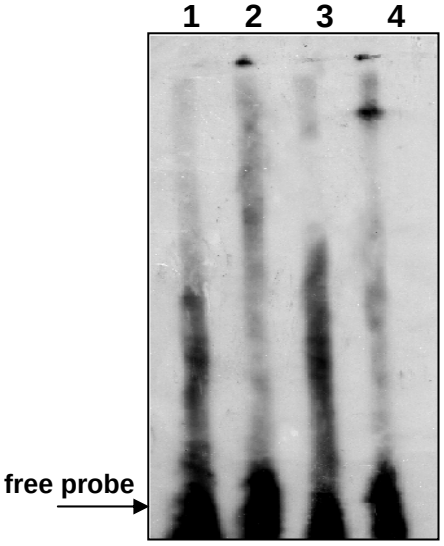


Fig. 5: Gel shift assays performed using the *PhdctA* upstream promoter region and total protein extract of *Ph*TAC125 cells grown in the presence and in the absence of L-malate.
Lane 1: non specific probe incubated with protein extracted from *Ph*TAC125 cells grown in the absence of L-malate; lane 2: specific probe incubated with protein extracted from *Ph*TAC125 cells grown in the absence of L-malate; lane 3: non specific probe incubated with protein extracted from *Ph*TAC125 cells grown in the presence of L-malate; lane 4: specific probe incubated with protein extracted from *Ph*TAC125 cells grown in the presence of L-malate. As negative control an undefined 120 bp DNA fragment was used.

The specificity of the binding was also demonstrated by competition EMSAs, incubating the specific radiolabelled probe with total protein extract and increasing quantity of unspecific and specific DNA competitor (data not shown).

These results demonstrated the occurrence of a fine transcriptional mechanism that regulates the expression of the *PhdctA* and *B* genes in *Pseudoalteromonas haloplanktis* TAC125.

Data obtained suggested that, in the presence of L-malate, the *Ph*MalR regulatory protein controls the expression of *PhdctAB* operon very likely through binding to the *cis*-acting region located upstream the outer membrane porin gene. The interaction of the regulatory protein with this DNA sequence probably strengthens the binding of RNA polymerase on the σ^{54} core promoter sequence and activates the transcription of downstream genes responsible for L-malate uptake. Nevertheless to demonstrate the direct involvement of *Ph*MalR in the

transcriptional activation of *PhdctAB* peron further experiments will be needed.

3. Materials and Methods

3.1 Bacterial strains, DNA constructs and media

Pseudoalteromonas haloplanktis TAC125 (*Ph*TAC125) [15] 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 minimal medium, containing 1 g/l KH_2PO_4, 1 g/l NH_4NO_3, 10 g/l NaCl, 0.2 g/l $MgSO_4$ x $7H_20$, 10 mg/l $FeSO_4$, 10 mg/l $CaCl_2$ x $2H_2O$, supplemented with casamino acid 0.5% w/v and 0.2% w/v L-malate as carbon source [7].

3.2 RT-PCR

Total RNA was extracted from *Ph*TAC125 cells grown at 15°C up to $3OD_{600}$ according to Tosco, A. *et al.* (2003) [16] in minimal medium in the presence and in the absence of L-malate. RNA has been reverse transcribed using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA was amplified using as primers specific oligonucleotides designed on *PhMalRS and PhdctAB* from *Ph*TAC125 respectively (table 1), and *Taq* polymerase (Promega, Madison, WI, USA) according to the manufacturer's instructions. The reaction mixture was amplified (denaturation at 95 C for 45 sec; annealing at 58° C for 45 sec; extension at 72 C for 1 min, 35 cycles), and the products were detected by gel electrophoresis.

3.3 Primer Extension

Total RNA for primer extension analysis was extracted from recombinant p(PSHA0363) *Ph*TAC125 cells [7] grown in minimal medium in the presence of L-malate. Primer extension experiments were performed as described by Tosco, A. *et al.*, 2003 [16], by using 18-21 nucleotide specific oligonucleotides as primers.

Table 1

Plasmids and oligonucleotides

Plasmid	Description
P(PSHAb0363)	pPLB containing the PSHAb0363 promoter region (3600 bp) [7].
Oligonucleotide	
PSHAbMalS RT-PCR Fw	5' GGTTTGGTCGAATACATGACTTAGAGC 3'
PSHAbMalS RT-PCR Rv	5' CATCAACCATAACAACCGAGGTAACTGC 3'
PSHAbMalR RT-PCR Fw	5' GATGAGGCGATGATCCGCGATTCG 3'
PSHAbMalR RT-PCR Fw	5' CTAAACCCACCTTCATTCACCAATGCC 3'
PSHAbdctA RT-PCR Fw	5' CCGATACAGGCGTTATGCGCTACGC 3'
PSHAbdctA RT-PCR Rv	5' CACTCTGAGTGTTGATACAAAGCACC 3'
PSHAbdctB RT-PCR Fw	5' GTAGCCGCGCCTTATGCTCATCC 3'
PSHAbdctB RT-PCR Rv	5' CCACTAAACTCGCCTAGCAAAGGT 3'
PSHAbMalSR RT-PCR Fw	5' CTAAGGCCGCACCCTTTGTCGGC 3'
PSHAbMalSR RT-PCR Rv	5' GTAACGTGCAACCAGCTCTTTGC 3'
PSHAbdctAB RT-PCR Fw	5' CCAAGCGGGTTTTGCCGCTACAC 3'
PSHAbdctAB RT-PCR Fw	5' GAGAGTAGTAAAGCTTTACCAAATCC 3'
P(PSHAb0363) PE Rev	5' CGTTAAGTGGGCTATGTGC 3'
PSHAbdctA bandshift Fw	5' GGGCATGTAGTAGTGTGCG 3'
PSHAbdctA bandshift Rv	5' GATGGCCAACTACTAAATAAAC 3'

3.4 Preparation of protein extracts

Protein extracts were performed as described by Papa, R. *et al.*, 2005 [7].

3.5 Electromobility shift assay

120 bp DNA fragment containing the repeat sequence located upstream *PhdctA* gene was obtained by PCR amplification by using specific oligonucleotides pairs indicated in Table 1. The obtained fragment was ^{32}P labelled. with Random primed DNA labelling kit (Roche) according to the manufacturer's instructions.

Electromobility shift assays were performed in 20 µl reaction volume, in binding buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.1mM PMSF,). Total protein extract (10 µg) was incubated with the ^{32}P-labelled DNA (2 ng, 50,000-100,000 cpm) in binding buffer, for 20 min at room temperature. Mixtures were then analysed by electrophoresis on 6% native polyacrylamide gel (29:1 cross-linking ratio) in 0.5x TBE (45 mM Tris-HCl pH 8.0, 45 mM boric acid, 1 mM EDTA). Electrophoreses were performed at room temperature at 200 V (20 V/cm). The gels were dried and analysed by autoradiography.

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Construction of an inducible expression system in the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125: functional characterization and biotechnological applications

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Abstract
Cold adapted microorganisms represent a powerful potential for biotechnological industry. The development of a shuttle genetic system for the transformation of the cold adapted Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 has made it possible the isolation of constitutive psychrophilic promoters and the construction of cold expression systems for the homologous/heterologous protein production at low temperatures.
To improve the potentiality of the cold expression system, we focused our attention on the isolation of regulated promoters and the development of efficiency induction strategies for recombinant protein production. In this paper we used a DNA genomic region from *Pseudoalteromonas haloplanktis* TAC125 containing a two-component system strongly regulated by the presence of L-malate to construct an inducible expression vector. We demonstrated that the inducible expression system was effective in the production of both psychrophilic and mesophilic proteins. The performances of the inducible and the constitutive system for the production of a psychrophilic β-galactosidase and a mesophilic α-glucosidase were also compared.

Keywords: Psychrophiles, protein production, cold inducible system

1. Introduction

One of the main problems often occurring during the heterologous protein production in bacteria is the incorrect folding of the nascent polypeptides resulting in their aggregation and accumulation as insoluble inclusion bodies. The study of inclusion bodies formation highlighted to rule played by hydrophobic interactions as mainly responsible of driving partially folded protein intermediates to stick and subtracting them from the productive folding pathway (Georgiou, G. *et al.*, 1996). To minimize this undesirable effect, many experimental approaches have been explored with some success, one of which consists in lowering the expression temperature till the physiologic limit allowed for the growth of mesophilic hosts (between 15 and 18°C for *Escherichia coli*). In fact, lowering the temperature has a pleiotropic consequence on the folding processes, minimizing the so-called hydrophobic effect. Although in some cases this approach has been reported to enhance the yield of recombinant protein production in soluble and active form, the exploitation of an industrial process implying a suboptimal growth of the expression host can be considered quite difficult. Starting from the above considerations, it seemed to us feasible the use of naturally cold-adapted bacteria as hosts for protein production at low temperature (even at around 0°C) as a rational alternative to mesophiles.
The development of a shuttle genetic system (Tutino M.L. *et al.*, 2001) for the transformation of cold adapted Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 (Birolo L. *et al.*,

2000) has made possible the isolation of constitutive psychrophilic promoters and the construction of cold expression systems for the homologous / heterologous protein production at low temperatures (Duilio A. *et al.*, 2004). The described expression system represented the first example of heterologous protein production based on a true cold-adapted replicon (Duilio, A. *et al.*, Uff. It. Brev. Marchi, 2003).

To improve the potentiality of cold expression system, we focused our attention on the isolation of regulated promoters and on development of efficiency induction strategies for recombinant protein production. In fact, the physical separation between the bacterial growth of the host and the production of heterologous protein can improves not only the productivity of the entire system, but can also plays an important role in the production of toxic proteins or/and that form insoluble aggregates.

Recently, using a proteomic approach and exploiting the information deriving from genome sequencing of *Ph*TAC125 (Medigue C. *et al.*, 2005) we isolated and characterized a functionally active two-component system (PSHAb0361-PSHAb0362) involved in the transcriptional regulation of the gene coding for an outer membrane porin (PSHAb0363), that is strongly induced by the presence of L-malate in the medium (Papa R. *et al.*, 2005).

In this paper we used the regulative region, comprising the two-component system located upstream the PSHAb0363 gene, to construct an inducible expression vector, named pUCRP.

The performances of the inducible and the constitutive system (Duilio A. *et al.*, 2004) for the production of the psychrophilic β-galactosidase from *Ph*TAE79 (Hoyoux A. *et al*., 2001) and the mesophilic α-glucosidase from *Saccharomyces cerevisiae* (Kopetzki, E. *et al*., 1989) were also compared. Data presented in this paper demonstrated that when these proteins are produced in *Ph*TAC125, they are completely soluble and catalytically competent in both expression system used.

2. Materials and methods

2.1 Bacterial strains, DNA constructs and media

Pseudoalteromonas haloplanktis TAC125 (*Ph*TAC125) (Birolo, L. *et al.*, 2000), a Gram-negative bacterium was grown in aerobic conditions at 4 and 15°C in minimal medium (1 g/l KH_2PO_4, 1 g/l NH_4NO_3, 10 g/l NaCl, 0.2 g/l $MgSO_4$ x $7H_20$, 10 mg/l $FeSO_4$, 10 mg/l $CaCl_2$ x $2H_2O$, supplemented with casamino acid 0.5% w/v and different concentration of L-malate when requested) and in TYP broth (16 gr/l yeast extract, 16 gr/l bacto tryptone, 10 gr/l marine mix) at pH 7.5, supplemented with 100μg/ml ampicillin if transformed.

2.2 Construction of the expression vectors

Plasmids are all reported in Table 1.

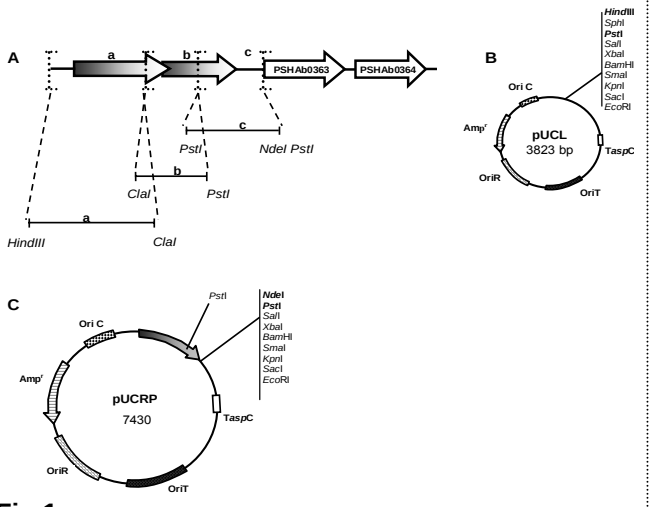

Fig.1
Construction of pUCRP expression vector.
A) *Ph*TAC125 DNA genomic fragment corresponding to the cold-active promoter of P(PSHAb0363) vector (Papa, R. *et al.*, 2005). B) pUCLT/Rterm promoter-less vector. C) pUCRP expression vector.

Genomic DNA preparation from *Ph*TAC125 was carried out as previously described (Tosco, A. *et al.*, 2003).

pP13 psychrophilic promoter-containing vector was isolated from a promoter-trap library (Duilio, A. *et al*., 2004), and the DNA fragment corresponding to the cold-active promoter was PCR amplified to generate pPM13 expression vector (Duilio, A. *et al.*, Uff. It. Brev. Marchi, 2003).

The pUCRP inducible cold-adapted gene-expression vector was constructed by cloning DNA fragment corresponding to the cold-active promoter of P(PSHAb0363) vector (Papa, R. *et al.*, 2005) into pUCLT/Rterm vector (fig. 1).

P(PSHAb0363) promoter region was inserted into pUCLT/Rterm vector by a three steps procedure as described in fig. 1. The first step consisted in the double *Hind*III/*Cla*I digestion of a 1700 bp fragment (PCR amplified by using the primers PSHAb0363a Fw and PSHAb0363a Rv and the *Ph*TAC125 DNA genomic as template). In the second step a 750 bp fragment was amplified using PSHAb0363bFw and PSHAb0363b Rv as primers and double *Cla*I/*Pst*I digested. Finally, the third fragment containing the 3' region of P(PSHAb0363) promoter region was amplified using PSHAb0363c Fw and PSHAb0363c Rv as primers and *Pst*I digested. The amplified fragments were cloned into pUCLT/Rterm corresponding sites by using two consecutive ligation reactions, and their nucleotide sequence checked to rule out the occurrence of any mutation during synthesis. The resulting vector was called pUCRP (table 1).

The amplifications were performed using in a mixture containing 60ng of template, 50pmoles of each oligonucleotide primer, 1.8mM, $MgCl_2$, 50mM KCl, 20mM Tris-HCl pH 8.3, 0.1% gelatine, 200μM dNTP in a final volume of 50μl. The mixtures were incubate at 95°C for 10 min, after which 1,25 units of Taq DNA polymerase were added. Twenty cycles of amplification (consisting of 1 min at 95°C, 1.5 min at 58°C and 1 min at 72°C) were carried out and were followed by a cycle in which the extension reaction at 72°C was prolonged for 10 min in order to complete DNA synthesis.

2.3 Construction of expression vectors for the production of *Saccharomyces cerevisiae* α-glucosidase in *Ph*TAC125.

The *Saccharomyces cerevisiae* GLUCP1 gene (*Sc*GLUCP1), encoding the α-glucosidase (Kopetzki, E. *et al.*, 1989) was inserted into the pPM13 expression vector generating the plasmid pPM13GLUCP1 (Tutino, M.L. and Duilio, A., personal communication). *Sc*GLUCP1 gene was inserted into the pUCRP expression vector by a single step procedure, consisting in the double *Nde*I/*Bam*HI digestion using pPM13GLUCP1 plasmid as template. The resulting vector was called pUCRPGLUCP1 (table 1).

Table 1: Plasmids and oligonucleotides

Plasmid	Description	References
P(PSHAb0363)	pPLB containing the PSHAb0363 promoter region	Papa, R. *et al.*, 2005
pP13	pPLB containing the PM13 promoter region	Duilio, A. *et al.*, 2004
pUCLT/Rterm	Vector deriving from the pUC18 plasmid, containing the T/R box and the transcription termination signal from *Ph*TAC125 *aspC* gene	Tutino, M.L. *et al*., 2002
pPM13	pUCLT/Rterm containing PM13 promoter region	Duilio, A. *et al.*, 2003
pUCRP	pUCLT/Rterm containing PSHAb0363 promoter region	This work
pPM13GLUCP1	pPM13 containing *Sc*GLUCP1 gene	Tutino *et al.*, personal comm.
pUCRPGLUCP1	pUCRP containing *Sc*GLUCP1 gene	This work
Oligonucleotide		
PSHAb0363a Fw	5' CAAAGCTAGGCAAAGCTTAATTATAC 3'	
PSHAb0363a Rv	5' CCTGGATCCAATATCGATAGTTTTACG 3'	
PSHAb0363b Fw	5'GATGGACGTCTAGAACTATCGATATTAG 3'	
PSHAb0363b Rv	5' CCTTCAATCTAGATATCTGCAGGAGTATC 3'	
PSHAb0363c Fw	5' GATACTCCTGCAGATATATTAATTG 3'	
PSHAb0363c Rv	5'GTTTAACGTGTCTGCAGTTTATCATATGGTGTCC 3'	

2.4 Enzymatic assays and analytical procedures

Duplicate cultures of recombinant *Ph*TAC125 cells were aerobically grown in liquid culture and cellular pellets (corresponding to 25 OD_{600nm}) were collected during the growth. The

cells were resuspended in 1 ml of Lysis Buffer (100 mM sodium phosphate, 5 mM EDTA, 2% Triton X-100, 1 mM PMSF, 1 mM DTT, and 5 mg/ml lisozyme, pH 7.8), and incubated for 20 min at 15°C. After a centrifugation step of 20 min at 4°C 9500 x *g*, the supernatant was collected.

Protein concentration was determined with the Bio-Rad protein assay (Bradford, M.M., 1976), using bovine serum albumine as standard.

Recombinant cold-active β-galactosidase was assayed spectrophotometrically at 25°C as previously reported (Hoyoux, A. *et al*., 2001), and calculation were performed on the basis of an extinction coefficient for *o*-nitrophenol of 3.5 mM^{-1} cm^{-1} at 410 nm and specific activity of 138.2 U/mg purified enzyme (Hoyoux, A. *et al.*, 2001).

α-Glucosidase production was assayed using 5 mM PNPG (*p*–nitrophenyl-α-D-glucopyranoside) as chromogenic substrate at 25°C in 100 mM potassium phosphate, 1 mM $MgCl_2$, 100 mM 2-mercaptoethanol, pH 6.8, and calculated on the basis of the *p*-nitrophenol ε_{410nm}=1.8 mM^{-1} cm^{-1} and specific activity of 130 U/mg purified enzyme (Kopetzki, E. *et al.*, 1989).

3. Results and Discussion

3.1 Analysis of the thermal-labile β-galactosidase production from *Pseudoalteromonas haloplanktis* TAE79 in *Ph*TAC125

The isolation and the structural and functional characterization of a L-malate inducible system from *Pseudoalteromonas haloplanktis* TAC125 was reported (Papa, R. *et al.*, 2005). This system, named P(PSHAb0363), showed to be able to direct the expression of a promoter-less *lacZ* gene from psychrophilic *Pseudoalteromonas haloplanktis* TAE79 (Hoyoux, A. *et al*., 2001); in particular, *Ph*TAC125 cells harbouring the PSHAb0363 promoter region showed a 13fold increase in the β-galactosidase activity when L-malate was added to the medium (Papa, R. *et al.*, 2005).

*Ph*TAC125 cells harboring β-galactosidase were grown in liquid culture in minimal medium in the presence and in the absence of L-malate at 15°C for till the reaching of the stationary phase. An aliquot of each culture was collected and their soluble protein content analyzed by SDS-gel electophoresis. Fig. 2 shows the Comassie brilliant blue-stained gels corresponding to the protein extracts from *Ph*TAC125 recombinant cells grown in the absence (lane 2) and in the presence (lane 3) of L-malate. Lane 3 is characterized by the presence of a 118KDa extra-band, whose electrophoretic mobility is similar to the purified protein (lane 1).

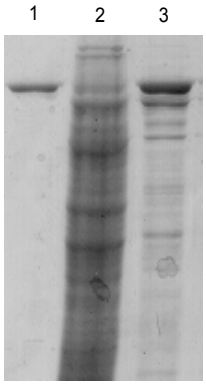

Fig. 2 Recombinant production of the thermal-labile β-galactosidase from *Ph*TAE79 in *Ph*TAC125 cells. 7.5% SDS PAGE gel electrophoresis of protein extract from *Ph*TAC125 cells harboring P(PSHAb0363) grown in minimal medium in the absence (lane 2) and in the presence (lane 3) of L-malate, in comparison with purified β-galactosidase (lane 1).

Data presented suggested that β-galactosidase is produced in *Ph*TAC125 under the control of inducible expression system only in the presence of L-malate, suggesting that P(PSHAb0363) vector possesses a basal expression level very low.

*Ph*TAC125 recombinant cells were grown in duplicate in liquid culture at 4°C

and 15°C for till the reaching of the stationary phase and the recombinant β-galactosidase activity was monitored, by catalytic assay, in the cell lysates as previously described by Hoyoux, A. *et al*., (2001). Since *Ph*TAC125 strain has not detectable β-galactosidase activity, pPLB vector (Duilio, A. *et al.*, 2004) recombinant cell lysate was used as negative control. The transcriptional activity of the system was further investigated by monitoring the β-galactosidase activity during the exponential and the late-stationary growth phase. These experiments demonstrated that its transcriptional activity is not growth-phase dependent (data not shown). The specific β-galactosidase activity produced by recombinant *Ph*TAC125 cells is reported in table 2. These data clearly demonstrate that the thermo-labile β-galactosidase is efficiently produced in *Ph*TAC125 in catalytically competent form; in contrast when this protein was produced in *E.coli* cells at 18°C it accumulated in insoluble aggregates and only 5% of it was soluble and active.

Table 2: β-galactosidase specific activities (mg/L) from *Ph*TAC125 cells harbouring P(PSHA0363) at 4° and 15°C in minimal medium (MM) in the presence and in the absence of L-malate 0.2%, respectively. The data shown are averages of 12 measurements. SD, standard deviation vectors.

β-galactosidase production (mg/L) ± SD		
	4°C	15°C
MM	4.11 ± 0.27	12.8 ± 0.3
MM+L-malate	52.2 ± 1.2	620± 24

In order to optimize the productivity of P(PSHAb0363) system the best induction conditions for PSHAb0363 promoter were found out. This procedure was divided in two consecutive steps: i) definition of optimal cellular growth phase in which induce the expression of heterologous gene (definition of t_0); ii) definition of optimal concentration of inductor to use to have high levels of expression of heterologous gene (C_0).

*Ph*TAC125 recombinant cells were grown in minimal medium (MM) in the absence of L-malate at 15°C. The induction of heterologous gene expression was tested by adding L-malate 0,2% at four different phases during cellular growth curve: 1) e 2) early and mid exponential phase, 3) e 4) early and mid stationary phase. The recombinant β-galactosidase activity was monitored before the addition of inductor (as negative control of the experiment), and 2-8-24 hours after the induction (t_1).

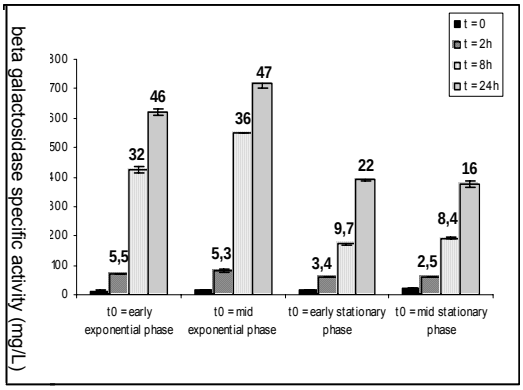

Fig. 3
Definition of optimal cellular growth phase to induce the expression of *PhlacZ* gene (t_0). β-galactosidase specific activities from *Ph*TAC125 cells harbouring P(PSHAb0363) grown in minimal medium (MM) by adding L-malate 0.2% at different time (t_0) during cellular growth curve (abscissae axis). The recombinant β-galactosidase activity was monitored before the addition of inductor (as negative control of the experiment), and 2-8-24 hours after the induction (t_1). Numbers above bars refer to induction ratio (IR) by L-malate adding (t_1/t_0). Means and standard deviations are derived from four independent assays.

In fig. 3 are reported the Induction Ratios (IR) achieved dividing the β-galactosidase production after the induction with that recorded before the induction (t_0). The best Induction Ratio (IR) was reached when L-malate was added during the exponential phase, in particular at t_0 = mid-exponential phase. High levels of reporter enzyme are

already detectable on cell lysates collected after 8 hours from induction, but maximal quantity of reporter enzyme was detectable at t_1 = 24 hours, probably due to accumulation of β-galactosidase in the cell (fig. 3).

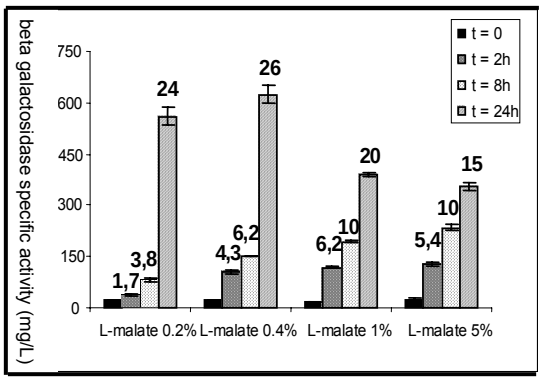

Fig. 4 Definition of optimal concentration of L-malate to induce the expression of *PhlacZ* gene (C_0). β-galactosidase specific activities from *Ph*TAC125 cells harbouring P(PSHAb0363) grown in minimal medium (MM) by adding L-malate at t_0 = mid-exponential phase at four different concentrations (C_0) (abscissae axis). The recombinant β-galactosidase activity was monitored before the addition of inductor (as negative control of the experiment), and 2-8-24 hours after the induction (t_1). Numbers above bars refer to induction ratio (IR) by L-malate adding (t_1/t_0). Means and standard deviations are derived from four independent assays.

The second step of procedure provided the definition of optimal concentration of inductor to use to have high levels of expression of heterologous gene (C_0) at t_0 = mid-exponential phase. Four different concentrations of L-malate were chosen: 0,2 - 0,4 - 1 - 5%. The Induction Ratios (IR) were reported in fig 4. The maximal yield was obtained using C_0 = 0,4% L-malate in cell lysates collected 24 hours after the induction.

Using L-malate concentration corresponding to 1%, and in particular to 5%, a strong reduction in Induction Ratio (IR) was observed, probably due an effect of dicarboxylic acid on osmotic equilibrium of the cell; in fact when L-malate 5% was added to recombinant cells a decrease of cellular growth was observed (data not shown).

3.2 Comparison between the performances of the inducible and the constitutive expression system for the β-galactosidase production

In this study we compared the performances of inducible system P(PSHAb0363) and the constitutive expression system pP13 (Duilio, A. *et al.*, Uff. It. Brev. Marchi, 2003) for the production of β-galactosidase at two different growth temperatures (4° and 15°C) in minimal and complex medium, in the presence and in the absence of L-malate 0,2% , respectively.

*Ph*TAC125 recombinant cells were grown in duplicate in liquid culture at 4°C and 15°C for till the reaching of the stationary phase. The specific β-galactosidase activity produced by recombinant *Ph*TAC125 cells is reported in table 3.

For the production of β-galactosidase in P(PSHAb0363), the induction conditions previously described were used (t_0 = mid-exponential phase; C_0 = 0,4% L-malate; t_1 = 24 hours).

Reporter enzyme production in pP13 was maximum when the recombinant cells were grown in complex medium (Typ medium) and was independent by the presence of L-malate (table 3). β-

Table 3 β-galactosidase production yields from *Ph*TAC125 cells harbouring P(PSHA0363) and pP13 grown at 4° and 15°C in minimal medium (MM) and in rich medium (Typ) in the presence and in the absence of L-malate, respectively. The data shown are averages of 12 measurements. SD, standard deviation vectors.

	β-galactosidase production yield (mg/L) ± SD							
Temperature	4°C				15°C			
Medium / Promoter	MM	MM+L-malate	Typ	Typ+L-malate	MM	MM+L-malate	Typ	Typ+L-malate
P(PSHA0363)	4.11± 0.27	52.2± 1.2	10.7±0.6	32.6± 0.3	12.8±0.3	620± 24	5.6±1.0	25.0± 0.9
pP13	85.1±3.8	97.6±1.1	322±3	341±7	98.7± 7.3	113± 3	180± 13	187± 11

galactosidase production in pP13 at 4°C in Typ medium was twofold higher than that recorded at 15°C; in contrast, the temperature effect observed on the reporter enzyme production when pP13 recombinant cells were grown in minimal medium (MM) is very low.

β-Galactosidase production in P(PSHAb0363) was maximum when cells where grown at 15°C in MM in the presence of L-malate.

Reporter enzyme production in P(PSHAb0363) recombinant cells grown in the presence of L-malate both in complex and minimal medium was always higher than that recorded for the recombinant cells grown in the absence of it, suggesting a specific L-malate induction; however the induction ratio was higher when recombinant cells were grown in MM (table 4), in particular at 15°C.

Table 4 Induction Ratio (IR) determined for β-galactosidase production yield from *Ph*TAC125 cells harbouring P(PSHA0363) and pP13 grown in the presence and in the absence of L-malate 0.2% at 4° and 15°C in minimal medium (MM) and in rich medium (Typ), respectively (see table 3).

	Ratio + L-malate / - L-malate			
Temperature	4°C		15°C	
Medium / Promoter	MM	Typ	MM	Typ
P(PSHA0363)	12.7	3.1	48	4.5
pP13	1.15	1.1	1.2	1.0

We can conclude that pP13 displayed maximum transcriptional activity at 4°C in Typ medium while P(PSHAb0363) exhibited maximum transcriptional activity at 15°C in minimal medium in the presence of L-malate as inductor.

In conclusion, the inducible expression system P(PSHAb0363) assures an higher β-galactosidase production yield than that obtained using the constitutive system pP13.

3.3 Analysis of the mesophilic α-glucosidase production from *Saccharomyces cerevisiae* in *Ph*TAC125

When alpha-glucosidase is produced heterologously in *Escherichia coli*, it aggregated in insoluble granules and the active soluble amount of protein was less than 1% of the total production (Le Thanh H. and Hoffmann F. 2005).

*Sc*GLUCP1 gene was inserted into the inducible expression vector pUCRP, as described in Materials and methods section.

Recombinant vector was mobilized into *Ph*TAC125 cells. Psychrophilic transconjugants named pUCRPGLUCP1, were grown in MM medium, in the absence and in the presence of L-malate at two temperatures (4°C and 15°C) till a late exponential growth phase and cell pellets were collected.

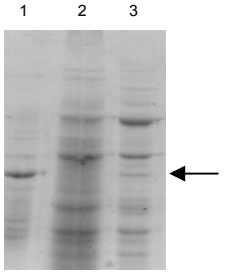

Fig. 3 Recombinant production of the mesophilic α-glucosidase from *Saccharomyces cerevisiae* in *Ph*TAC125 cells. 7.5% SDS PAGE gel electrophoresis of protein extract from *Ph*TAC125 cells harboring pUCRPGLUCP1 grown in minimal medium in the absence (lane 2) and in the presence (lane 3) of L-malate, in comparison with α-glucosidase inclusion bodies produced by recombinant *E. coli* cells (lane 1).

Soluble protein fractions were analysed by 7.5% SDS-polyacrilamide gel electrophoresis, and compared to the yeast α-glucosidase produced by pKK-177GLUCP1 recombinant *E. coli* cells as inclusion bodies. As shown in fig. 3, recombinant soluble fraction extracted from *Ph*TAC125 cells grown in the presence of L-malate, obtained from 15°C-grown cultures (lanes 3), is

characterized by the presence of an extra-band showing an electrophoretic mobility similar to that of α-glucosidase (lane 1). In the lane corresponding to recombinant soluble fraction extracted from *Ph*TAC125 cells grown in the absence of L-malate is not possible to evidence any band with an electrophoretic mobility similar to that of α-glucosidase (lane 2).

Furthermore, the analysis of the corresponding cellular insoluble fractions ruled out the occurrence of any accumulation of the heterologous enzyme as inclusion bodies in the cold-adapted host (data not shown). As already evidenced for β-galactosidase production, α-glucosidase is produced in *Ph*TAC125 under the control of inducible expression system only in the presence of L-malate.

Since wild-type *Ph*TAC125 cells are devoid of any α-glucosidase activity, the production of the recombinant enzyme in the soluble fractions was monitored by following the *p*-nitrophenol release from *p*–nitrophenyl-α-D-glucopyranoside (PNPG) substrate at 410nm (Kopetzki, E. *et al*., 1989).

For the production of α-glucosidase in pUCRP system, we used the induction conditions previously described for β-galactosidase production (t_0 = mid-exponential phase; C_0 = 0,4% L-malate; t_1 = 24 hours).

As reported in table 5, recombinant α-glucosidase activity was recorded in all conditions tested, and the higher α-glucosidase specific activity was observed in lysates from cells grown at 15°C in the presence of L-malate.

Table 5: α-glucosidase specific activities (mg/L) from *Ph*TAC125 cells harbouring pUCRPGLUCP1 at 4° and 15°C in minimal medium (MM) in the presence and in the absence of L-malate 0.2%, respectively. The data shown are averages of 12 measurements. SD, standard deviation vectors.

α-glucosidase activity (mg/L) ± SD		
	4°C	**15°C**
MM	**0.86 ± 0**	**0.64 ± 0**
MM+L-malate	**8.5± 0.8**	**26.7 ± 1.8**

3.2 Comparison between the performances of the inducible and the constitutive expression system for the α-glucosidase production.

In this study we also compared the α-glucosidase production yields in *Ph*TAC125 by using two different expression systems: the inducible system pUCRPGLUCP1 and the constitutive expression system pPM13GLUCP1 (Tutino, M.L. and Duilio, A., personal communication).

*Ph*TAC125 recombinant cells were grown in duplicate in liquid culture at 4°C and 15°C in minimal and complex medium, in the presence and in the absence of L-malate respectively, for till the reaching of the stationary phase. Recombinant α-glucosidase produced in *Ph*TAC125 cells, in both expression systems, resulted to be totally soluble and active (data not shown). The specific α-glucosidase activity produced by recombinant *Ph*TAC125 cells is reported in table 6.

α-Glucosidase production in pPM13 was

Table 6. α-glucosidase specific activities from *Ph*TAC125 cells harbouring pUCRPGLUCP1 and pPM13GLUCP1 grown at 4° and 15°C in minimal medium (MM) and in rich medium (Typ) in the presence and in the absence of L-malate, respectively. The data shown are averages of 12 measurements. SD, standard deviation vectors.

	α-glucosidase specific activity (mg/L) ± SD							
Temperature	4°C				15°C			
Medium / Promoter	MM	MM+L-malate	Typ	Typ+L-malate	MM	MM+L-malate	Typ	Typ+L-malate
pUCRPGLUCP1	0.86 ± 0	8.5± 0.8	0.40± 0.05	2.05± 0.03	0.64 ± 0	26.7 ± 1.8	0.43± 0.04	9.22± 0.04
pPM13GLUCP1	0	2.80± 0.03	21.4±0.9	22.6±0.5	0	3.30± 0.05	11.7±0.6	12.1±0.5

maximum when the recombinant cells were grown in complex medium (Typ medium) at 4°C and it was independent by the presence of L-malate (table 6).; when pPM13GLUCP1 recombinant cells were grown in minimal medium (MM) no temperature effect was observed on the enzyme production. In contrast, α-Glucosidase production in pUCRP was maximum when cells where grown at 15°C in MM in the presence of L-malate.
Also in this case the induction ratio for pUCRPGLUCP1 is higher when recombinant cells were grown in MM (table 7), while no induction effect is reported for pPM13GLUCP1.

Table 7 Induction Ratio (IR) determined for α-glucosidase specific activities from *Ph*TAC125 cells harbouring pUCRPGLUCP1 and pPM13GLUCP1 grown in the presence and in the absence of L-malate at 4° and 15°C in minimal medium (MM) and in rich medium (Typ), respectively (see table 6).

	Ratio + L-malate / - L-malate			
Temperature	4°C		15°C	
Medium / Promoter	MM	Typ	MM	Typ
pUCRPGLUCP1	9.9	5.1	41.2	21.4
pPM13GLUCP1	-	1.0	-	1.1

However the maximal production yield obtained for α-glucosidase enzyme in *Ph*TAC125 is significantly lower than that obtained for β-galactosidase, presumably because α-glucosidase is an eukaryotic gene and its expression in a bacterium could be influenced by the different codon usage between eukaryotes and bacteria.
About the performances of the inducible cold expression system, we obtained similar α-glucosidase production yields in comparison with the best constitutive cold expression system at our disposition; nevertheless the possibility to have an inducible expression system allows to separate the bacterial growth phase from the production phase, which could be particularly useful for the production of proteins that result toxic or dangerous for bacterial growth and survival.
In conclusion, on the contrary to the heterologous production in mesophilic host *Escherichia coli*, the recombinant production of the α-glucosidase from *Saccharomyces cerevisiae* and of the β-galactosidase from *Pseudoalteromonas haloplanktis* TAE79 in cold loving microrganism *Ph*TAC125 results to be totally soluble and active.

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1.3 Conclusions

An *in silico* analysis performed on the *Ph*TAC125 genome sequence (Medigue, C. *et al*., 2005) showed that, in contrast to many γ-proteobacteria (including vibrios), this cold adapted microorganism does not possess any member of the classical PTS (Kornberg, H.L. 2001; Tchieu, J.H. *et al.*, 2001; Plumbridge, J. 2002; Siebold, C. *et al.*, 2001), dedicated to the transport and modification steps needed for carbohydrates metabolism. Moreover the genes encoding key enzymes of the Embden-Meyerhoff pathway could not be identified. In contrast, the *Ph*TAC125 genome does contain the genes coding for the enzymes of the alternative metabolic Entner-Doudoroff pathway.

In order to define the favourite carbon source for *Ph*TAC125 we analyzed its growth behaviour in minimal medium both in the presence and in the absence of four different carbon sources: D-glucose, D-fructose, D-xylose and L-malate. Data obtained showed that *Ph*TAC125 i) is unable to grow on fructose and xylose; ii) glucose is metabolized very slowly; whereas iii) L-malate is degraded at a considerable rate during cellular growth (fig. 1, chapter 1.2.1).

The analysis of the proteome of *Ph*TAC125 was performed using two-dimensional gel (2-D) on the basis of the genome sequence (Medigue, C. *et al*., 2005), and the differences in the protein expression profile of cells grown in minimal medium in the presence and in the absence of L-malate as carbon source were identified.

Approximately 270 protein spots were identified in the gels and a classification into functional groups of the proteins identified was carried out. Among these, approximately 40 enzymes involved in glycolysis and the TCA cycle, 36 enzymes involved in amino acid metabolism and 10 aminoacyl-tRNA synthetases were identified. Moreover, approximately 60 proteins involved in cell envelope and membrane-associated cellular processes were detected, some of them associated to the cell wall and others involved in membrane bioenergetics and motility. Only 10% of those were reported as unknown in the genome annotation (table 1, appendix). Using this approach, a total of 31 proteins differently expressed in the presence of L-malate was identified (table 2,chapter 1.2.1).

Our attention has been focused on the gene PSHAb0363 coding for a putative outer membrane porin (named *Phdct*A). In fact, the upstream region of the gene coding for the putative outer membrane porin has a rather specific organization. Two CDSs encoding a putative C4-dicarboxylates sensor kinase and a putative C4 response regulator were respectively located upstream of the porin gene. Furthermore, a CDS coding for a periplasmic transporter of dicarboxylic acids (named *Phdct*B) was found downstream of the porin gene. This association strongly suggests the occurrence of a two-component sensor regulatory system.

The transcriptional fusion experiments, performed using the complete genome region upstream of the porin gene (i.e. the genes coding for sensor kinase and C4 response regulator), demonstrated that this

regulatory element is remarkably influenced by the presence of L-malate in the growth medium (fig. 4, chapter 1.2.1).
These results demonstrated that *Ph*TAC125 possesses a functionally active two-component regulatory system for sensing C4-dicarboxylic acids in the environment (named *Ph*MAlRS)(Papa, R. *et al.*, 2005)
The DNA region located upstream the *PhdctA* porin gene was further investigated. The transcriptional regulation of the *PhMalRS* and *PhdctAB* loci was first investigated by RT-PCR experiments. The results reported indicated that each gene in both the *Ph*MalRS and *Ph*DctAB loci is transcribed by its own promoter. However, functional differences exist among the different promoter. The promoter element located upstream the *PhMalS* gene is only active in the presence of L-malate whereas the promoter located upstream the *PhdctA* gene is able to dictate transcription of *PhMalA* in both conditions but it is strongly induced by L-malate. On the contrary, the presence of L-malate appeared to be irrelevant on *PhdctB* transcription (fig. 3, chapter 1.2.2).
The promoter region upstream *PhdctA* region was investigated by primer extension experiments. The results of these analyses revealed two different transcriptional start sites, a proximal start site P1 and a distal start site P2 (fig. 4, chapter 1.2.2) A putative σ^{70} promoter element, according to the σ^{70}-dependent consensus sequence for *Ph*TAC125, was identified upstream the proximal start site, while a putative σ^{54} promoter element was inferred upstream the distal transcriptional start site, on the basis of a consensus sequence deriving from a collection of 186 promoters from 47 different bacterial species (Studholme, D.J. and Dixon, R., 2003).
77 bp located upstream the distal transcriptional start site P2 was also identified a 53 bp inverted repeat sequence (fig. 4, chapter 1.2.2). Functional analysis of this element showed that it was specifically recognised by protein extracts of *Ph*TAC125 only when the cells were grown in the presence of L-malate, suggesting that this DNA region represents the *cis*-acting region requested for the binding of transcriptional regulatory proteins leading to the induction of the *PhdctAB* operon expression (fig. 5, chapter 1.2.2).
In conclusion, we used the regulative region, comprising the two-component system located upstream the PSHAb0363 gene, to construct an inducible expression vector, named pUCRP (fig. 1, chapter 1.2.3).
We demonstrated that the inducible expression system was effective in the production of the psychrophilic β-galactosidase from *Ph*TAE79 and mesophilic α-glucosidase from *Saccharomyces cerevisiae*. The performances of the inducible and the constitutive system (Duilio, A. *et al.*, 2004) for the production of a psychrophilic β-galactosidase and a mesophilic α-glucosidase were also compared (tables 3-6, chapter 1.2.3). Data presented demonstrated that when these proteins are produced in *Ph*TAC125, they are completely soluble and catalytically competent in both expression systems used.

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PART 2

Engineering of a psychrophilic microorganism for the degradation of aromatic compounds

2.1 Introduction

2.1.1 Microbial degradation of aromatic pollutants: a paradigm of metabolic versatility

Environmental pollutants are compounds that are toxic to living organisms; they are released into the ecosystem at high concentrations, usually as a consequence of human activities. Contaminants are either compounds of industrial origin that present chemical structures alien to the biosphere (xenobiotics), e.g. polychlorobiphenyls (PCBs), polichlorodioxins, trinitrotoluene (TNT) and azo dyes, or natural compounds that have been mobilized to a bioavailable form that is toxic to organisms, e.g. hydrocarbons present in fossil fuels and heavy metals present in minerals (Fig. 1). Major sources of pollution are: (i) chemical and pharmaceutical industries that produce a wide array of xenobiotics and synthetic polymers; (ii) pulp and paper bleaching, which are the main sources of chlorinated organic compounds in the environment; (iii) mining, which releases heavy metals into biogeochemical cycles; (iv) fossil fuels (coal and petroleum), which may be accidentally released in large amounts into the ecosystem (oil spills) and whose combustion increases significantly CO_2 atmospheric levels (green-house effect) and causes deposition of nitric and sulfuric acids (acid rain and smog); and (v) intensive agriculture, which releases massive amounts of fertilizers, pesticides, and herbicides (Dua, M. *et al.*, 2002 ; Rieger, P.G. *et al.*, 2002).

The removal of pollutants from the environment via natural physico-chemical and biological processes (natural attenuation) is, in general, a slow and unpredictable way of counteracting anthropogenic pollution and irreversible damage to the biosphere. Therefore, the main, if not the only, successful strategy to fight pollution is the use and manipulation of the detoxification abilities of living organisms (bioremediation) (Fig. 1) (Lovley, D.R. 2003; Dua, M. *et al.*, 2002; Wackett, L.P. 2003; Wackett, L.P. *et al*, 2000; Watanabe, M.E. 2001).

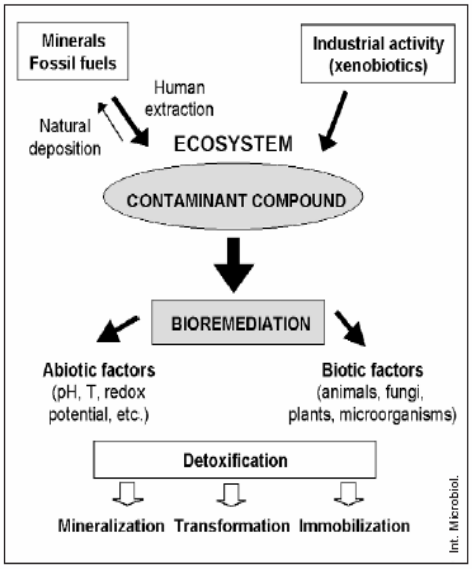

Fig. 1. Main sources of pollution in the ecosystem and the factors that influence bioremediation processes.

Although most organisms are endowed with detoxification abilities, i.e mineralization, transformation and/or immobilization of pollutants, microorganisms, particularly bacteria, have been the most well-studied and the most frequently used for bioremediation strategies. Bacteria, which evolved more than three billion years ago, have developed strategies for obtaining energy from virtually every compound. They play a crucial role in sustainable development of the biosphere and in biogeochemical cycles. The abundance of microorganisms, together with their great ability for

horizontal gene transfer and their high growth rates, allows them to evolve quickly and to adapt to environmentally changing conditions, even to extreme environments that do not allow proliferation of other living organisms. The great genetic diversity of microorganisms accounts for their great metabolic versatility (De Lorenzo, V. 2001; Lovley, D.R. 2003; Timmis, K.N. *et al.*, 1999).

2.1.2 Microbial utilization of aromatic compounds

Next to glucosyl residues, the benzene ring is the unit of chemical structure most widely spread in nature. Moreover, the thermodynamic stability of the benzene ring increases its persistence in the environment; therefore, many aromatic compounds are major environmental pollutants (Dagley, S. 1986). By expressing different catabolic (biodegradative) pathways, microorganisms can use a wide array of aromatic compounds as sole carbon and energy sources (Harayama, S. *et al.*, 1992). The general ability of bacteria to use such compounds is related to the fact that most of these compounds are commonly present in the environment as a result of the recycling of plant-derived material (Harwood, C.S. *et al.*, 1996). Human-made xenobiotic compounds, by contrast, have been in contact with the microflora only for about 100 years; therefore, some of them are still poorly degraded, if at all. Oxygen is the most common final electron acceptor for microbial respiration, and aerobic processes provide the highest amount of energy to cells (Fig. 2) (Field, J.A. *et al.*, 1995). In chemotrophic reactions, a portion of the substrate is oxidized to obtain energy and another part is assimilated into cell mass. In aerobic respiration, oxygen not only is the electron acceptor but also participates in activation of the substrate via oxygenation reactions.

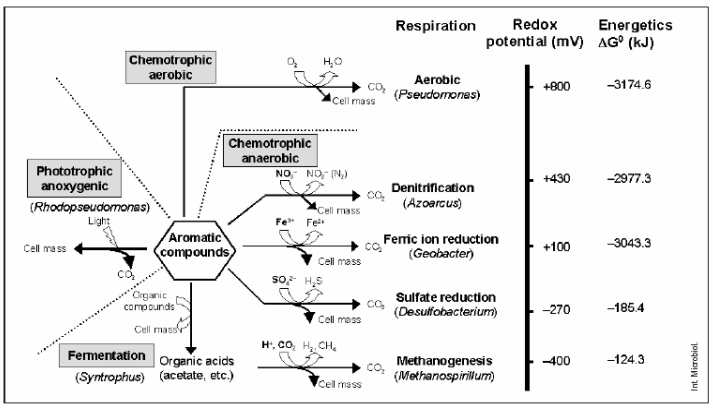

Fig. 2.
Microbial utilization of aromatic compounds. The different terminal electron acceptors in respiration are indicated in bold and they are aligned with the redox potential bar. The energetics (free-energy changes) of the aerobic and anaerobic degradation of a model aromatic compound, benzoate, are indicated on the right. Methanogenesis needs to be coupled to fermentation reactions. Bacterial genera representative of each type of metabolism are shown in parentheses. Modified from (Widdel, F. *et al.*, 2001).

Although a wide phylogenetic diversity of microorganisms is capable of aerobic degradation of contaminants, *Pseudomonas* species and closely related organisms have been the most extensively studied owing to their ability to degrade so many different contaminants (Wackett, L.P. 2003). However, many polluted environments are often anoxic, e.g., aquifers, aquatic sediments and submerged soils. In such environments, biodegradation is carried out by either strict anaerobes or facultative microorganisms using alternative electron

acceptors, such as nitrate (denitrifying organisms), sulphate (sulfate reducers), Fe(III) (ferric-ion reducers), CO_2 (methanogens), or other acceptors (chlorate, Mn, Cr, U, etc.) (Fig. 2) (Gibson, J. *et al.*, 2002; Lovley, D.R. 2003; Widdel, F. *et al.*, 2001).

Aerobic and anaerobic biodegradation of aromatic compounds have several common features. Structurally diverse compounds are degraded through many different peripheral pathways to a few intermediates that are further channelled via a few central pathways to the central metabolism of the cell. In the aerobic catabolic funnel, most peripheral pathways involve oxygenation reactions carried out by monooxygenases and/or hydroxylating dioxygenases that generate dihydroxy aromatic compounds (catechol, protocatechuate, gentisate, homoprotocatechuate, homogentisate, hydroquinone, hydroxyquinol). These intermediate compounds are the substrates of ring-cleavage enzymes that use molecular oxygen to open the aromatic ring between the two hydroxyl groups (ortho cleavage, catalyzed by intradiol dioxygenases) or proximal to one of the two hydroxyl groups (meta cleavage, catalyzed by extradiol dioxygenases) (Fig. 3) (Harayama, S.*et al.*, 1992). Aerobic bacterial degradation of aromatic hydrocarbons is generally divided into two major routes (Arenghi, F.L. *et al.*, 2001; Harayama, S. *et al.*, 1989), the so-called upper pathway, which leads to the formation of partially oxidized aromatic intermediates, and a lower pathway, which uses dihydroxylated aromatic molecules. These activated aromatic compounds undergo ring cleavage reactions and are further processed to give molecules that can eventually enter the citric acid cycle (Cafaro, V. *et al.*, 2004).

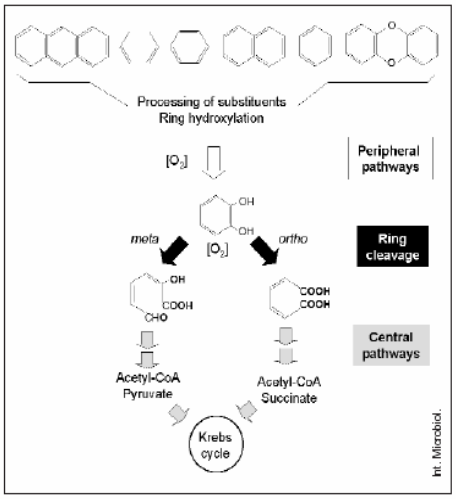

Fig. 3. The catabolic funnel for the aerobic degradation of aromatics. **White arrows**, peripheral pathways, **black arrows**, cleavage of the ring; **gray arrows**, central pathways.

2.1.3 Genetics and evolution of biodegradation pathways

The genes responsible for biodegradation pathways are usually arranged in clusters that comprise: (i) catabolic genes encoding the enzymatic steps of the catabolic pathway; (ii) transport genes responsible for active uptake of the compound; and (iii) regulatory genes that adjust expression of the catabolic and transport genes to the presence of the compound to be degraded (Diaz, E. *et al.*, 2000). Bacteria thriving in the environment are exposed to a range of physical and chemical signals that need to be processed to achieve a positive or negative physiological response. For instance, bacteria are usually confronted with alternative carbon sources and they must “decide” which of them will be preferentially consumed before metabolizing less preferred substrates, such as pollutants, to guarantee, therefore, a satisfactory metabolic return. In order to achieve this goal, bacteria have developed a physiological response (superimposed

regulation) that controls and adjusts the specific regulation of catabolic operons to the physiological and metabolic state of the cells (Cases, I. *et al.*, 2001). The catabolic clusters are usually present in mobile genetic elements, such as transposons and plasmids, which facilitate their horizontal transfer of the respective genes and, therefore, rapid adaptation of microorganisms to the presence of new pollutants in a particular ecosystem (Tan, H.M. 1999; Van der Meer, J.R. *et al.*, 1992).
Although microorganisms have acquired the ability to use pollutants as carbon and energy sources, their efficiency at removing such pollutants might not be optimal for cleaning up present-day pollution. In fact, microorganisms have evolved towards ecological fitness rather than biotechnological efficiency; thus, it would take a long time for bacteria capable of cleaning up anthropogenic pollution to evolve by natural selection. Hence, studying the physiology, biochemistry and genetics of the catabolic pathways becomes crucial to recreate and accelerate natural processes in the test tube as well as to accomplish their rational manipulation to design more efficient biocatalysts for different biotechnological applications. These include: (i) bioremediation of polluted sites, (ii) biotransformation of toxic compounds into fine chemicals and other high added-value products (green chemistry), and (iii) development of *in situ* biomonitoring devices and biosensors to monitor pollutant bioavailability (De Lorenzo, V. 2001; Schmid, A. *et al.*, 2001, Timmis, K.N. *et al*, 1999).

2.1.4 *Pseudomonas* species

Prokaryotes from archaea to eubacteria possess the capacity to biotransform aromatic compounds either as pure culture or in consortia. However, the aerobic aromatic catabolic pathways of pseudomonads (and closely related microorganisms) (table 1) together with those of their associated plasmids are the most extensively studied workhorses of environmental bioremediation of this abundant class of pollutants.

Table 1: List of *Pseudomonas* strains degrading aromatic compounds (Shingler, V. 2003)

Pathway	Host	Reference
(Methyl)phenols	*Pseudomonas* sp.CF600 (pVI50)	Shingler *et al*. (1993)
2-Hydroxybiphenyl	*P. azelaica* HBP1	Jaspers *et al*. (2000)
Phenol	*Acinetobacter calcoaceticus* NCIB8250	Schirmer *et al*. (1997)
Phenol	*P. putida* H (pPGH1)	Burchhardt *et al*. (1997)
Phenanthrene/naphthalene	*Burkholderia* sp. RP007	Laurie and Lloyd-Jones (1999)
Toluene	*Ralstonia pickettii* PKO1	Byrne and Olsen (1996)
Toluene	*P. stutzeri* OX1	Arenghi *et al*. (1999)
Toluene/xylene	*P. putida* mt-2 (TOL pWW0)	Inouye *et al*. (1988)
Biphenyl/PCB	*Rhodococcus* sp. strain M5	Labbe *et al*. (1997)
Styrene	*P. putida* CA-3	Velasco *et al*. (1998)
Toluene	*P. putida* F1	Lau *et al*. (1997)
4-Hydroxybenzoate	*Pseudomonas putida* WCS358	Bertani *et al*. (2001)
Toluene	*P. putida* mt-2 (TOL pWW0)	Spooner *et al*. (1986)
Benzoate pathway	*P. putida*	Cowles *et al*. (2000)
Catechol	*P. putida*	Rothmel *et al*. (1990)
Chlorocatechol	*P. putida* (pAC27)	Coco *et al*. (1993)
Naphthalene/salicylate	*P. putida* (pNAH7)	You *et al*. (1988)
2,4-Dichlorophenoxyacetic acid	*Ralstonia eutropha* JMP134 (pJP4)	Leveau and van der Meer (1996)

At >6 million bp, *Pseudomonas* genomes are among the largest of the ≈ 70 bacterial genomes sequenced to date. Consistent with their ability to

thrive in diverse environments and execute efficient catabolism of a broad spectrum of carbon sources, *Pseudomonas* genomes contain the highest proportion of regulatory genes observed, and a high proportion of genes dedicated to the catabolism, transport and efflux of organic compounds (Nelson, K.E. *et al* ., 2002).

2.1.4.1 *Pseudomonas stutzeri* OX1

Several bacteria isolated so far can degrade *m*-xylene and *p*-xylene through the progressive oxidation of a methyl group (Assinder, S.J. *et al.*, 1990). *o*-Xylene cannot enter in this pathway, and in the few *o*-xylene-degrading bacteria described so far, this isomer appears to be oxidized via pathways involving either mono- or dioxygenation reactions of the aromatic ring without any processing of the methyl groups (Bertoni, G. *et al.*, 1996).

Table 2: The range of substrates and the reaction products of the catabolic enzymes from *P.stutzeri* OX1 (Barbieri, P. *et al.*, 2001).

Toluene/*o*-xylene monooxygenase		Phenol hydroxylase		Catechol 2,3-dioxygenase
Benzene	Phenol	*	Catechol	+
Toluene	*o*-cresol	*	3-Methylcatechol	+
	m-cresol	*	3-Methylcatechol	+
o-Xylene	2,3-Dimethylphenol	*	4-Methylcatechol	+
	3,4-Dimethylphenol	*	3,4-Dimethylcatechol	+
m-Xylene	2,4-Dimethylphenol		3,4-Dimethylcatechol	+
p-Xylene	2,5-Dimethylphenol		3,5-Dimethylcatechol	-
Ethylbenzene	2-Ethylphenol		3,6-Dimethylcatechol	-
	3-Ethylphenol		NT	NT
	4-Ethylphenol		NT	NT
Styrene	2-Vinylphenol		NT	NT
	3-Vinylphenol		NT	NT
Naphtalene	α-Naphtol		NT	NT
	β-Naphtol		NT	NT
			NT	NT

P. stutzeri OX1 was isolated from activated sludges of a mixed civic and industrial wastewater treatment plant. It has the ability to utilize *o*-xylene, the most recalcitrant of the xylene isomers, as the only carbon and energy source (Baggi, G. *et al.*, 1987), and can also grow on toluene, cresols, and some dimethylphenols (table 2) (Barbieri, P. *et al.*, 1989).

Another feature that contributed to the adaptation of this strain to a polluted environment is its resistance to organic and inorganic mercury compounds. In *P.stutzeri* OX1, mercury resistance is plasmid encoded whereas the genes involved in the hydrocarbon catabolism are located on the chromosome (Barbieri, P. *et al.*, 1989).

Several physiological evidences had previously suggested that the catabolism of aromatic compounds proceeds through the same catabolic route and involves oxygenation reactions of the aromatic ring to form mono- and dimethylcatechols which are subsequently catabolized by a *meta*-cleavage pathway (Baggi, G. *et al.*, 1987; Barbieri, P. *et al.*, 1989).

Two monooxygenases have been found in the genome of *P.stutzeri* OX1, phenol hydroxylase (PH) (Arenghi, F.L. *et al.*, 2001) and toluene *o*-xylene monooxygenase (ToMO) (Bertoni, G. *et al.*, 1996; Bertoni, G. *et al.*,1998) (Fig. 4).

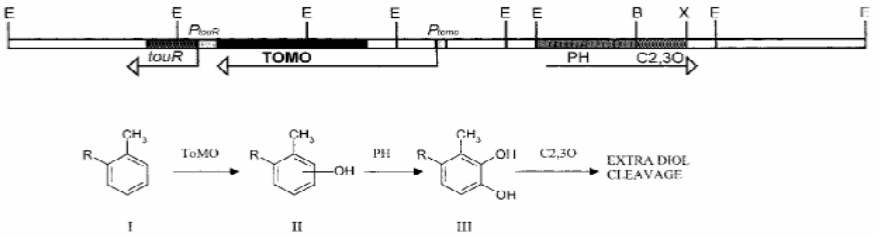

Fig. 4
The map of the *P. stutzeri* OX1 region encoding the functions involved in the degradation of toluene (R=H) and *o*-xylene (R=CH3) and the reactions catalyzed by the catabolic enzymes. The hydrocarbons (I) are converted to mono- or dimethylphenols (II) through the action of toluene/*o*-xylene monooxygenase (ToMO) and subsequently to mono- or dimethylcatechols (III) by phenol hydroxylase (PH). Catechol 2,3-dioxygenase (C2,3O) then catalyzes the cleavage of the aromatic ring.

Monooxygenases are key enzymes in the upper pathway and catalyze hydroxylation of the aromatic ring at different positions (Olsen, R.H. *et al.*, 1994; Shields, M.S. *et al.*, 1989; Yen, K.M. *et al.*, 1991).
Recently (Notomista, E. *et al.*,2003), it has been recognized that bacterial multicomponent monooxygenases (BMMs) constitute a family of enzymes which can be divided into six distinct groups, each with a characteristic subunit composition (table 3).

Table 3: BMM classification according to operon composition and structure (Notomista, E. *et al.*, 2003).

Organism	Operon	BMMP[a]	N of subunits
Comamonas testosteroni R5	phcKLMNOP	PH	6
Comamonas testosteroni TA441	aphKLMNOP	PH	6
Pseudomas sp. JS150	tbmABCDEF	T2BMO	6
Ralstonia sp. KN1	phyZABCDE	PH	6
Ralstonia eutropha ReuE26	poxABCDEF	PH	
Pseudomonas putida P35X	phhKLMNOP	PH	6
Pseudomonas sp. CF600	dmpKLMNOP	PH	6
Pseudomonas putida H	phlABCDEF	PH	6
Acinetobacter sp. 20B	dsoABCDEF	DMSO	6
Acinetobacter calcoaceticus	orfl23456	PH	6
Burkholderia cepacia G4	tomA012345	T2MO	6
Burkholderia cepacia JS150	tbclABCDEF	MO	6
Burkholderia kururensis		MO	6
Ralstonia metallidurans CH34	—	MO[b]	6
Alcaligenes eutrophus JMP134	phlKLMNOP	MO	6
Burkholderia picketii PKO1	tbuA1UBVA2C	T3MO	6
Pseudomonas aeruginosa JI104	bmoABCD + 2ORF?	BMO	6?
Burkholderia cepacia AA1	tbhABCDEF	T3MO	6
Pseudomonas stutzeri OX1	touABCDEF	TOMO	6
Pseudomonas mendocina KR1	tmoABCDEF	T4MO	6
Rhodococcus sp. AD45	isoABCDEF	IMO	6
Xanthobacter sp. Py2x	amoABCDEF	AMO	
Burkholderia cepacia JS150	tbc2ABCDEF	MO	6
Ralstonia metallidurans CH34	—	MO[b]	6
Methylocystis sp. M	mmoXYBZC	MMO	5
Methylosinus trichosporium OB3B	mmoXYBZC	MMO	5
Methylococcus capsulatus Bath	mmoXYBZC	MMO	5
Methylomonas sp. KSPIII	mmoXYBZC	MMO	5
Nocardia corallina B-276	amoABCD	AMO	4
Pseudonocardia sp. K1	thmADBC	THF MO	4
Rhodobacter sphaeroides 2.4.1	—	MO[b]	4
Sulfolobus solfataricus P2—	MO	MO[b]	?

a
PH, phenol hydroxylase;
MMO, methane monooxygenase;
TMO, toluene monooxygenase;
BMO, benzene monooxygenase;
AMO, alkene monooxygenase;
DMSO, dimethylsulde oxygenase;
IMO, isoprene monooxygenase;
TOMO, toluene o-xylene monooxygenase;
THF MO, tetrahydrofuran monooxygenase
TBMO, toluene/ benzene monooxygenase
b
Hypothetical

BMMs are transcribed from single operons that code for four to six polypeptides. Analysis of the sequences from nucleotide and protein databases indicates that most bacterial strains possess only one BMM, but a few cases (3 out of 31) of bacterial genomes coding for more than one monooxygenase have been found (Notomista, E. *et al.*, 2003).

2.1.4.2 Toluene/*o*-xylene-monooxygenase from *P. stutzeri* OX1

Toluene/o-xylene-monooxygenase (ToMO) from P*seudomonas stutzeri* OX1 is endowed with a broad spectrum of substrate specificity, and the ability to hydroxylate more than a single position of the aromatic ring in two consecutive monooxygenation reactions (Bertoni, G. *et al.*, 1996). Thus ToMO is able to oxidize *o*-, *m*- and *p*-xylene, 2,3- and 3,4-dimethylphenol, toluene, cresols, benzene, naphthalene, ethylbenzene, styrene (Bertoni, G. *et al.*, 1996), trichloroethylene, 1,1-dichloroethylene, chloroform (Chauhan, S. *et al.*, 1998) and tetrachloroethylene (Ryoo, D. *et al.*, 2000). This makes the complex unique with respect to other known monooxygenases, and potentially useful for its use in bioremediation strategies (Chauhan, S. *et al.*, 1998; Ryoo, D. *et al.*, 2000; Sullivan, J.P. *et al.*, 1998) and/or the synthesis of commercially valuable compounds (Gallagher, S.C. *et al.*, 1997). The nucleotide sequence revealed six CDSs, named *tou* A, B, C, D, E and F (*tou*, for toluene/*o*-xylene utilization), which showed relevant similarities to the subunits of several enzymatic complexes involved in the oxygenation of aromatic compounds (Bertoni, G. *et al.*, 1998). On the basis of homology studies of the coding gene sequence it has been hypothesized that the gene products of the cluster form an electron transfer complex in which ToMO F, a NADH oxidoreductase, is the first member of the electron transport chain. ToMO F is able to transfer electrons from NADH to ToMO C, which is a Rieske-type ferredoxin that tunnels electrons to the terminal oxygenase, the ToMO H subcomplex composed by the *tuo*A, *tou*B and *tou*E gene products. Finally, another member of the complex is subunit ToMO D, for which a regulatory function has been suggested (Bertoni, G. *et al.*, 1998; Scognamiglio, R. *et al.*, 2001) (Fig. 5).

Recombinant ToMO has been expressed and reconstituted in vitro and a catalytic characterization has been carried out (Cafaro, V. *et al.*, 2002).

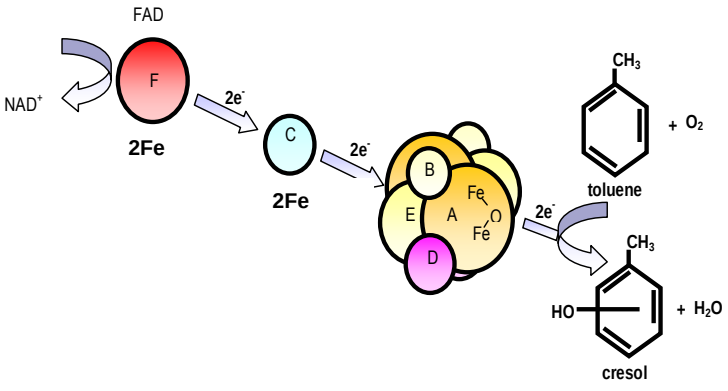

Fig. 5: Schematic representation of reaction mechanism proposed for ToMO.

Toluene/*o*-xylene-monooxygenase is able to convert phenolic intermediates into catechols. As could be predicted, *o*-cresol, *p*-cresol, and 2,3- dimethylphenol are converted into 3-methylcatechol, 4-methylcatechol, and 3,4-dimethylcatechol,

respectively. *m*-Cresol and 3,4-dimethylphenol are converted mainly into 4-methylcatechol and supposedly 4,5-dimethylcatechol, respectively. Thus, according to these results, the early stages of toluene and *o*-xylene oxidation in *P. stutzeri* OX1 consist of two subsequent monooxygenations has been proposed (Cafaro, V. *et al*., 2004). First, toluene or *o*-xylene is converted into a mixture of all the possible methylphenols, and then the methylphenols are converted into methylcatechols. In the latter reaction, the regioselectivity of hydroxylation appears more restricted, with a preference for the position more distant from the methyl group(s).

2.1.5 Microbial degradation of aromatic pollutants at low temperatures

The idea of using microorganisms to reduce environmental contamination, such as in soils and waste waters, is not new but appears to be feasible alternative to physicochemical methods (Timmis, K.N. *et al.*, 1999).

Although many pollution problems occur in sea waters and in effluents of industrial processes which are characterised by low temperatures, considerable effort has been directed toward the genetic manipulation of mesophilic bacteria to create or improve their ability to degrade various pollutants. In temperate regions, large seasonal variations in temperature reduce the effectiveness of microorganisms in degrading organic pollutants. However, bioaugmentation and inoculation of contaminated environments with specific cold adapted microorganisms should be helped to improve the biodegradation of recalcitrant chemicals (Gerday, C. *et al.*, 2000).

With the aim to investigate the degradation of aromatic compounds at low temperatures the Antarctic psychrophilic bacterium, *Pseudoalteromonas haloplanktis* TAC125 (see part 1, Introduction) was considered the likely candidate for the production of a recombinant aromatic degradation activity in a broad range of temperatures. The recent development of a shuttle genetic system for the transformation of cold adapted Gram-negative bacterium *Ph*TAC125 has made possible the isolation of psychrophilic promoters and the construction of a cold expression system (see part 1, Introduction). Therefore, *Ph*TAC125 was used as representative recipient for a biodegradative gene belonging to the upper pathway of *Pseudomonas stutzeri* OX1, the gene coding for the toluene/o-xylene-monooxygenase.

A first characterization of recombinant *Ph*TAC125 cells expressing *tou* cluster was already carried out (Siani, L. PhD Thesis, 2004). Here an extensive analysis of ToMO catalytic performances in a broad range of temperature on different aromatic substrates has been reported.

Data obtained support the possibility of developing specific degradative capabilities for the bioremediation of chemically contaminated marine environments and of industrial effluents characterised by low temperatures, using a naturally cold adapted bacterium.

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2.2 Results and Discussion

Recombinant expression of Toluene *o*-Xylene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1 in a marine Antarctic bacterium: *Pseudoalteromonas haloplanktis* TAC 125

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Abstract

A psychrophilic bacterium, *Pseudoalteromonas haloplanktis* TAC 125, isolated from seawater, was used as recipient for a biodegradative gene of the mesophile *Pseudomonas stutzeri* OX1. *tou* cluster, coding for toluene-*o*-xilene monooxygenase, was successfully cloned into a cold expression vector. Catalytic parameters of the recombinant microorganisms have been determined. Production of catalytically efficient TAC/*tou* microorganisms supports the possibility of developing specific degradative capabilities for the bioremediation of chemically contaminated marine environments and of industrial effluents characterised by low temperatures.

1. Introduction

Microbial degradation of aromatic hydrocarbons has been extensively studied with the aim of developing applications for the removal of toxic compounds from contaminated environments. Degradation of aromatic compounds by aerobic bacteria is generally divided into an upper pathway, which produces dihydroxylated aromatic intermediates by the action of monooxygenases, and a lower pathway that processes these intermediates down to molecules that can enter the citric acid cycle [1]. Although many contamination problems occur in sea waters and in effluents of industrial processes which are characterised by low temperatures, efforts have been directed only toward genetic manipulation of mesophilic bacteria to create or improve their ability to degrade various pollutants.

An antarctic psychrophilic microorganism, *Pseudoalteromonas haloplanktis* TAC 125 [2], was chosen as a likely candidate for the production of a recombinant strain able to degrade aromatic compounds to be used in a broad range of temperatures. The recent development of a shuttle genetic system for the transformation of cold adapted Gram-negative bacterium *Ph*TAC125 has made possible the isolation of psychrophilic promoters and the construction of a cold expression system [3]. Therefore, *Ph*TAC125 was used as representative recipient for a biodegradative gene belonging to the upper pathway of *Pseudomonas stutzeri* OX1. *Pseudomonas stutzeri* OX1, a mesophilic bacterium isolated from activated sludge [1], is able to grow on a wide spectrum of aromatics, including phenol, cresols, and dimethylphenols, but also on non-hydroxylated molecules such as toluene, *o*-xylene, and benzene [1].

In this paper we report the construction of a cold expression system and the cloning in this vector of the *tou* cluster from *P.stutzeri* OX1, coding for toluene-*o*-xilene monooxygenase (ToMO), that catalyses the hydroxylation of aromatic rings. Moreover, a preliminary characterization of the recombinant strain is also reported.

2. Materials and Methods

2.1 Bacterial strains and microbiological techniques.

The Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) [2], collected in

1992 from seawater near the French Antarctic Station Dumont d'Urville (60°40'; 40°01'E) during several summer campaigns of the Expeditions Polaires Francaise in Terre Adélie, was grown under aerobic conditions at 4°, 15° and 25°C in TYP broth (16 gr/l yeast extract, 16 gr/l bacto tryptone, 10 gr/l marine mix) at pH 7.5, supplemented with 100μg/ml ampicillin when transformed.

E. coli strain S17-1(λ*pir*) was used as donor in intergeneric conjugation experiments [4]. *E. coli* cells were routinely grown in LB broth [5]; 100 μg/ml of ampicillin was used as selection marker. Antarctic bacteria transformation was achieved by interspecific conjugation between transformed *E. coli* S17-1(λ*pir*) cells (donor strain) and the Antarctic host cells (recipient strain) following the procedure previously described [6].

Plasmid pBZ1260 containing a 6Kb fragment coding for monooxygenase activity isolated from *P. stutzeri* OX1 was kindly provided by Dr.P.Barbieri (Dipartimento di Biologia strutturale e funzionale, Universià dell'Insubria, Varese-Italy). Both wild type and recombinant microorganisms were grown in TYP broth supplemented with ampicillin when transformed. Mid-logarithmic-phase cells were used to inoculate identical flasks to a standard optical density value of 0.05-0.1. At different times, growth rates were measured spectrophotometrically (Cary UV lamp) at 600nm.

2.2 Construction of expression vector (pPM4) for the production of Toluene *o*-Xylene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1 in *Ph*TAC125.

The pPM4 cold-adapted gene-expression vector was constructed by cloning the P4 promoter from *Ph*TAC125 [3] into pUCLT/Rterm vector, which represents a promoter-less intermediate during the pFF vector construction [7]. The pP4 psychrophilic promoter-containing vector was isolated from a promoter-trap library [3], and the DNA fragment corresponding to the cold-active promoter was PCR amplified by using a universal primer pair designed on the vector and inserting *Hind*III (pPLBfw) and *Nde*I *Pst*I (pPLBrv) restriction sites, respectively. Amplification was performed in a mixture containing 60 ng of template, 50 pmoles of each oligonucleotide primer, 1.8 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl pH 8.3, 0.1% gelatine, 200 μM dNTP in a final volume of 50 μl. The mixture was incubated at 95°C for 10 min, after which 1,25 units of Taq DNA polymerase were added. Twenty cycles of amplification (consisting of 1 min at 95°C, 1.5 min at 55°C and 1 min +5sec/cycle at 72°C) were carried out and were followed by a cycle in which the extension reaction at 72°C was prolonged for 15 min in order to complete DNA synthesis.

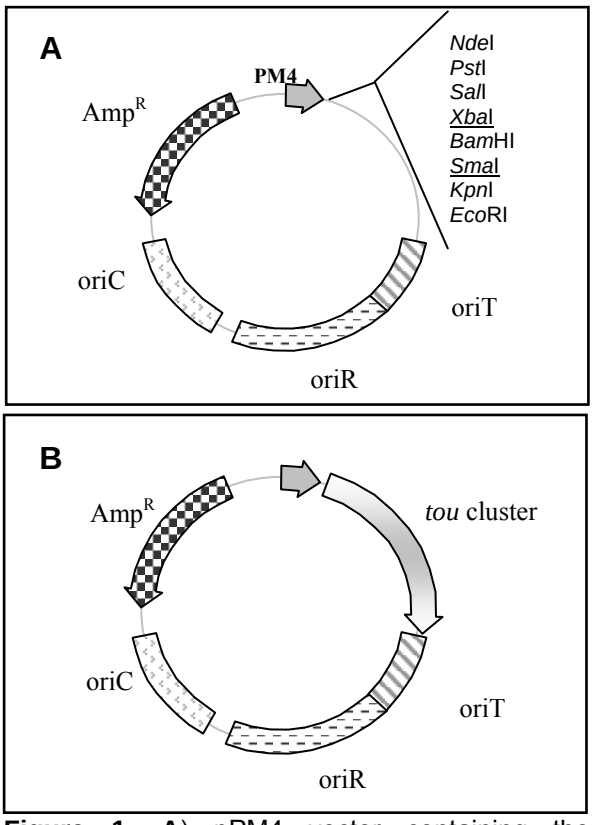


Figure 1: A) pPM4 vector containing the psychrophilic promoter PM4; **B)** pLS16274 vector containing *tou* cluster under the control of PM4 promoter.

The amplified fragment was double digested by *Hind*III/*Pst*I and cloned into pUCLT/Rterm corresponding sites, and their nucleotide sequence checked to rule out the occurrence of any mutation during synthesis. The resulting vector was named pPM4 (figure 1A). *Xba*I-*Fsp*I fragment coding for toluene-o- xylene monooxygenase from pBZ1260 plasmid was cloned into *Xba*I/*Sma*I pPM4 plasmid of *Ph*TAC125 to generate LS16274 expression vector (figure 1B, table 1).

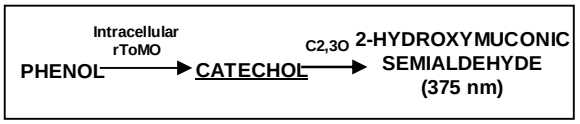


Figure 2: Scheme of the coupled assay between intracellular rToMo and the purified C2,3O

Table 1: plasmids and oligonucleotides

Plasmid	Description	Reference
pUCLT/Rterm	Vector deriving from the pUC18 plasmid, containing the T/R box and the transcription termination signal from *Ph*TAC125 *aspC* gene	7
pPLB	Promoter-trap cold-adapted vector containing the promoter-less *Ph*TAE79 *lacZ* gene	3
pP4	pPLB containing the P4 *Ph*TAC125 promoter (422bp, Accession number AJ557253)	3
pBZ1260	Ap^r *tou*ABCDEF orfA 6Kb *Not*I-*Dra*I fragment of the *P.stutzeri* OX1 DNA (Accession number AJ005663)	1
pLS16274	pPM4 containing *Xba*I-*Fsp*I fragment of *tou* cluster	this work
Oligonucleotide		
pPLBfw	5'-ATAGGGGAAGCTTTAGATAT-3'	this work
pPLBrv	5'-TGTCTGCAGGTCATATGTATTCCTG-3'	this work

semialdehyde (figure 2), which can be monitored at 375 nm (ε_{375} = 33,00 M^{-1} cm^{-1}). Cells at late-exponential phase (1.2 OD/ml) were used for determination of the catalytic parameters on phenol; assays were performed using 10 OD/ml of recombinant cells. One unit of enzyme activity in TAC/*tou* cells is defined as the amount of enzyme required to form 1μmol of cleavage product per minute under the assay conditions. Specific activity of the enzyme was expressed as mU/OD of cells.

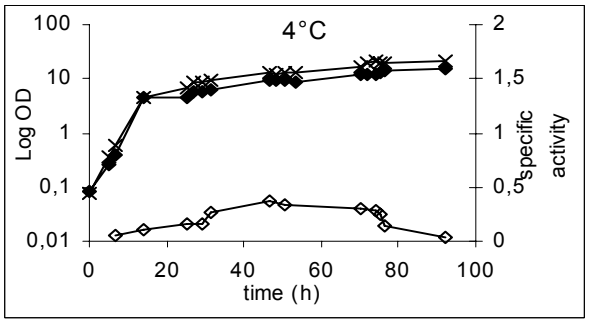
2.3 Enzymatic assays on cells on phenol.

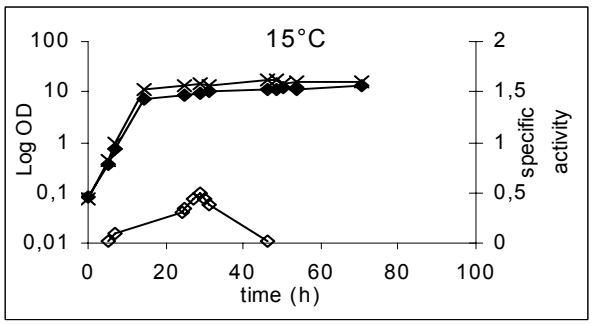
Assays were performed using TAC/*tou* cells expressing ToMO activity. Cells were grown in TYP containing 100 μg of ampicillin/ml at 4°, 15° and 25°C. The specific activity of cells on phenol was determined during cellular growth at each temperature. 10 OD of TAC/*tou* cells were centrifuged at 6,000 rpm for 15 minutes at 4°C, and cellular pellet was resuspended in 1 mL Tris-HCl 50mM pH 7.5 and incubated with 1mM phenol as substrate at room temperature for 15 minutes. ToMO activity was assayed by determining the catechol produced by hydroxylation of phenol. Catechol amount was measured in a coupled assay with recombinant catechol 2,3-dioxygenase from *P.stutzeri* OX1 [8], which cleaves the catechol ring and produces 2-hydroxymuconic

3. Results and Discussion

3.1 Construction of the psychrophilic gene-expression vector pPM4 and its use for the recombinant production of the toluene-o-xylene monooxygenase from *Pseudomonas stutzeri* OX1.

The cold-active promoter contained into the above mentioned pP4 vector was PCR amplified by using a universal primer pair (pPLBfw and pPLBrv, table 1) designed on the vector backbone. The latter oligonucleotide was designed to introduce a *Nde*I restriction site at an optimal distance from the natural *PhlacZ* Shine-Dalgarno sequence and overlapping its start codon. The amplified





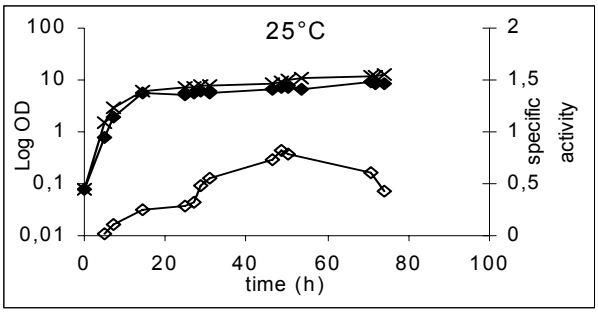


Figure 3: *Ph*TAC125 wild type ( ) and TAC/*tou* ( ) growth behavior at 4-15-25°C. rTomo specific activity in TAC/*tou* cells during cellular growth ( ).

promoter-containing fragment was inserted in pUCLT/Rterm [7] corresponding sites as described in the materials and methods section. The *Pseudomonas stutzeri* OX1 *tou* cluster encoding the complex of toluene-o-xylene monooxygenase [1] was inserted into the pPM expression vector as described in the materials and methods section. The mating experiment performed using *E. coli* strain S17-1(λ*pir*) (previously transformed with LS16274 vector) and *Ph*TAC125 produced psychrophilic transconjugants named TAC/t*ou*.

3.2 Growth curves and rToMo activity detection.

Recombinants were grown in TYP medium at three different temperatures (4-15-25°C). As shown in figure 3 the growth of TAC/*tou* cells at 4-15-25°C is similar to that of non-recombinant PhTAC125, suggesting that presence of pLS16274 does not affect cellular growth. rToMO activity was measured on TAC/tou cells using phenol as substrate. Data in figure 3 also indicate that an active rToMO is produced at all temperatures starting from mid-exponential phase up to late stationary phase. The maximum activity was detectable in late exponential phase for each condition and it seems to be constant for each temperature tested (figure 3).

3.3 Catalytic parameters of rToMO

Catalytic parameters of rToMO were determined at two growth temperatures (4-25°C). As shown in table 2, K_M values of rToMO on phenol determined for TAC/*tou* cells grown at 25 and 4°C, are similar to the value determined for *E.coli* cells as reported in Cafaro *et al*. 2004 [9], suggesting that the physiological properties of the hosts do not change ToMO specifity for phenol.

Table 2: Catalytic parameters determined on phenol using TAC/*tou* cells in comparison with *E.coli*/*tou*.

	K_M (μM)	mU/OD
E.Coli /*tou* grown at 37°C	2.01	10.00
TAC/*tou* grown at 25°C	1.89	0.82
TAC/*tou* grown at 4°C	2.04	0.38

On the other hand, the “specific activity” (mU/OD) determined for rToMO in TAC/*tou* is ten-fold lower than the value determined for *E.coli*/*tou*. This is probably due to different strength of the inducible mesophilic promoter in

comparison with the cold constitutive promoter [9,3].
This is the first step towards the construction of a cold loving microorganism endowed with biodegradative capabilities toward aromatic molecules. Further experiments will be performed with the aim of cloning the entire *P.stutzeri* lower pathway genes, that are necessary for the complete degradation of aromatic compounds.

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Recombinant expression of Toluene *o*-xylene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1 in the marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

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Abstract:
The psychrophilic bacterium, *Pseudoalteromonas haloplanktis* TAC125, isolated from seawater, was used as recipient for a biodegradative gene of the mesophile *Pseudomonas stutzeri* OX1. *tou* cluster, coding for toluene-o-xilene monooxygenase (ToMO), was successfully cloned into a cold expression vector. Catalytic parameters of the recombinant microorganisms on three different substrates were determined and compared with those exhibited by *Escherichia coli* recombinant cells expressing ToMO. Production of catalytically efficient TAC/*tou* microorganisms supports the possibility of developing specific degradative capabilities for the bioremediation of chemically contaminated marine environments and of industrial effluents characterised by low temperatures .

Keywords: Monooxygenases; Antarctic psychrophilic bacterium; *Pseudoalteromonas haloplanktis* TAC125; *Pseudomonas stutzeri* OX1.

1. Introduction

Microbial degradation of aromatic hydrocarbons has been extensively studied with the aim of developing applications for the removal of toxic compounds from contaminated environments. Degradation of aromatic compounds by aerobic bacteria is generally divided into an upper pathway, which produces dihydroxylated aromatic intermediates by the action of monooxygenases, and a lower pathway that processes these intermediates down to molecules that enter in the citric acid cycle.The characterization of the catabolic properties of micro organisms that are able to grow on toluene as the sole carbon and energy sources showed that different biochemical routes for the mineralization of toluene evolved among *Pseudomonas* (Bertoni, G. *et al.*, 1996; Bertoni, G. *et al.*, 1998; Arenghi, F.L. *et al.*, 2001; Harayama, S. *et al.*, 1989). Although many pollution problems occur in sea waters and in effluents of industrial processes which are characterised by low temperatures, considerable effort has been directed toward the genetic manipulation of mesophilic bacteria to create or improve their ability to degrade various pollutants. With the aim of investigating the degradation of aromatic compounds at low temperatures (seawater, rivers and groundwater, etc.) the Antarctic bacterium, *Pseudoalteromonas haloplanktis* TAC125 (Birolo, L *et al.*, 2000), originally isolated from a natural marine environment, was chosen as the likely candidate for the expression of a degrading activity of aromatic

compounds. The development of a shuttle genetic system for the transformation of the cold adapted Gram negative bacterium *Ph*TAC125 (Tutino, M.L. *et al.*, 2001) made possible the isolation of psychrophilic promoters and the construction of cold expression systems (Duilio, A. *et al.*, 2004). Therefore, *Ph*TAC125 was used as recipient for a biodegradative gene belonging to the upper pathway of *Pseudomonas stutzeri* OX1. *Pseudomonas stutzeri* OX1, a mesophilic bacterium isolated from activated sludge (Bertoni, G. *et al.* 1996), is able to grow on a wide range of aromatics, including phenols, cresols, and dimethylphenols, but also on non-hydroxylated molecules such as toluene, *o*-xylene, and benzene (Bertoni, G. *et al.*, 1996; Bertoni, G. *et al.*, 1998). The pathway for the degradation of these compounds in *P.stutzeri* OX1 is chromosomally encoded. Degradation of aromatic molecules is initiated by mono- and dioxygenases which produce dihydroxylated intermediates (*upper pathway*), which are subsequently catabolized through a *meta*-cleavage pathway (Bertoni, G. *et al.*, 1996). In order to construct a recombinant *Ph*TAC125 able to metabolize aromatic compounds in a broad range of temperature, *tou* cluster from *P.stutzeri* OX1, coding for toluene-*o*-xilene monooxygenase (ToMO), an enzyme of the upper pathway catalysing the hydroxylation of aromatic rings, was cloned into an expression vector under the control of a constitutive psychrophilic promoter (Duilio, A. *et al.*, 2004). ToMO is endowed with a broad spectrum of substrate specificity and with the ability to hydroxylate different positions of the aromatic ring in two consecutive monooxygenation reactions (Cafaro, V. *et al.*, 2004). It's also able to oxidize *o*-, *m*-, and *p*-xylene, 2,3- and 3,4-dimethylphenol, toluene, cresols, benzene, naphthalene, and styrene (Cafaro, V. *et al.*, 2004).

In this paper we report cloning, expression and characterization of rToMO in *Pseudoalteromonas haloplanktis* TAC125. The production of recombinant TAC/*tou* cells endowed with the ability of degrading phenol, *o*- and *p*-cresol at low temperatures indicate that such a strategy can be used to develop engineered psychrophilic bacteria endowed with the ability of degrading aromatic pollutants.

2. Materials and Methods

2.1 Bacterial strains and microbiological techniques

The Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) (Birolo, L. *et al.*, 2000) was collected in 1992 from seawater near the French Antarctic Station Dumont d'Urville (60°40'; 40°01'E); it was grown in aerobic conditions at 4°, 15° and 25°C in minimal medium (1 g/l KH_2PO_4, 1 g/l NH_4NO_3, 10 g/l NaCl, 0.2 g/l $MgSO_4$ x $7H_20$, 10 mg/l $FeSO_4$, 10 mg/l $CaCl_2$ x $2H_2O$) with L-malate 0.4% at pH 7, supplemented with 100μg/ml ampicillin when transformed.

E.coli strain S17-1(λ*pir*) was used as donor in intergeneric conjugation experiments (Tascon, R.I. *et al.*, 1993). *E. coli* cells were routinely grown in LB broth (Sambrook, J. and D.W. Russell, 2001) containing 100 μg/ml of ampicillin if transformed. Antarctic bacterium transformation was achieved by intergeneric conjugation between the transformed *E. coli* S17-1(λ*pir*) cells

Table 1: plasmids and oligonucleotides

Plasmid	Description	Reference
pUCLT/Rterm	Vector deriving from the pUC18 plasmid, containing the T/R box and the transcription termination signal from *Ph*TAC125 *aspC* gene	Tutino, M.L. *et al.* (2002)
pPLB	Promoter-trap cold-adapted vector containing the promoter-less *Ph*TAE79 *lacZ* gene	Duilio, A. *et al.* (2004)
pP4	pPLB containing the P4 *Ph*TAC125 promoter (422bp, Accession number AJ557253)	Duilio, A. *et al.* (2004)
pBZ1260	Ap^r *tou*ABCDEF orfA 6Kb *Not*I-*Dra*I fragment of the *P.stutzeri* OX1 DNA (Accession number AJ005663)	Bertoni, G. *et al.* (1996)
pLS16274	pPM4 containing *Xba*I-*Fsp*I fragment of *tou* cluster	This work
Oligonucleotide		
pPLBfw	5'ATAGGGGAAGCTTTAGATAT	
pPLBrv	5'TGTCTGCAGGTCATATGTATTCCTG	

(donor strain) and the Antarctic host cells (recipient strain) following the procedure previously described (Duilio, A. *et al.,* 2001). Mid-logarithmic-phase cells were used to inoculate flasks to a standard optical density value to 0.05-0.1, and at various times the resultant growth rates were measured spectrophotometrically (Cary UV lamp) at 600nm.

2.2 Construction of expression vector (pPM4) for the production of Toluene *o*-Xylene monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1 in *Ph*TAC125

The pPM4 cold-adapted gene-expression vector was constructed by cloning P4 promoter from *Ph*TAC125 (Duilio, A. *et al.,* 2004) into pUCLT/Rterm vector, which represents a promoter-less intermediate during the pFF vector construction (Tutino, M.L. *et al.,* 2002). The pP4 psychrophilic promoter-containing vector was isolated from a promoter-trap library (Duilio, A. *et al.,* 2004), and the DNA fragment corresponding to the cold-active promoter was PCR amplified by using a universal primer pair designed on the vector and inserting *Hind*III (pPLBfw) and *Nde*I *Pst*I (pPLBrv) restriction sites, respectively (Siani, L. *et al.*, 2005).

The amplification was performed in a mixture containing 60 ng of template, 50 pmoles of each oligonucleotide primer, 1.8 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl pH 8.3, 0.1% gelatine, 200 μM dNTP in a final volume of 50 μl. The mixture was incubated at 95°C for 10 min, after which 1,25 units of Taq DNA polymerase were added. Twenty cycles of amplification (consisting of 1 min at 95°C, 1.5 min at 55°C and 1 min +5sec/cycle at 72°C) were carried out and were followed by a cycle in which the extension reaction at 72°C was prolonged for 15 min in order to complete DNA synthesis. The amplified fragment was double digested by *Hind*III/*Pst*I and cloned into pUCLT/Rterm corresponding sites, and their nucleotide sequence checked to rule out the occurrence of any mutation during synthesis. The resulting vector was called pPM4. *Xba*I-*Fsp*I fragment coding for toluene-o-xylene monooxygenase from pBZ1260 plasmid was cloned in *Xba*I/*Sma*I pPM4 plasmid of *Ph*TAC125 to generate pLS16274 expression vector (Fig. 1, Table 1) (Siani, L. *et al.*, 2005).

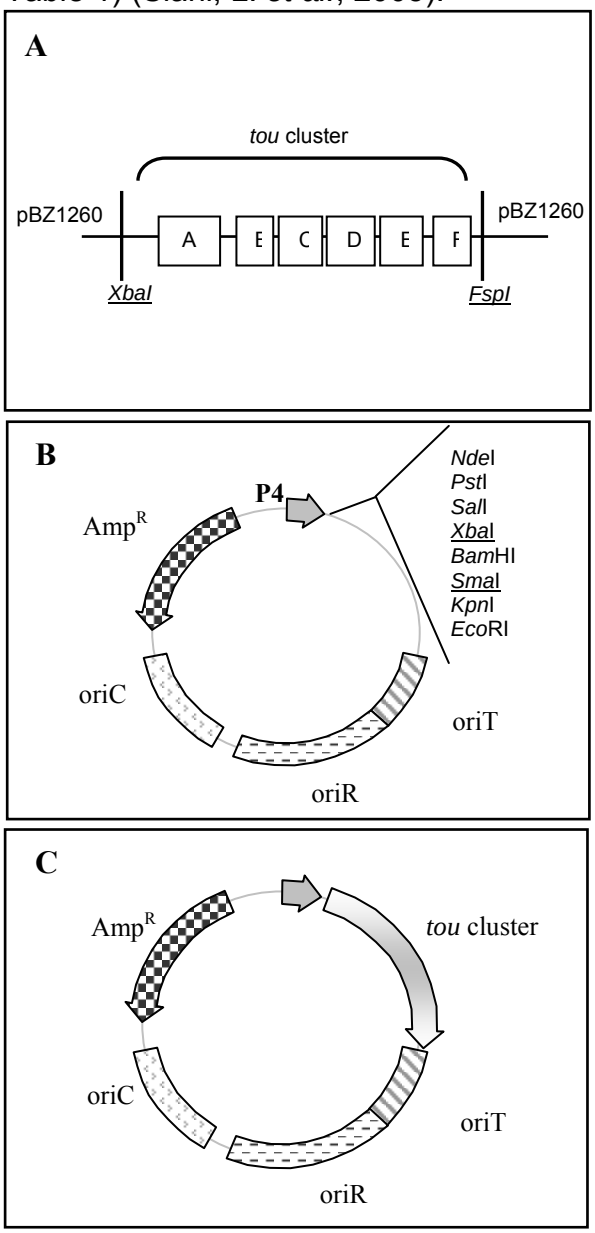

Fig. 1 - Schematic representation of pPM4 expression vector construction for the production of ToMO from P.stutzeri OX1 in PhTAC125.
A: pBZ1260 plasmid containing *tou* cluster from *P.stutzeri* OX1; **B**: pPM4 vector containing the psychrophilic promoter P4; **C**: pLS16274 vector containing *tou* cluster under the control of P4 promoter.

2.3 Enzymatic assays on whole cells

Assays were performed using TAC/*tou* cells expressing ToMO activity. One unit of enzyme activity was defined as the amount of recombinant cells required to form 1 μmol of cleavage product per min under the assay conditions. Specific activity of the enzyme was expressed as mU/OD of cells. Catalytic parameters were calculated using the program GraphPad Prism (GraphPad Software) as reported in Cafaro, V. *et al.*, 2004.

Cells were grown in minimal medium containing L-malate 0.4% and 100 μg/ml of ampicillin at 4°, 15° and 25°C until late-exponential phase (1.2 OD_{600}/ml) was reached. Specific activity of cells was determined during cellular growth at each temperature. 10 OD of TAC/*tou* cells were centrifuged at 6,000 rpm for 15 minutes at 4°C and resuspended in 1 mL Tris-HCl 50mM pH 7.5. Cells were then incubated with the appropriate substrate at 1 mM final concentration for 15 minutes at room temperature under agitation. ToMO activity was assayed by determining the amount of product formed as the result of substrate hydroxylation in a coupled assay using recombinant catechol 2,3-dioxygenase from *P.stutzeri* OX1 (Cafaro, V. *et al.,* 2002). When phenol, *o*- and *p*-cresol are used as substrates, catechol, 3-methyl and 4-methyl catechol, are produced, respectively. Catechol, 3-methyl and 4-methyl catechol are cleaved by C2,3O (Viggiani, A. *et al.,* 2003) yielding 2-hydroxymuconic semialdehyde (ε_{375} = 33,000 M^{-1} cm^{-1}), 3-methyl,2-hydroxymuconic semialdehyde (ε_{388} = 11,900 M^{-1} cm^{-1}), and 4-methyl,2-hydroxymuconic semialdehyde (ε_{382} = 28,1000 M^{-1} cm^{-1}), respectively.

2.4 Determination of phenol and catechol concentration

Total amount of phenol and catechol in the medium were calculated by their absorption at 271 nm (ε=1.62 mM^{-1} cm^{-1}) and 275 nm (ε=2.34 $mM^{-1}\cdot cm^{-1}$), respectively, after baseline subtraction.

3. Results

3.1 TAC/*tou* cells growth curves and *Ph*-rToMO activity detection

The psychrophilic expression vector pPM4 was used for the recombinant production of toluene-*o*-xylene monooxygenase from *P.stutzeri* OX1 in *Pseudoalteromonas haloplanktis* TAC125, after its transformation as described in Materials and Methods. Recombinant TAC/*tou* cells were grown in minimal medium supplemented with 0.4% L-malate as carbon source at three different temperatures (4, 15, and 25°C). As shown in Fig. 2 TAC/*tou* cells growth curves do not differ significantly, at all temperatures tested, from the growth

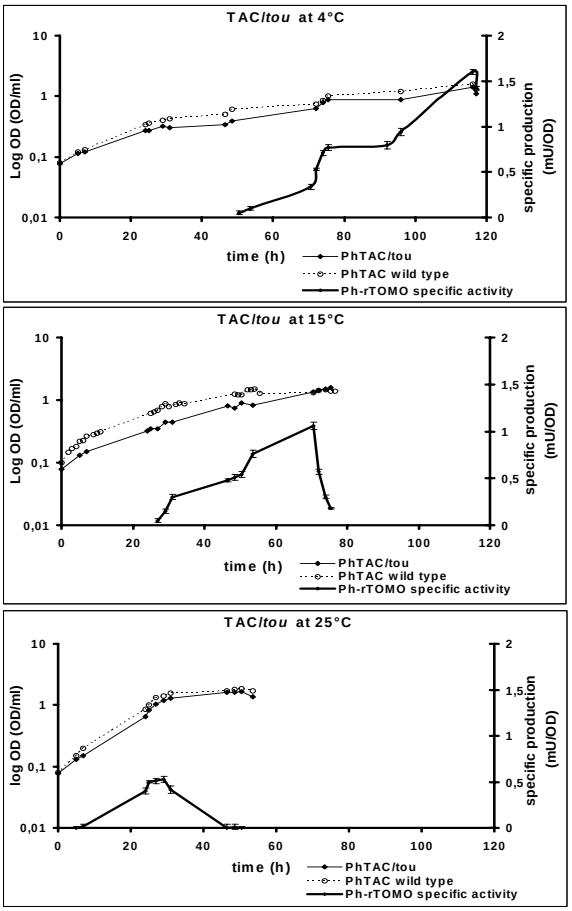

Fig. 2 - TAC/*tou* cells growth curves and *Ph*-rToMO activity detection

*Ph*TAC125 wild type and TAC/*tou* growth curves at three different temperatures (4-15-25°C) in minimal medium supplemented with L-malate 0.4%. *Ph*-rToMO specific activity (mU/OD) on 1mM phenol as substrate was determined. Enzymatic assays were performed on TAC/*tou* cells according to Cafaro, V. *et al.*, (2002).

curves of the non-recombinant *Ph*TAC125, thus suggesting that the recombination does not affect cellular growth.

Enzymatic activity of rToMO expressed in TAC/*tou* cells (*Ph*-rToMO) was measured on phenol as substrate by monitoring the production of catechol in a discontinuous coupled assay as already described in Cafaro, V. *et al*., (2002). Data in Fig. 2 show that active *Ph*-rToMO was produced at all temperatures, starting from mid-exponential phase up to late stationary growth phase. Maximum activity was detected in TAC/*tou* cells grown at 4°C in late stationary phase (Fig. 2).

TAC/*tou* cells were also grown in the presence and in the absence of phenol at 15°C (Fig. 3). Phenol proved to be non toxic for TAC/*tou* cells at least at used concentration (1mM) as it doesn't affect cellular growth. Relative consumption and accumulation of phenol and catechol in the medium during cellular growth confirm the production of an active enzyme from the mid-exponential phase. Cellular death observed during the late exponential phase could be justified by increase toxicity due to catechol accumulation.

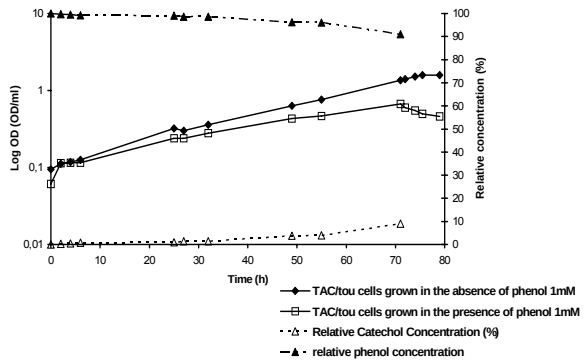

Fig. 3 - TAC/*tou* cells growth curves in the presence and in the absence of 1mM phenol.

TAC/*tou* growth curves at 15°C in minimal medium supplemented with L-malate 0.4% in the presence and in the absence of 1 mM phenol are reported. Relative consumption and accumulation of phenol and its catechol respectively, during cellular growth in the medium was determined.

3.2 *Ph*-rToMO catalytic parameters

Catalytic parameters of *Ph*-rToMO on phenol, *o*- and *p*-cresol were determined at 25°C on cells grown at 15°C and compared with those determined for rToMO produced in *E.coli* cells (*Ec*-rToMO) (Cafaro, V. *et al*., 2004). Results (Table 2) indicate that K_M values of *Ph*-rToMO on phenol and *p*-cresol are always lower than those measured using of *Ec*-rToMO, whereas the K_M value for *o*-cresol is one order of magnitude higher than that measured for *Ec*-rToMO.

The k_{cat} values for *Ph*-rToMO on the three substrates are always lower than those measured for *Ec*-rToMO. The k_{cat} values of *Ph*-rToMO for phenol and *p*-cresol are 30- and 50-fold lower than those determined for *E.coli* cells expressing ToMO, respectively. k_{cat}/K_M ratios were also determined (Table 2).

Table 2: Catalytic parameters determined for *Ph*-rToMO in comparison with *Ec*-rToMO on phenol, p-cresol and o-cresol.

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($10^3 s^{-1}\mu M^{-1}$)
***Ph*-rToMO on phenol**	0.98	0.03	31
***Ec*-rToMO on phenol**	2.18	1.00	460
***Ph*-rToMO on p-cresol**	7.69	0.013	1.69
***Ec*-rToMO on p-cresol**	13.26	0.63	47.5
***Ph*-rToMO on o-cresol**	59.63	0.13	2.18
***Ec*-rToMO on o-cresol**	6.0	0.90	150

Ph-rToMO constants were measured on cells according to Cafaro *et al.*, (2004). K_M values for *Ph*-rToMO were determined using GraphPad software; k_{cat} for *Ph*-rToMO were determined using Lineweaver Burk equation. R^2 values calculated for constants were always higher then 0.92. K_M and k_{cat} values for *Ec*-rToMO according to Cafaro *et al.,* (2004). Errors were always lower than 6%, as reported in Cafaro, V. *et al.,* (2004).

3.3 *Ph*-rToMO thermophilicity

TAC/*tou* cells grown at 15°C were used for assaying rToMO activity, on three different substrates, as function of temperature. Data reported in Fig. 4 indicate that maximal activity can be measured between 30-37°C. However it is worth to note that the system is active in a very broad range of temperatures (5-37°C) displaying more

than 50% of its maximal activity at 20°C and retaining more than 20% of its activity also at lower temperatures (5-15°C).

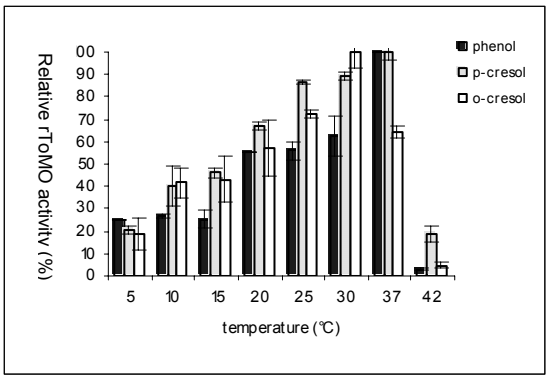

Fig. 4 – *Ph*rToMO thermophilicity on phenol, *p*-cresol and *o*-cresol.
TAC/*tou* cells grown at 15°C were used to assay relative rToMO activity (%) on 1mM phenol, 1mM *p*-cresol and 1mM *o*-cresol as function of temperature (5-42°C). Enzymatic assays were performed on TAC/*tou* cells according to Cafaro *et al.*, (2002).

4. Discussion

To investigate the degradation of aromatic compounds at low temperatures, the Antarctic psychrophilic bacterium, *Pseudoalteromonas haloplanktis* TAC125 (Birolo, L *et al.,* 2000), originally isolated from a natural marine environment, was chosen as candidate for the expression of an aromatic degradation activity. *tou* cluster from *Pseudomonas stutzeri* OX1, coding for toluene-*o*-xilene monooxygenase (ToMO), an enzyme of the upper pathway catalysing the hydroxylation of aromatic rings (Bertoni, G. *et al.* 1996), was cloned into a "cold" expression vector under the control of the constitutive psychrophilic promoter P4 (Duilio, A. *et al.*, 2004) (Fig. 1).
Recombinants, named TAC/*tou*, were grown in minimal medium in a broad range of temperatures (4-25°C) and their growth profiles were compared with those of non-recombinant *Ph*TAC125 (Fig. 2) confirming that the recombination doesn't affect cellular growth.
An active *Ph*-rToMO is produced at all temperatures tested, starting from mid-exponential phase up to late stationary growth phase and maximal activity is detected in TAC/*tou* cells grown at 4°C in the late stationary growth phase (Fig. 2). This result may be related to: i) the temperature effect already observed for the psychrophilic promoter P4: that possesses a transcriptional efficiency 2.3 fold higher at 4°C than that at 15°C (Duilio, A. *et al.*, 2004); ii) a different phenol permeability of cellular membrane as function of temperature.
Phenol proved to be non toxic for TAC/*tou* cells, at least at 1mM concentration. Active *Ph*-rToMO, produced starting from mid-exponential phase, is able to hydroxylate phenol to catechol and catechol accumulation in the medium gives rise an increase toxicity and is responsible for cellular death in the late exponential phase (Fig. 3).
Catalytic parameters of *Ph*-rToMO on phenol, *o*- and *p*-cresol were determined and compared with those determined for *Ec*-rToMO (Cafaro, V. *et al*., 2004). K_M values of *Ph*-rToMO for phenol, *p*-cresol are always lower than those of *Ec*-rToMO whereas K_M value for *o*-cresol is one order of magnitude higher than that determined for *Ec*-rToMO. *Ph*-rToMO K_{cat} values on the three substrates are always lower than those of *Ec*-rToMO; in particular *Ph*-rToMO K_{cat} values for phenol and *p*-cresol are 30 and 50 fold lower than those determined for *E.coli* cells expressing ToMO (table 2).
k_{cat}/K_M was also determined; both *Ph*-rToMO and *Ec*-rToMO hydroxylate *p*-cresol more efficiently than *o*-cresol, which, on the other hand, is hydroxylated more efficiently than phenol. Catalytic parameters determined for *Ph*-rToMO are considerably lower than those determined for *Ec*-rToMO suggesting an effect of temperature on ToMO activity when the enzyme is produced in TAC/*tou* cells. Also in this case a different permeability of *Ph*TAC125 and *E.coli* cellular membranes could justify the

higher *Ec*-rToMO k_{cat}/K_M values observed. The decrease of hydrophobic interactions at low temperatures, with the concomitant increase of structural flexibility of enzymes (Feller, G. and Gerday, C., 2003) could also result in an higher flexibility of the catalytic site, and therefore the higher catalytic efficiency, of ToMO produced in *Ph*TAC cells instead *E.coli* cells.

Finally, we have determined *Ph*-rToMO activity as function of different incubation temperature using three different substrates (phenol, *o*-cresol and *p*-cresol). As shown in fig.4, rToMO retains about 40% of its activity on *p*-cresol and *o*-cresol in a broad range of temperature (10-37°C) wherease it retains about 50% of its activity on phenol between 20-37°C and only 20% between 5-15°C.

The data we have collected confirm the production of catalytically efficient TAC/*tou* micro organisms and represent the first step to confer biodegradative capabilities in a cold loving micro organism with the perspective to create a recombinant cold adapted bacterium able to completely metabolize aromatic compounds, that could be useful for the bioremediation of chemically contaminated marine environments and of industrial effluents characterised by low temperatures.

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2.3 Conclusions

The idea of using microorganisms to reduce environmental contamination, such as in soils and waste waters, is not new but appears to be feasible alternative to physicochemical methods (Timmis, K.N. *et al.*,1999).

Bioaugmentation and inoculation of contaminated environments with specific cold adapted microorganisms should be helped to improve the biodegradation of recalcitrant chemicals that occur in sea waters and in effluents of industrial processes which are characterised by low temperatures (Gerday, C. *et al.*, 2000).

With the aim to investigate the degradation of aromatic compounds at low temperatures the Antarctic psychrophilic bacterium, *Pseudoalteromonas haloplanktis* TAC125 (Birolo, L. *et al.*, 2000) was used as representative recipient for the biodegradative gene belonging to the upper pathway of *Pseudomonas stutzeri* OX1 coding for the toluene/*o*-xylene-monooxygenase (ToMO) (Bertoni, G. *et al.*, 1996).

Moreover, a preliminary characterization of the recombinant strain is also reported (Siani, L. PhD Thesis, 2004).

Recombinants, named TAC/*tou*, were grown in rich and minimal medium in a broad range of temperatures (4-25°C) and their growth profiles were compared with those of non-recombinant *Ph*TAC125 (fig. 3, chapter 2.2.1; fig. 2, chapter 2.2.2); this analysis confirmed that the recombination doesn't affect cellular growth. An active rToMO, able to hydroxylate phenol to catechol, was detectable at all temperatures tested starting from mid-exponential phase up to late stationary phase with a maximum peak in late exponential phase (fig. 3, chapter 2.2.1; fig. 2, chapter 2.2.2). Then phenol proved to be non toxic for TAC/*tou* cells, at least at 1mM concentration (figure 3; chapter 2.2.2); however the catechol accumulation in the medium gives rise an increase toxicity and is responsible for cellular death in the late exponential phase.

Catalytic parameters of *Ph*-rToMO on phenol, *o*- and *p*-cresol were determined and compared with those determined for *Ec*-rToMO (Cafaro, V. *et al*., 2004) (table 2, chapter 2.2.1; table 2, chapter 2.2.2). Both *Ph*-rToMO and *Ec*-rToMO hydroxylate *p*-cresol more efficiently than *o*-cresol, which, on the other hand, is hydroxylated more efficiently than phenol. Catalytic parameters determined for *Ph*-rToMO are considerably lower than those determined for *Ec*-rToMO suggesting an effect of temperature on ToMO activity when the enzyme is produced in TAC/*tou* cells. A different permeability of *Ph*TAC125 and *E.coli* cellular membranes could justify the higher *Ec*-rToMO k_{cat}/K_M values observed (table 2, chapter 2.2.2). The decrease of hydrophobic interactions at low temperatures, with the concomitant increase of structural flexibility of enzymes (Feller, G. and Gerday, C., 2003) could also result in an higher flexibility of the catalytic site, and therefore the higher catalytic efficiency, of ToMO produced in *Ph*TAC125 cells instead *E.coli* cells.

TAC/*tou* cells grown at 15°C up to late exponential phase were used to assay rToMO activity on three different substrates (phenol, *o*-cresol and *p*-cresol) as function of different incubation temperature (figure 4; chapter 2.2.2). rToMO retains about 40% of its activity on *p*-cresol and *o*-cresol in

a broad range of temperature (10-37°C); although it retains about 50% of its activity on phenol between 20-37°C and only 20% between 5-15°C.
Data obtained support the possibility of developing specific degradative capabilities for the bioremediation of chemically contaminated marine environments and of industrial effluents characterised by low temperatures, using a naturally cold adapted bacterium.
This is the first step towards the construction of a cold loving microorganism endowed with biodegradative capabilities toward aromatic molecules. Further experiments will be performed with the aim of cloning the entire metabolic pathways necessary for the complete degradation of aromatic compounds.

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Appendix

Supplementary materials
Chapter 1.2.1
Table 1

Proteins identified in the 2D-gel analysis of *Pseudoalteromonas haloplanktis* in the presence and in the absence of L-malate, respectively. Proteins were clustered into functional groups according to the genome annotation(Medigue, C. *et al.*, 2005).
[a)] MM, Minimal Medium
[b)] MM + L-malate, Minimal Medium with L-malate.

Spot	Function	pI	MW	Accession number	MM[a]	MM + MAL[b]
	1.Cell envelope and membrane-associated cellular processes					
	1.1 Cell Wall					
1	GTP-binding protein TypA	5,15	67,2	PSHAa0167	+	
2	outer membrane protein	4,76	40,2	PSHAa0183	+	+
3	Long-chain fatty acid transport protein precursor	4,82	45,7	PSHAa2426	+	+
4	putative outer membrane antigen	4,94	92,6	PSHAa2028	+	+
5	Rod shape-determining protein	4,97	37,3	PSHAa2682	+	
6	UDP-N-acetyl-muramate:alanine ligase, L-alanine adding enzyme	5,78	53,2	PSHAa2503	+	
7	putative membrane-associated protein with TPR-like domain	4,87	22,3	PSHAb0136		+
8	phosphoglucosamine mutase	5,74	47,4	PSHAa0872	+	
9	L-glutamine:D-fructose-6-phosphate aminotransferase	5,56	66,9	PSHAa2994	+	
10	bifunctional ADP-L-glycero-D-manno-heptose synthase	5,22	50,5	PSHAa2323	+	
	1.2 Transport/binding proteins and lipoproteins					
12	putative outer membrane porin	4,5	43,9	PSHAa1575	+	+
13	putative outer membrane porin	4,55	34,4	PSHAa2567		+
14	putative enzyme with a phosphatase-like domain	6,52	101,9	PSHAa2478		+
15	Na(+)-translocating NADH-quinone reductase subunit alpha	5,52	48,6	PSHAa2241		+
16	Magnesium and cobalt efflux protein CorC	4,5	33	PSHAa1043	+	
17	putative outer membrane receptor for ferric iron uptake	4,89	80,5	PSHAa0695	+	
18	oxaloacetate decarboxylase, alpha subunit	5.04	64,2	PSHAa0539	+	
19	putative TonB-dependent receptor	4,59	77,2	PSHAa0108	+	
20	putative ABC transporter, periplasmic substrate-binding protein	5,23	28,3	PSHAa2562	+	
21	putative Hemin receptor protein HmuR	5,25	71,2	PSHAb0072	+	+
22	Iron-regulated outer membrane virulence protein homolog	4,81	73,3	PSHAb0251		+
23	putative outer membrane protein with a TonB box	4,95	117,8	PSHAb0340	+	
24	putative outer membrane protein with a TonB box	4,95	117,8	PSHAb0341	+	+
25	putative outer membrane porin	4,98	38,4	PSHAb0363		+
26	outer membrane protein W (OmpW) family	4,98	23,5	PSHAa2155	+	+
27	Apolipoprotein N-acyltransferase	5,1	37,7	PSHAa0722	+	
28	putative Ton-B dependent protein (could be involved in iron transport)	4,81	90,4	PSHAb0512	+	
29	ABC transporter ATP-binding protein	5,2	62,3	PSHAa2225	+	
30	putative transport protein (ABC superfamily, ATP_binding)	5.06	60	PSHAa1574	+	
	1.3 Sensors (signal transduction)					
31	putative TonB-dependent outer membrane receptor	5,13	66,8	PSHAa2275	+	+
32	TonB dependent outer membrane receptor	4,49	91,7	PSHAb0286	+	+
33	putative TonB-dependent receptor	4,38	91,9	PSHAa1840		+
34	putative TonB-dependent receptor	5,5	86,2	PSHAa1271	+	+
35	putative outer membrane TonB-dependent receptor	8,17	86,6	PSHAb0254	+	+
36	TonB-dependent receptor protein	6,12	109	PSHAa1987	+	+
37	Outer membrane receptor protein	5,96	84,3	PSHAa2180	+	+
38	Outer membrane receptor	5,04	78,8	PSHAb0279	+	+
	1.4 Membrane bioenergetics (electron transport chain and ATP synthase)					
39	putative peroxiredoxin/glutaredoxin family protein	5.03	27,9	PSHAa0759	+	+
40	ATP synthase F1, alpha subunit	5,38	55,1	PSHAa3010	+	+

41	membrane-bound ATP synthase, F1 sector, beta-subunit	4,7	50	PSHAa3008	+	+
42	membrane-bound ATP synthase, F1 sector, epsilon-subunit	6,08	15,1	PSHAa3007	+	+
43	Ubiquinol-cytochrome c reductase, iron-sulfur subunit	7,7	20,9	PSHAa2530		+
	1.5 Motility and chemotaxis					
44	putative ferric enterobactin receptor	4,9	84,4	PSHAa1824	+	
45	Probable chemotaxis protein (MotB)	5,36	32,1	PSHAa0815	+	+
46	chemotaxis protein CheA	4,68	79	PSHAa0812	+	
47	chemotaxis protein CheZ	4,53	27,8	PSHAa0811	+	
48	putative Sigma-54 dependent response regulator (FlaM)	4,99	51,5	PSHAa0790	+	
49	flagellin	4,94	40	PSHAa0781	+	+
50	flagellar biosynthesis; hook protein	4,54	47,2	PSHAa0772	+	
51	purine-binding chemotaxis protein	4,43	18,2	PSHAa0818	+	
52	putative flagellin (FliC-like)	4,64	40,2	PSHAa0782		+
	1.6 Protein secretion					
53	Conserved hypothetical protein	8,65	27,2	PSHAa1869	+	
54	preprotein translocase, ATPase secretion component (GSP)	5,39	103	PSHAa2743	+	
	1.7 Cell division					
55	aerobic respiration control protein ArcA (Dye resistance protein)	5,71	27,3	PSHAa0551	+	+
56	Cell division protein	5,26	44,4	PSHAa2500	+	
57	cell division inhibitor, membrane ATPase, activates MinC	5,42	29,4	PSHAb0506	+	+
58	60 kDa chaperonin (Protein Cpn60) (GroEL protein)	4,71	57,1	PSHAa0259	+	+
5	Rod shape-determining protein	4,97	37,3	PSHAa2682	+	
59	GTPase involved in cell partioning; multicopy suppresssor of ftsJ (rrmJ)	4,73	42,9	PSHAa2654	+	
	2. Intermediary metabolism					
	2.1 Metabolism of carbohydrates and related molecules					
60	putative ALDOLASE	5,91	50,2	PSHAb0559	+	+
61	transketolase 1 (TK 1)	5,8	71,9	PSHAa0671	+	+
62	bifunctional protein [aconitate hydratase 2; 2-methylisocitrate dehydratase]	4,96	93,9	PSHAa0184		+
63	Fructose-1,6-bisphosphatase	5.19	35,3	PSHAa0463	+	+
64	enolase	4,97	48,1	PSHAa0742	+	
65	Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49) (G6PD)	6.08	55,3	PSHAa1140	+	
66	Glyceraldehyde 3-phosphate dehydrogenase	6,4	52,3	PSHAa1900	+	+
67	2-methylisocitrate lyase	5.03	32,6	PSHAa1775	+	
68	methylcitrate synthase (citrate synthase 2)	6.17	41,4	PSHAa1774	+	+
69	aconitate hydratase 1 (anaerobic respiration)	5,2	94,4	PSHAa1773	+	
70	Isocitrate dehydrogenase	5,7	80,5	PSHAa1727	+	
71	citrate synthase	5,74	48,1	PSHAa1653	+	+
72	Major NAD(P)H-flavin oxidoreductase	5,87	27,5	PSHAa1234	+	
73	Succinate dehydrogenase flavoprotein subunit	5,84	64,6	PSHAa1649		+
74	succinate dehydrogenase, Fe-S protein	6,87	26,6	PSHAa1648	+	+
75	succinyl-CoA synthetase, beta subunit	5,18	41,4	PSHAa1645	+	+
76	succinyl-CoA synthetase, alpha subunit, NAD(P)-binding	5,7	29,7	PSHAa1644	+	+
77	acetyl-CoA acetyltransferase with thiolase domain	5,85	40,7	PSHAa1454	+	
78	succinyl-CoA transferase, subunit A	5,44	25,1	PSHAa1447		+
79	succinyl-CoA transferase, beta subunit	4,76	23,3	PSHAa1446	+	+
80	fumarate hydratase, class I	5,06	54,7	PSHAa1166	+	+
81	dihydrolipoamide dehydrogenase	5,79	50,6	PSHAa0393	+	+
82	pyruvate dehydrogenase, decarboxylase subunit, thiamin-binding	5,21	99,7	PSHAa0391	+	
83	phosphoglycerate mutase III, cofactor-independent	5.07	55,6	PSHAa0366	+	+
84	aconitate hydratase	4,89	101,8	PSHAa0159	+	
85	fumarase C (fumarate hydratase Class II)	4,93	48,7	PSHAa0048	+	
9	L-glutamine:D-fructose-6-phosphate aminotransferase	5,56	66,9	PSHAa2994	+	
86	Tryptophanyl-tRNA synthetase	6,19	36,8	PSHAa2708	+	
87	malate dehydrogenase, NAD(P)-binding	5,37	32,1	PSHAa2658	+	+
88	transaldolase B	5,24	35	PSHAa2559		+

89	malate synthase G	5,47	78,7	PSHAb0061	+	
90	Isocitrate lyase 2	5,63	58,7	PSHAb0062	+	
91	putative NADP-dependent malic enzyme	4,92	46,2	PSHAa2725	+	
92	phosphoenolpyruvate carboxylase	5,57	100,2	PSHAb0314	+	
93	putative fructose-bisphosphate aldolase	5,56	33,1	PSHAb0548		+
94	phosphoenolpyruvate carboxykinase	5,56	55,9	PSHAa0228	+	+
	2.2 Metabolism of amino acids and related molecules					
	2.2.1 Amino acid biosynthesis and salvage					
95	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	5,35	29,9	PSHAa2039	+	+
96	serine hydroxymethyltransferase	5,97	45,4	PSHAa2376	+	+
97	Argininosuccinate synthase	4,92	44,3	PSHAa2288	+	+
98	Bifunctional protein ArgHA	5,3	69,4	PSHAa2287	+	
99	methionine aminopeptidase; contains a divalent metal, usually cobalt	6.13	28,9	PSHAa2037	+	
100	O-acetylserine sulfhydrylase, PLP-dependent enzyme	5,8	34,4	PSHAa1918	+	
101	6-oxohexanoate dehydrogenase, Succinate-semialdehyde dehydrogenase	4,67	50,2	PSHAa1590	+	
102	Tryptophan synthase alpha chain	5,64	28,7	PSHAa1288	+	
103	carbamoyl phosphate synthase, large subunit	4,96	117,9	PSHAa1229	+	+
104	carbamoyl phosphate synthetase, glutamine amidotransferase small subunit	5,62	40,9	PSHAa1228	+	+
105	aspartate aminotransferase, PLP-dependent	5,52	43,8	PSHAa0956	+	
106	D-3-phosphoglycerate dehydrogenase	5,85	44,3	PSHAa0666	+	
107	ATP-sulfurylase, subunit 2 (ATP:sulfate adenylyltransferase)	6.04	35	PSHAa0212	+	
108	ATP-sulfurylase, subunit 1 (ATP:sulfate adenylyltransferase)	5.06	51,8	PSHAa0211	+	+
109	glutamine synthetase	4,82	51,6	PSHAa0166	+	+
110	sulfite reductase, alpha subunit (flavoprotein)	5,12	68,1	PSHAa0154	+	
111	shikimate kinase I	5,3	19,1	PSHAa2715	+	+
112	putative cystathionine gamma-synthase or beta-lyase, PLP-dependent	5,8	46	PSHAb0477	+	+
113	imidazole glycerol phosphate synthase, subunit with HisH;	4,91	28,4	PSHAb0483	+	
114	putative Branched-chain amino acid aminotransferase	5,8	36,1	PSHAa1270	+	
115	probable isopropylmalate dehydrogenase	6,14	35,7	PSHAb0465		+
116	3-phosphoshikimate 1-carboxyvinyltransferase	4,82	46,2	PSHAa1423	+	
117	Aspartate-semialdehyde dehydrogenase	4,67	37	PSHAa2078		
	2.2.3 Degradation of amino acids and related molecules					
118	putative peptidase, M23/M37 family	8,75	53,6	PSHAa0544	+	
119	Aminopeptidase N	8,41	95,9	PSHAa2492	+	
120	glycine cleavage complex protein T	5,13	39,6	PSHAa2475	+	
121	carboxy-terminal protease for penicillin-binding protein 3	5,57	76,4	PSHAa1678	+	
122	Isovaleryl-CoA dehydrogenase	5,56	42,1	PSHAa1452	+	+
123	urea carboxylase (alpha subunit)	5,28	75,6	PSHAa1449	+	+
124	Leucine dehydrogenase	5,77	37,1	PSHAa1167	+	+
125	oligopeptidase A	4,93	77	PSHAa0361	+	
126	succinylglutamic semialdehyde dehydrogenase	6.07	51,6	PSHAa0196	+	+
127	acetylornithine transaminase, PLP-dependent	5,43	43,2	PSHAa0194	+	+
128	probable cold-active aminopeptidase; secreted using a signal peptide	5,4	71,3	PSHAa2915	+	
129	succinate-semialdehyde dehydrogenase I, NADP-dependent	5,74	53	PSHAa2665	+	
130	Fumarylacetoacetase	5,86	49	PSHAa2169	+	
131	4-hydroxyphenylpyruvate dioxygenase	4,64	38,8	PSHAa2168		+
132	Cytosol aminopeptidase	5,7	54,7	PSHAa2388	+	
	2.3 Metabolism of nucleotides and nucleic acid					
	2.3.1 Biosynthesis and salvage of purines, pyrimidines, nucleosides, and nucleotides					
133	putative GTP-binding protein with nucleoside triP hydrolase domain	4,75	39,5	PSHA_1_1466	+	
134	Phophoribosylformylglycinamidine synthase (FGAM	4,93	140,9	PSHAa2330	+	+

	synthetase)					
135	adenylosuccinate lyase	5,62	50,8	PSHAa1692	+	
	30S ribosomal subunit protein S1; site specific RNA					
136	helicase	4,89	61,1	PSHAa1425	+	+
103	carbamoyl phosphate synthase, large subunit	4,96	117,9	PSHAa1229	+	+
137	conserved protein, ETFP adenine nucleotide-binding domain	5,09	34,5	PSHAa1851	+	
	carbamoyl phosphate synthetase, glutamine					
104	amidotransferase small subunit	5,62	40,9	PSHAa1228	+	+
138	dihydro-orotase, DHOase	5,89	37,8	PSHAa1183	+	
139	phosphoribosylpyrophosphate synthetase	5,36	34,3	PSHAa1054	+	+
	polynucleotide phosphorylase; part of the degradosome					
140	(cold shock protein)	5,05	76,5	PSHAa1001	+	+
141	CTP synthetase	6,41	64,8	PSHAa0741	+	
142	GMP synthetase (glutamine aminotransferase)	5	57,7	PSHAa0649	+	+
143	IMP dehydrogenase	8,12	52	PSHAa0648		+
	putative hypoxanthine-guanine					
144	phosphoribosyltransferase(hpt)	5,79	21,3	PSHAa0609	+	
145	bifunctional purine biosynthesis protein purH	4,98	57,3	PSHAa0345	+	+
146	phosphoribosylglycinamide synthetase (GAR synthetase)	4,57	45,8	PSHAa0343	+	+
147	Uridine kinase	7.05	24,3	PSHAb0080	+	
148	nucleoside diphosphate kinase	5,5	15,5	PSHAb0142		+
96	serine hydroxymethyltransferase	5,97	45,4	PSHAa2376	+	+
149	Argininosuccinate synthase (Citrulline--aspartate ligase)	5,52	55,7	PSHAa2322	+	+
	2.3.3 Sugar nucleotide biosynthesis and conversions					
8	phosphoglucosamine mutase	5,74	47,4	PSHAa0872	+	
61	transketolase 1 (TK 1)	5,8	71,9	PSHAa0671	+	+
142	GMP synthetase (glutamine aminotransferase)	5	57,7	PSHAa0649	+	+
143	IMP dehydrogeanse	8,12	52	PSHAa0648		+
145	bifunctional purine biosynthesis protein purH	4,98	57,3	PSHAa0345	+	+
150	putative regulator of nucleoside diphosphate kinase (rnk)	4,77	14,6	PSHAa0258	+	
	2.4 Metabolism of lipids					
96	serine hydroxymethyltransferase	5,97	45,4	PSHAa2376	+	+
	Alpha keto acid dehydrogenase complex, E1 component,					
151	alpha subunit	6,39	43,6	PSHAa1632	+	+
152	Alpha keto acid dehydrogenase complex, beta subunit	5,13	35,4	PSHAa1631		+
153	Alpha keto acid dehydrogenase complex, E2 component	5,32	57,3	PSHAa1630		+
154	Enoyl-CoA hydratase/carnithine racemase	5	28	PSHAa1457	+	+
123	urea carboxylase (alpha subunit)	5,28	75,6	PSHAa1449	+	+
155	putative beta-ketoacyl synthase	5,75	68,8	PSHAa1382	+	
156	biotin carboxylase (A subunit of acetyl-CoA carboxylase)	6,42	49,2	PSHAa0266	+	+
157	multifunctional 3-hydroxybutyryl-CoA epimerase	5,87	78,1	PSHAa0011	+	
158	enzyme adding cmnm(5) to tRNA-s(2)U34	4,79	49,2	PSHAa3021	+	
159	acetylCoA carboxylase, carboxytransferase subunit alpha	5,48	35,5	PSHAa2014	+	
160	malonyl-CoA-[acyl-carrier-protein] transacylase	4,95	32,3	PSHAa1809		+
161	short-chain alcohol dehydrogenase family protein	5.01	43,5	PSHAa2242	+	+
162	3-oxoacyl-[acyl-carrier-protein] synthase I	5,2	42,6	PSHAa2080		+
163	3-oxoacyl-[acyl-carrier-protein] reductase	5,35	25,9	PSHAa1808	+	
	2.5 Metabolism of coenzymes and prosthetic groups					
164	Glutamyl-tRNA synthetase (Glutamate-tRNA ligase)	5,6	56,5	PSHAa0635	+	
165	Uroporphyrinogen-III synthase HemD	4,75	42	PSHAa0099	+	
166	5-aminolevulinate dehydratase (porphobilinogen synthase)	5,65	37,1	PSHAa2935	+	+
167	uroporphyrinogen decarboxylase	5,36	39,2	PSHAa2899	+	
168	Electron transfer flavoprotein beta-subunit (Beta-ETF)	9,01	26,4	PSHAa1618	+	+
169	Electron transfer flavoprotein alpha-subunit (Alpha-ETF)	5,03	31,4	PSHAa1617	+	+
170	Lactoylglutathione lyase (Glx I)	5	20,2	PSHAa1601	+	
171	6,7-dimethyl-8-ribityllumazine synthase	4,99	16,3	PSHAa2371	+	+
	2.6 Metabolism of sulfur					
	methionine aminopeptidase; contains a divalent metal,					
99	usually cobalt	6.13	29	PSHAa2037	+	
101	O-acetylserine sulfhydrylase, PLP-dependent enzyme	5,8	34,4	PSHAa1918	+	

107	ATP-sulfurylase, subunit 2 (ATP:sulfate adenylyltransferase)	6.04	35	PSHAa0212	+	
108	ATP-sulfurylase, subunit 1 (ATP:sulfate adenylyltransferase)	5.06	51,8	PSHAa0211	+	+
110	sulfite reductase, alpha subunit (flavoprotein)	5,12	68,1	PSHAa0154	+	
158	enzyme adding cmnm(5) to tRNA-s(2)U34	4,79	49,2	PSHAa3021	+	
172	co-chaperone protein Hsc20, involved in assembly of Fe-S clusters	4,75	19,6	PSHAa2668	+	
173	chaperone (Hsp70 family), involved in assembly of Fe-S clusters	4,67	66,1	PSHAa2667	+	
112	putative cystathionine gamma-synthase or beta-lyase, PLP-dependent	5,8	46	PSHAb0477	+	+
	3. Information transfer pathways					
	3.1 DNA metabolism					
174	putative transposase (IS605)	9.12	43,3	PSHAa0582	+	
175	nucleoid-associated protein	5,46	34,5	PSHAa2381		+
176	multifunctional DNA polymerase I	5	99,8	PSHAa2757	+	
177	DNA gyrase, subunit A, type II topoisomerase	4,97	97	PSHAa1421	+	
	3.2 RNA metabolism					
178	conserved protein with nucleic acid-binding protein-like domain	6,22	85	PSHAa2854		+
48	putative Sigma-54 dependent response regulator(FlaM)	4,99	51,5	PSHAa0790	+	
179	Bacterial transcriptional regulator LuxR family	7,77	31,1	PSHAa2414	+	
180	negative regulatory gene for the psp opreon, phage shock protein	5,32	25,6	PSHAa2049	+	
136	30S ribosomal subunit protein S1; site specific RNA helicase	4,89	61,1	PSHAa1425	+	+
181	putative PhoP (RR in two-component regulatory system with PhoQ)	5,09	26,2	PSHAa1283	+	
182	transcription elongation factor GreA	4,92	17,8	PSHAa1230	+	+
183	putative methyltransferase	8,28	46,9	PSHAa1147	+	
184	ribosome-binding factor A, role in processing of 16S rRNA	5,29	15,1	PSHAa0998	+	
185	transcription elongation protein (N utilization substance protein A)	4,49	55,3	PSHAa0996	+	+
56	aerobic respiration control protein arcA (Dye resistance protein)	5,71	27,3	PSHAa0551	+	+
186	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	5,56	39,3	PSHAa0318	+	
187	adenylosuccinate synthetase	5,38	47,6	PSHAa0275		+
188	component in transcription antitermination	5,58	20,8	PSHAa0217	+	
158	enzyme adding cmnm(5) to tRNA-s(2)U34	4,79	49,2	PSHAa3021	+	
189	stationary-phase regulator of Sigma D	5,01	18,5	PSHAa2900	+	
191	DNA-directed RNA polymerase omega chain	4,87	10,2	PSHAa2791		+
192	stringent starvation protein A, regulator of transcription	5,14	24,5	PSHAa2527	+	+
193	putative RNA-binding protein with unique protein fold	5,19	20,4	PSHAa0028	+	
194	protein with Cold-shock DNA-binding domain	6,05	22,4	PSHAa1600	+	+
195	putative ATP-dependent RNA helicase	9,32	48	PSHAb0411		+
196	Adenylate kinase	5,57	23,1	PSHAa1208	+	
	3.3 Protein synthesis, maturation and folding					
197	tryptophan synthase, alpha protein	5,57	20,9	PSHAa1298	+	+
198	30S ribosomal subunit protein S6	5,36	14,5	PSHAa2443	+	+
199	50S ribosomal subunit protein L9	5,19	15,7	PSHAa2439	+	+
200	peptidyl-prolyl cis-trans isomerase B (rotamase B)	5.51	18,2	PSHAa2066	+	
201	cysteine tRNA synthetase	5,82	52,3	PSHAa2065	+	
202	peptidyl-prolyl cis/trans isomerase (trigger factor)	5	48	PSHAa2063	+	+
203	putative Peptidyl-prolyl cis-trans isomerase D	4,89	67,5	PSHAa2058	+	+
204	protein chain elongation factor EF-Ts	5,14	30,8	PSHAa2035	+	+
205	ribosome releasing factor	6.04	20,6	PSHAa2033	+	
206	aspartate tRNA synthetase	5.09	65,6	PSHAa1938	+	
207	phenylalanine tRNA synthetase, alpha-subunit	5,38	37,3	PSHAa1904		+
208	thioredoxin reductase, FAD/NAD(P)-binding	4,8	34,5	PSHAa1720	+	+

209	serine tRNA synthetase	5,39	48,6	PSHAa1712	+	+
210	asparaginyl-tRNA synthetase	4,93	52,6	PSHAa1616	+	
136	30S ribosomal subunit protein S1; site specific RNA helicase	4,89	61,1	PSHAa1425	+	+
211	FKBP-type 22KD peptidyl-prolyl cis-trans isomerase (rotamase)	4,42	21,8	PSHAa1414	+	+
212	threonyl-tRNA synthetase	5,52	73,8	PSHAa1396	+	
213	Hsp 24 DnaK nucleotide exchange factor	4,62	22,7	PSHAa1222	+	
214	50S ribosomal protein L25	5,01	23,1	PSHAa1053	+	+
215	ATP-dependent protease, Hsp 100	5,37	95,6	PSHAa0935	+	
216	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase)	4,61	15,3	PSHAa0920	+	
217	arginyl-tRNA synthetase	6.03	64,7	PSHAa0656	+	
164	Glutamyl-tRNA synthetase (Glutamate-tRNA ligase)	5,6	56,5	PSHAa0635	+	
218	Tyrosyl-tRNA synthetase II	5,79	44	PSHAa0545	+	
219	lysyl-tRNA synthetase	5.05	57,6	PSHAa0518	+	+
58	60 kDa chaperonin (Protein Cpn60) (GroEL protein)	4,71	57,1	PSHAa0259	+	+
220	heat shock protein 33, redox regulated chaperone	4,66	32,5	PSHAa0229	+	
221	protein chain elongation factor EF-Tu; GTP-binding factor	4,97	43,2	PSHAa0227	+	+
222	protein chain elongation factor EF-G, GTP-binding	4,86	77,5	PSHAa0226	+	+
223	50S ribosomal subunit protein L7/L12	4,56	122,5	PSHAa0221	+	
224	50S ribosomal subunit protein L4	9,76	21,9	PSHAa0145		+
225	putative second protein chain elongation factor EF-G	5,02	76,5	PSHAa2940	+	+
226	30S ribosomal subunit protein S4	10,1	23,4	PSHAa2807		+
172	co-chaperone protein Hsc20, involved in assembly of Fe-S clusters	4,75	19,6	PSHAa2668	+	
173	chaperone (Hsp70 family), involved in assembly of Fe-S clusters	4,67	66,1	PSHAa2667	+	
227	peptidyl-prolyl cis-trans isomerase (PPIase)	5,67	48,9	PSHAa2633	+	+
228	histidine tRNA synthetase	5,33	47,5	PSHAb0137	+	
229	Chaperone protein DnaK (Heat shock protein 70)	4,59	68,7	PSHAb0357		+
230	Peptidyl-prolyl cis-trans isomerase	4,52	17,2	PSHAa0721	+	
231	protein chain elongation factor EF-Tu; possible GTP-binding factor	4,97	43,2	PSHAa2911	+	+
232	elongation factor P (EF-P)	4,85	20,7	PSHAa0474	+	
	4. Other functions					
	4.1 Adaption to atypical conditions					
233	putative Glutathione S-transferase	6.33	32,4	PSHAa2192	+	
213	Hsp 24 DnaK nucleotide exchange factor	4,62	22,7	PSHAa1222	+	
56	aerobic respiration control protein ArcA (Dye resistance protein)	5,71	27,3	PSHAa0551	+	+
220	heat shock protein 33, redox regulated chaperone	4,66	32,5	PSHAa0229	+	
234	DNA-binding DPS protein	5.06	18,3	PSHAa2954	+	+
235	organic solvent tolerance protein	4,77	87,6	PSHAa2632	+	
236	periplasmic serine endoprotease	5,95	46,6	PSHAa2536	+	+
192	stringent starvation protein A, regulator of transcription	5,14	24,5	PSHAa2527	+	+
237	putative succinylglutamic semialdehyde dehydrogenase	6.07	51,6	PSHAb0426	+	
238	Oxidoreductase, FMN-binding	5,23	39,1	PSHAa1863	+	
239	putative acetylornithine transaminase	5,43	43,2	PSHAb0428	+	
	4.2 detoxitication					
240	Antioxidant, AhpC/Tsa family (partial match)	5,37	22,9	PSHAa0839	+	+
233	putative Glutathione S-transferase	6.33	32,4	PSHAa2192	+	
241	iron superoxide dismutase	5,24	21,5	PSHAa1215	+	
242	Catalase (EC 1.11.1.6)	6,13	57,7	PSHAa1737	+	
	4.3 Antibiotic production					
243	putative enzyme for maturation of Microcin B17 and degradation of CcdA	5,4	48,4	PSHAa2747	+	
244	putative peptidase for maturation of Microcin B17 and degradation of CcdA	5,26	51	PSHAa2675	+	
	4.4 Phage-related functions					
245	conserved protein of unknown function; could be a phage	6,24	25,2	PSHAa0042	+	

	protein					
	5 Unknown proteins					
246	putative secreted protein	4,59	42,5	PSHAa2968	+	
247	Conserved hypothetical protein	5,36	34,1	PSHAa2436	+	
248	ThiJ/PfpI family protein	4,79	23,7	PSHAa2190	+	
249	Pterin-4-alpha-carbinolamine dehydratase	5,53	12,9	PSHAa2042	+	+
250	conserved protein of unknown function	4,54	95,8	PSHAa1989	+	
251	conserved protein of unknown function	5,84	30,3	PSHAa1949	+	
252	conserved protein of unknown function	5,32	39,6	PSHAa1906	+	
253	conserved protein of unknown function	4,79	45,6	PSHAa1899	+	
254	conserved protein of unknown function	5,29	25,2	PSHAa1833	+	
255	conserved protein of unknown function	4,8	45	PSHAa1782	+	
256	Pirin-related protein	5,14	33,6	PSHAa1755	+	
257	conserved protein of unknown function	4,77	22,1	PSHAa1751	+	+
258	ORF215	7,73	25,2	PSHAa1464	+	
259	conserved protein, nucleotide triphosphate hydrolase domain	5,53	74,2	PSHAa1214	+	
260	conserved protein of unknown function	4,83	25	PSHAa1187	+	+
262	conserved protein of unknown function	5,81	24,3	PSHAa0975	+	+
263	putative orphan protein	4,94	39,9	PSHAa0664	+	+
264	putative orphan protein	5,22	27,8	PSHAa0397	+	+
265	conserved protein of unknown function	8,88	27,9	PSHAa0394		+
266	putative acyltransferase	9,27	35,2	PSHAa0174	+	
267	conserved protein of unknown function	4,96	27	PSHAa0125	+	+
268	conserved protein of unknown function	4,44	66,9	PSHAa0078	+	
269	conserved protein of unknown function, possibly secreted	6,98	19,4	PSHAa2971	+	+
270	conserved protein of unknown function	6,43	27,3	PSHAa2853	+	
271	conserved protein of unknown function	7,92	20,8	PSHAa2755	+	
272	putative orphan protein	5,99	24,5	PSHAb0053	+	
273	conserved protein of unknown function	5.03	24,2	PSHAb0060	+	

Experiences in foreign laboratories

During my PhD training, I attended for three months the laboratory of Dr Thomas Schweder in the Institute of Microbiology and Molecular Biology, directed by the professor Michael Hecker, at the Ernst Moritz Arndt University of Greifswald (Germany).

Pubblications Index

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- Rosanna Papa, Gennaro Marino e Angela Duilio. La regolazione dell'espressione genica in microrganismi adattati al freddo: aspetti molecolari ed applicazioni biotecnologiche. Giornate Scientifiche Interpolo 2005, Napoli, pag. 516.
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- Loredana Siani, Rosanna Papa, Gennaro Marino and Giovanni Sannia Recombinant Antarctic bacteria for degradation of chemical contaminants in marine environments and/or at low temperatures Giornate di Chimica e Biotecnologie delle Fermentazioni 2005, Latina, pag. 25.
- Angela Duilio, Rosanna Papa and Gennaro Marino. Regulation of gene expression in cold loving micro organisms: molecular aspects and biotechnological applications. 8th National Biotechnology Congress 2005, Siena, pag. 53.
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Extremophiles. 2005 Sep 23; [Epub ahead of print]

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

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Running title: **Disulfide bond formation in *Pseudoalteromonas haloplanktis* TAC 125**

Abstract

In prokaryotes, protein disulfide bond oxidation, reduction and isomerization are catalysed by members of the thioredoxin superfamily, characterised 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 bond formation in the periplasmic space.
This paper addresses the question of disulphide 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 *E.coli* and characterized.
Results reported in this paper provides some insights into disulphide bonds formation in a micro organism isolated in the Antarctic sea water.

Keywords:*Pseudoalteromonas haloplanktis*; gene regulation; cold adaptation; thiol disulphide oxidoreductase pathways; protein folding; DsbA.

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 catalysed 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, J.C. *et al.*, 1991; Schallreuter, K.U. *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, A. *et al*., 2004). The initial oxidative event is catalysed by DsbA, which interacts with reduced protein substrates and catalyses oxidation of their cysteine residues to disulfide bonds. Homologues of DsbA, characterised by the conserved -C-P-H-C- motif of the catalytic site, have been found in a wide range of bacteria (Hiniker, A. *et al*., 2004), suggesting the conservation of the mechanisms involved in disulphide 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, C.W. *et al.*, 2003). This DsbA-like protein, named SrgA, is characterised 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, J.M. *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, S., *et al.*, 2004; Tinsley, C.R., *et al*., 2004).

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

Experimental

Bacterial strains, DNA constructs and media

Plasmids are all reported in Table I.

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, L., *et al.*, 2000).

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

E.coli cells were routinely grown in LB (Sambrook, J. *et al*., 2001) containing 100 μg/ml of ampicillin, when transformed. *E.coli* JCB570 and JCB571 were used for motility assays (Bardwell, J.C. *et al.*, 1991). *E.coli* JCB816 and JCB817 were used for Lac^- phenotype assays (Grauschopf, U. *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) was used for the construction of the *Ph*TAC125 DNA genomic library. pPLB vector was used for transcriptional analysis (Duilio, A. *et al.*, 2004). pET22b(+) (Novagen) was used for the expression of recombinant proteins (*Ph*DsbA and *Ph*DsbA2).

Cloning and sequencing of *PhdsbA* locus

Genomic DNA preparation from *Ph*TAC125 was carried out as previously described (Tosco, A. *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, D. *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 radiolabelled terminator cycle sequencing kit (Amersham Pharmacia Biotech.), 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.

Construction of the expression plasmids

For the complementation assays, the *PhdsbA, PhdsbA2* and *EcdsbA* genes were amplified (Tosco, A. *et al.*, 2003) and cloned into the commercial expression vector pTRC99A, generating the pT(*PhdsbA*), pT(*PhdsbA2*) and pT(*EcdsbA*) plasmids, respectively (Table I).
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 I).
For the transcriptional fusion experiments, the promoter regions upstream of *PhyihE* and *PhdsbA2* genes were amplified and cloned into the reporter vector pPLB (Duilio, A. *et al.*, 2001), generating P(*yihE*) and P(*dsbA2*) plasmids (table I). 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 I).
All the resulting plasmids were used to transform *Ph*TAC125 cells by interspecific conjugation (Duilio, A. *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, J.C. *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, J. *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 $3OD_{600}$) and northern blotting analysis were performed as described by Tosco, A. *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, A. *et al.*, 2003, by using 18-21 nucleotide specific oligonucleotides as primers.
Reporter assays of transcriptional fusions measured activity of a cold-adapted β−galactosidase as described by Duilio, A. *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 (30mM Tris-HCl pH 8, 20% sucrose, 1mM EDTA pH 8) and incubated at room temperature for 20 min. The shocked cells were collected by centrifugation at 13000 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 13000 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.) 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, A., 1979). The fractions containing the active protein were pooled, concentrated, dialyzed against 20 mM Tris-HCl pH 8, 1M 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, 1M 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, M.M., 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, J.B. *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 3 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, J.B. *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, J. *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 1:1000 and 1.3000 respectively, and peroxidase-conjugated anti-rabbit secondary antisera (1:20000) (A9169, Sigma). 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), 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 (θ) = 100 θ_{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 the equation (1):

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

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

Thermal unfolding transitions were analyzed with the two-state N ⇌ 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 nonlinear 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, J.C. *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, E. *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, P. *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 figure 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, G. 1985), contain at the N-terminus a putative translocational signal, typical of the periplasmic proteins.

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-labelled DNA fragments internal to *PhyihE, PhdsbA* and *PhdsbA2* genes were used as probes. As shown in Fig.2A, P1 probe recognised a 1.5 kb transcript corresponding to the co-transcription of the *PhyihE* and *PhdsbA* genes. P2 probe hybridises with two different transcripts: i) a 1.5 kb transcript corresponding in size to *PhyihE* and *PhdsbA* genes together; ii) a 1.2 kb transcript that was attributed to the co- transcription of the *PhdsbA* and *PhdsbA2* genes. Finally, P3 probe detects two transcripts: i) a 1.2 kb transcript corresponding in size to the co-transcription of *PhdsbA* and *PhdsbA2* genes; ii) a 0.65 kb transcript corresponding to the *PhdsbA2* monocistronic message. All probes recognise 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: i) an active promoter element located upstream of the *PhyihE* gene that is responsible for the full-length locus transcription and *PhyihE-PhdsbA* co-expression; ii) a promoter element upstream of the *PhdsbA* gene, responsible for the *PhdsbA* and *PhdsbA2* co-transcription; iii) 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.

Fig. 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 -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.

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 bp and 300 bp, respectively) were individually fused to a promoter-less *lacZ* gene contained in a pPLB plasmid (Duilio, A. *et al.*, 2001), generating the P(*yihE*) and P(*dsbA2*) vectors (Table I). 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. Fig. 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 I). 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.

_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, A. 1979), as described in the Material 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 oxidised 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, A. *et al.*, 1993; Moutiez, M. *et al.*, 1999).

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 Material 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 disulphide bond formation in the P-ring protein (Dailey, F.E. *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 (panel 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, J.C. *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 disulphide bond formation causes the fused protein to be entrapped in the cytoplasmic membrane. pT(*PhdsbA*) and pT(*PhdsbA2*) JCB817 transformed cells did not developed 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 disulphide isomerases from the cold-adapted Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 provides some insights into disulphide 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 characterised 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, A. *et al.*, 1993; Andersen, C.L. *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, L.W. *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 oxidising 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, A. *et al.*, 1999). Experiments aimed at determining this parameter by chromatographic analysis (Siedler, F. *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, C.W. *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, P. *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, C.W. *et al.*, 2003; Rodriguez-Penap, J.M. *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, likely 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, M. *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, A. *et al.*, 2004). The P1 promoter shows significative 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, H. *et al.*, 2004).

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Table I.

Plasmids constructed in this study

Plasmid	Description*
pT(*PhdsbA*)	pTRC99A Δ(*Nco*I-*Eco*RI) Ω(*PhdsbA* gene)
pT(*PhdsbA2*)	pTRC99A Δ(*Nco*I-*Eco*RI) Ω(*PhdsbA*2 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)

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

Table 2:

Thermodynamic parameters of the thermal unfolding of oxidised 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

```
                1
PhDsbA          MLKKLKLSL LLLCLPFA A..LAANFEV GNQYTVIDIE KSTTPQVTEY
PhDsbA2         MIKLVKAGL LAVLLPFA ATSFAATFEE GVHYEVVSER ATKKPEVKEF
EcDsbA           MKKIWLAL AGLVLAFS A.SAA.QYED GKQYTTLEKP VAGAPQVLEF
                                   :.:*. *..*..:... ....*.*.*:
           50
PhDsbA          FSFYCPHCFK FEP...VAHA IEENLPAGAV FIKNHVNFLG GVSPQTQSNL
PhDsbA2         FSFYCPACNN MEP...LVAE IKPMLDKGVK FKKSHVDFVG VRDTEHQQMI
EcDsbA          FSFFCPHCYQ FEEVLHISDN VKKKLPEGVK MTKYHVNFMG G...DLGKDL
                ***:****.. .*....:... :...*..*:. ..*.**.*.* :........:
           100
PhDsbA          SLAYLVAKKH GQADTITDKI FKSIHVQRAP LTEIKDLKKL LDINGISSDT
PhDsbA2         SQALATAEVL PQKDKIIAAI FSHIHTKRAN FNELADVKDV FVAQGVDGDK
EcDsbA          TQAWAVAMAL GVEDKVTVPL FEGV.QKTQT IRSASDIRDV FINAGIKGEE
                :.*...*... ...*.:...: *..:*..... .....*::.: ....**..:.
           150
PhDsbA          FDQDIASMPI IAAEQAMQDK QNKYSKLGAL TGVPTFIVND KYKINLNTIK
PhDsbA2         FDKLFKSFSV RTLSSKMKRD QDYFKEKGAL RGVPTFIVNG KYKLLLGRE.
EcDsbA          YDAAWNSFVV ....KSLVAQ QEKAAADVQL RGVPAMFVNG KYQLNPQGMD
                :*....*..: ......:... *......:.* .***...**. **.*......
           189
PhDsbA          ......SQEE LDEVSFLLAL.....
PhDsbA2         .....SGISE PADITKLINY LASK
EcDsbA          TSNMDVFVQQ YADTVKYLSE KK..
                ........ ..:....:.. ....
```

Fig. 1

Sequence alignment of DsbA homologues (*Ph*DsbA, *Ph*DsbA2, Fig.1) 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.

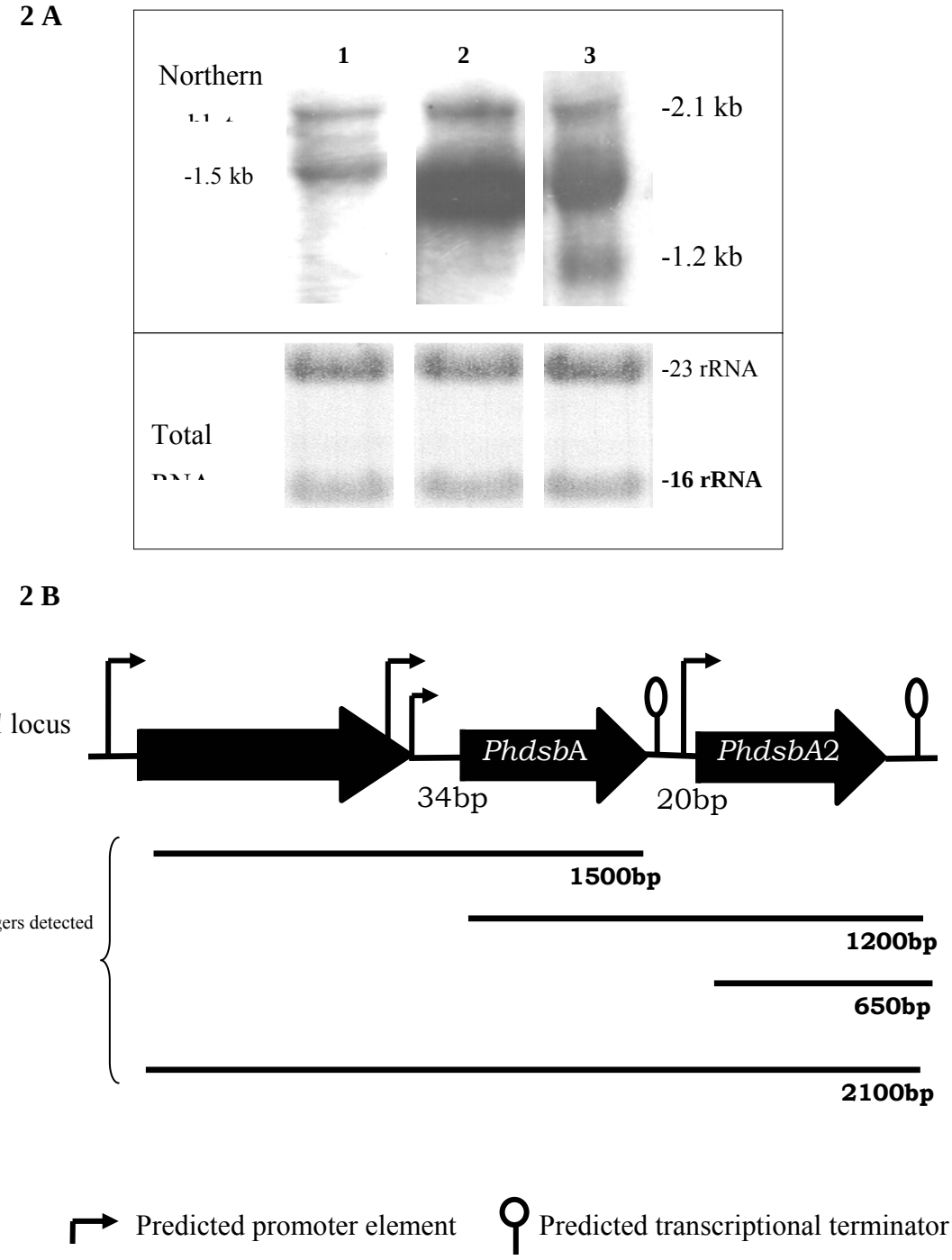
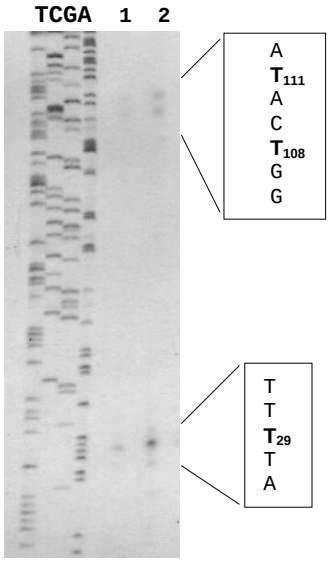


Fig. 2
A. Northern blot analysis (top panel) and visualization of the same samples in an agarose-formaldehyde gel (bottom panel) of *Ph*TAC125 total RNA. Total RNA samples (30μg), isolated from mid-logarithmic-grown cells, were separated onto the 1.2% agarose gel, blotted to nitrocellulose membrane and hybridized with probes P1 (internal to *PhyihE,* lane 1), P2 (internal to *PhdsbA,* lane 2), and P3 (internal to *PhdsbA2,* lane 3).
B. A schematic representation of the *PhdsbA* locus and its transcriptional organization.

3 A



3 B

5′ **-35** P1 **-10** **+1**

ATGGAGCGATGGCTTTTGTGCGGA**ACTTTTCATGGTTTGCT**GATGAC**AAAT**$\underline{\text{}}$**111ACT**$\underline{\text{}}$**108GGGAGCAACAAATTTT**

-35 P2 **-10** **+1**

AGCACTTAAAGAGCAGCTTGCCGCATTGCAA**GAAGCACCGCTAAAAT**TATTGC**CATAACATT**$\underline{\text{}}$**29TATTCAAAAA**

SD ***PhdsbA***

TATGTACAAC*GAGA***GACTA**ATG**CTTAAAAAATTAAAACTGAGCCTACTACTACTTC**

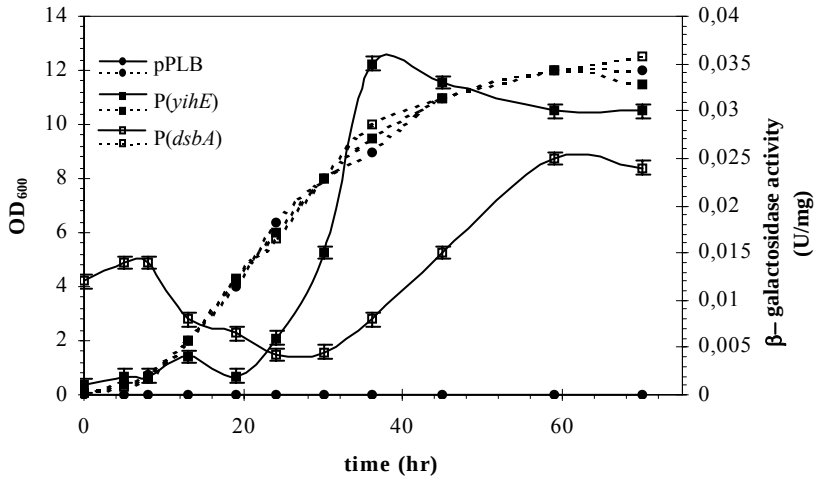
3′

Fig. 3

A. Primer extension analysis of the *PhdsbA* transcript. A 18 bp oligonucleotide was annealed to 10 μg (lane 1) and 30 μg (lane 2) of PhTAC125 total RNA and extended using AMV reverse transcriptase. The nucleotide sequence of the upstream region was determined using the same oligonucleotide as a primer (lane 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 *PhdsbA* start codon is indicated as bold.

4 A



4 B

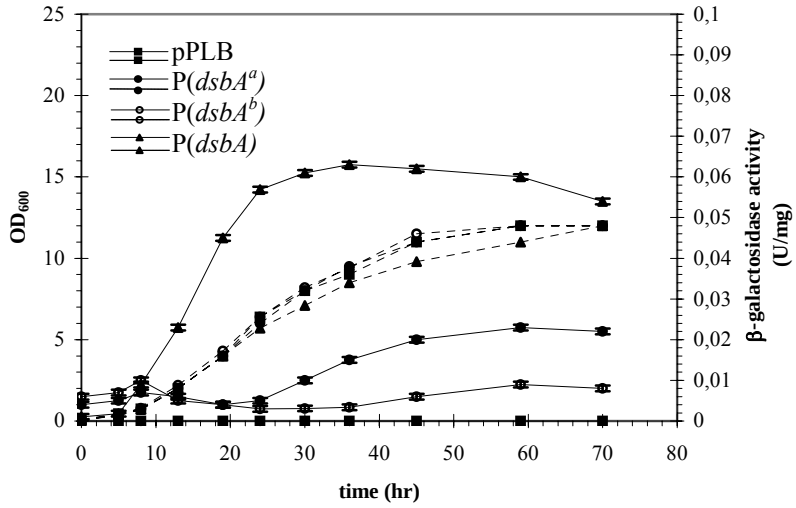


Fig. 4
Profiles of growth in aerated Typ cultures at 15°C and expression of *Phdsb::lacZ* transcriptional fusion constructs in *Ph*TAC125. Broken lines indicate the growth curves, solid lines indicate the promoter activity. Each data point represents an average value from two samples.
A. pPLB (negative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

5

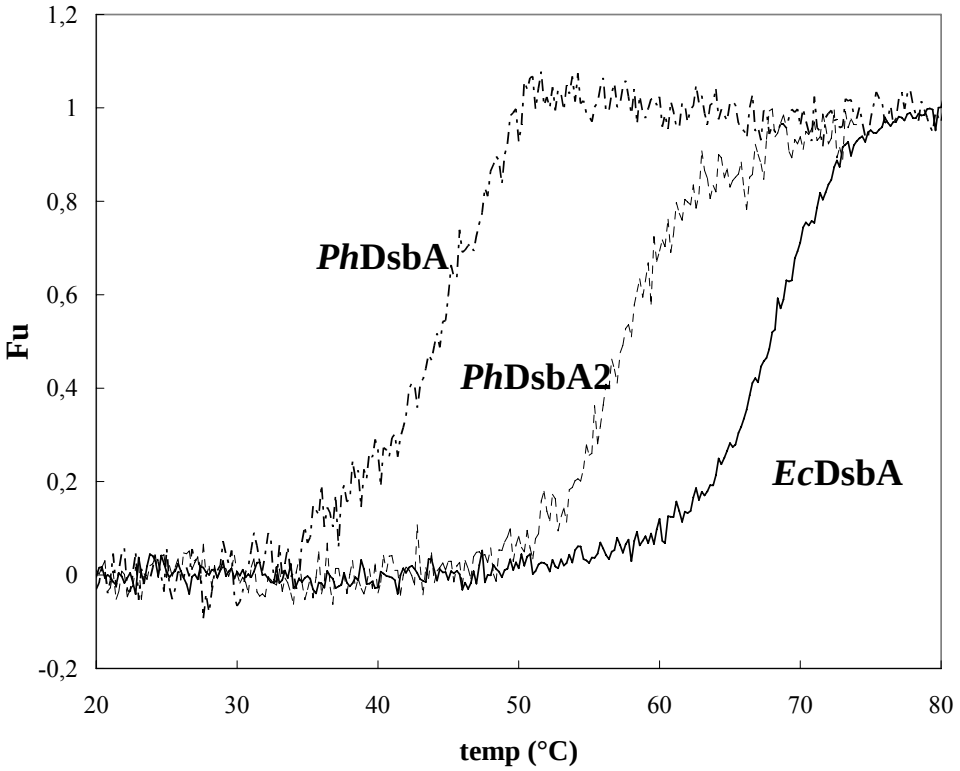


Fig. 5
The melting of secondary structure of DsbAs. Enzyme concentration was 10 μM in 50 mM Tris-HCl pH 7.0. Thermal transitions were followed at 220 nm as temperature was varied at a rate of 1°C min^{-1}. The fraction of unfolded protein was calculated from experimental data as described in the experimental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

6

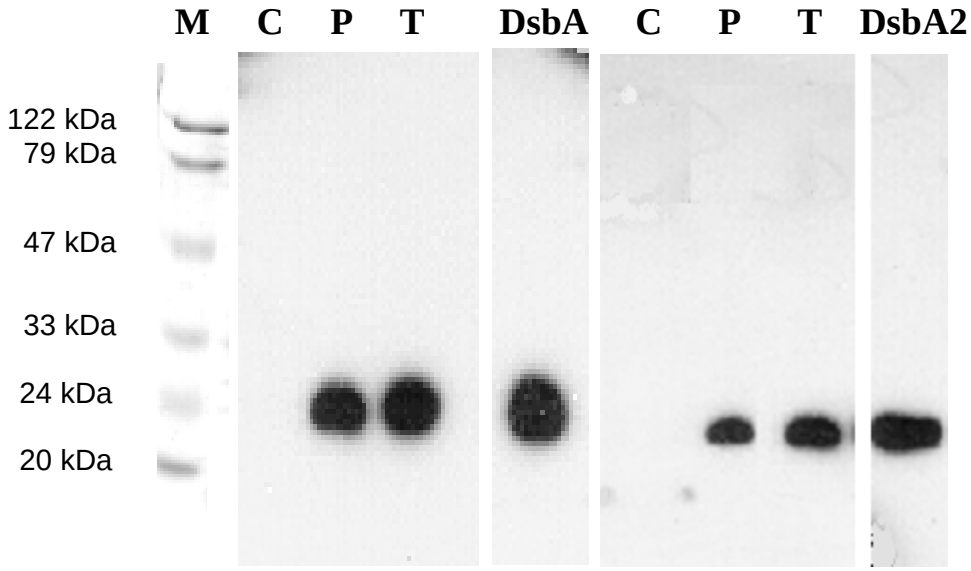


Fig. 6
Western immunoblot showing the cellular localisation of *Ph*PDsbA and *Ph*DsbA2 proteins.
C, soluble cytoplasm fraction; P, periplasmic fraction; T, whole-cell lysate.
DsbA: purified protein; DsbA2: purified protein.

7

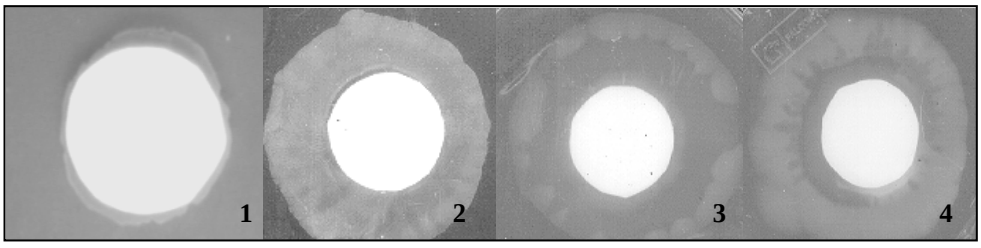


Fig. 7
Complementation assay of *E.coli dsbA*$^{-}$ strain JCB571
1: non recombinant pTRC99A vector (negative control)
2: pT(*EcdsbA*) JCB571 recombinant cells (positive control)
3: pT(*PhdsbA*) and pT(*PhdsbA2*) JCB571 recombinant cells
4: pT(*PhdsbA2*) JCB571 recombinant cells