# Novel expression systems for recombinant protein production at low temperatures

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One of the main limitations experienced while producing proteins in conventional bacterial mesophilic systems is the need to operate at their optimal growth temperature (usually 37 ºC) for the production process. Since temperature has a general negative impact on protein folding due to the strong temperature dependence of hydrophobic interactions that mainly drive the aggregation reaction, the production of recombinant proteins at low temperatures represents an exciting model to improve the quality of the products. Recombinant protein production in psychrophilic bacteria, i.e. at temperature as low as 4°C, may minimise undesired hydrophobic interactions during protein folding, desirably resulting in enhancing the yield of soluble and correctly folded products. In this context, a few cold adapted species are under early but intense exploration as cold cell factories, among them, *Pseudoalteromonas haloplanktis* being a representative example. The efficiency of cold-adapted expression systems was tested by fully soluble and biologically competent production of several thermal-labile and aggregation-prone products in *Ph*TAC125 such as the mature human nerve growth factor and a yeast α-glucosidase Furthermore, with respect to *E. coli*, *Ph*TAC125 is extremely efficient in secreting proteins in the culture medium. By the use of a psychrophilic α-amylase as secretion carrier for the extra-cellular targeting of recombinant proteins an efficient gene-expression system was set up. Observed efficiency of the cold-adapted system (secretion yield was always above 80%) placed it amongst the best heterologous secretion systems in Gram-negative bacteria reported so far.

## 1. Introduction

The number of candidate proteins to be used as biopharmaceuticals or in industrial processes is rapidly increasing in recent years (Pavolu *et al.*, 2005). However, efficient expression of genes in homologous/heterologous expression systems and rapid purification steps are actually major bottlenecks. In fact, although many recombinant proteins have been successfully produced by common prokaryotic (*Escherichia coli*) and eukaryotic (yeasts and CHO cells) hosts, these conventional systems have often proved to be unproductive due to the special properties of the protein to be produced. Indeed, beside the obvious impossibility of achieving a large scale production of thermally labile proteins at the normal *E. coli* growth temperature, degradation of the product by the host proteases and the incorrect folding of the nascent polypeptides, resulting in the protein aggregation and accumulation as insoluble inclusion bodies, are sometimes observed (Speed *et al*., 1996). To overcome the above mentioned limits of *E. coli* as host for recombinant protein production, a rational experimental approach has

consisted in lowering the cultivation temperature (Baneyx, 1999), since this change has a pleiotropic consequence on the folding process. Inclusion bodies formation is a process mainly driven by hydrophobic interactions which are directly dependent on temperature (Kiefhaber *et al*., 1991). There are many examples in literature describing the effectiveness of enhancing solubility of a number of difficult proteins by this approach (Vasina and Baneyx, 1997). The growth of *E. coli* below 37 ºC has been often explored to minimise aggregation but without consistent, protein-irrespective results. The major drawback in *E. coli* cultivation at sub-optimal temperatures is, however, the decrease in biomass production which reduces the global process productivity. Therefore, the use of psychophilic bacteria as alternative expression hosts is the compelling choice towards the exploitation of industrial processes at temperatures as low as 0°C.

## 2. The psychophilic host: *Pseudoalteromonas haloplanktis* TAC125

*P. haloplanktis* TAC125 is a Gram-negative bacterium isolated from an Antarctic coastal seawater sample collected in the vicinity of the French Antarctic station Dumont D'Urville, Terre Adélie. It can be classified as a Eurypsychrophile (i.e. a bacterium growing in a wide range of low temperatures; Atlas and Bartha, 1993) and was the first Antarctic Gram-negative bacterium of which the genome was fully sequenced and carefully annotated (Médigue *et al*., 2005). Genomic and metabolic features of this bacterium, accounting for its remarkable versatility and fast growth compared with other bacteria from aqueous environments, were discovered by combining genome sequencing and further *in silico* and *in vivo* analyses. *P. haloplanktis* TAC125 is able to duplicate in a wide range of temperatures (0-30°C), with an apparent optimal growth temperature a t 20°C, where the observed duplication time in rich medium is 31 minutes (Tutino *et al.,* 1999). However, the bacterium still duplicates at fast speed even at lower temperatures (at 4°C, one cell division is completed in about 100 min; unpublished results from this laboratory) and, when provided with sufficient nutrients and aeration, it grows to very high density (up to $A_{600}$=20) under laboratory settings, even at 0°C. This growth performance makes it one of the faster growing psychrophiles so far characterised. Fast growth rates, combined with the ability of *P. haloplanktis* TAC125 to reach very high cell densities even under laboratory growth conditions and to be easily transformed by intergeneric conjugation (Duilio *et al*., 2004a), made this bacterium an attractive host for the development of an efficient gene expression system at low temperatures.

## 3. Genetic tools for recombinant protein production at low temperatures

### 3.1 The psychrophilic expression vector

A few other reported examples of recombinant protein production in psychrophiles made use of molecular signals (such as the origin of replication and the transcriptional promoter) derived from mesophiles. A different philosophy inspired the construction of our gene-expression systems, which derived from the proper assembly of true phychrophilic molecular signals into a modified *E. coli* cloning vector. By combining

mesophilic and psychorophilic genetic signals a collection of psychophilic gene-expression vectors was set up to produce recombinant proteins in *P. haloplanktis* TAC125. The mesophilic signals consist of the pUC18-derived origin of replication (OriC) and a selection marker gene (a β-lactamase encoding gene), allowing the plasmid to replicate either in *E. coli* or in the phychrophilic host. Another crucial mesophilic signal is represented by the OriT sequence, the conjugational DNA transfer origin from the broad host range plasmid pJB3 (Blatny *at al*., 1997). Structural and functional studies led to the isolation of the psychrophilic origin of replication (OriR) from the *P. haloplanktis* TAC125 endogenous plasmid pMtBL (Tutino *et al*., 2001).

### 3.2 Phsychrophilic promoters

*3.2.1 Constitutive expression*

The structural/functional characterisation of *P. haloplanktis* TAC125 promoters was carried out by random cloning of genomic DNA fragments and identification of promoter sequences by evaluating their capability to express a promoter-less reporter gene (Duilio *et al*., 2004). By this promoter-trap strategy, a collection of constitutive psychrophilic promoters showing different strengths at different temperatures was identified. The implementation of the above described psychrophilic promoters in the pMtBL-derived shuttle vectors resulted in the set up of cold-adapted gene-expression systems, characterised by the constitutive production of the recombinant protein.

*3.2.2 Regulated expression*

Sometimes efficient production can only be achieved by fine tuning the recombinant gene expression. This goal can be reached by using regulated promoters and efficient induction strategies. Recently, by using a differential proteomic approach, we isolated and characterised a two-component system. This regulatory system is responsible for the transcriptional regulation of the gene coding for an outer protein porine, and it is strongly induced by the presence of L-malate in the medium (Papa *et al*., 2006). The regulative region of the porine gene was used for the construction of an inducible cold expression vector, where the recombinant protein expression is under L-malate control.

### 3.3 Molecular signals for protein addressing

*3.3.1 Periplasmic secretion*

Although the production of recombinant protein in the host cytoplasm is the preferred strategy many processes due to higher production yields, this approach cannot be pursued when the wanted product requires the correct formation of disulphide bonds to attain its catalytic competent conformation. Indeed as for all Gram-negative bacteria, *P. haloplanktis* TAC125 cytoplasm is a reducing environment and the formation of disulphide bridges is confined in the periplasmic space. From the genome analysis, we know that *P. haloplanktis* TAC125 contains all the canonical periplasmic export machineries (Medigue *et al*., 2005). Therefore, gene fragments encoding two signal peptides from psychrophilic secreted proteins following different translocation mechanisms have been cloned in the psychrophilic expression vectors, under the control of different promoters in order to allow the signal peptides N-terminal fusion for periplasmic addressing of recombinant proteins.

*3.3.2 Extra-cellular secretion*

In order to combine the effects of low temperatures on the recombinant product solubility with the advantages linked to extra-cellular protein targeting, a gene expression system for the production and extra-cellular secretion of recombinant proteins in psychrophilic bacteria was set up. The novel system makes use of the psychrophilic α-amylase from *P. haloplanktis* TAB23 (Feller *et al*., 1992) as a secretion carrier. This exo-protein is produced and secreted as a larger precursor with a long C-terminal propeptide that is not mandatory for the α-amylase secretion when it is produced by recombinant cold-adapted bacteria the propeptide (Tutino *et al*., 2002; Cusano *et al.,* 2006a). Starting from the latter observation, the secretion of chimeric proteins obtained by the replacement of α-amylase C-terminal propeptide with a passenger protein was studied (Cusano *et al.,* 2006b). The novel genetic system allows the easy in-frame cloning of any gene downstream of the mature psychrophilic α-amylase encoding region (Figure 1). The spacer between the carrier and passenger proteins contains the motif -Ala-Ser-Ser-Thr- recognised and cleaved by a *P. haloplanktis* TAC125 secreted protease that allows the separation of the protein of interest from the secretion carrier when it reaches the extra-cellular medium.

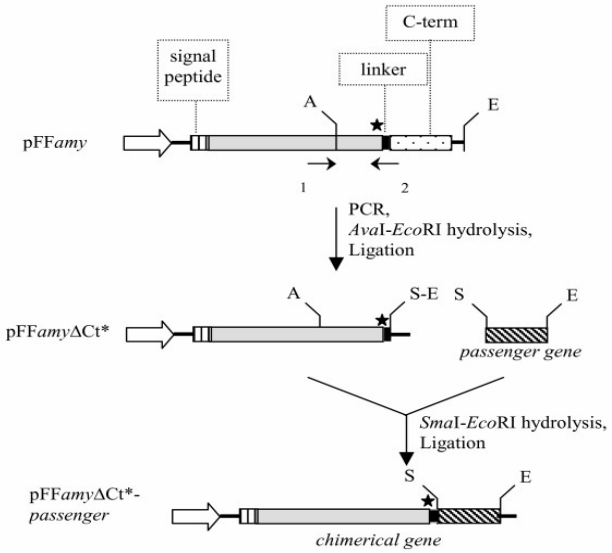


**Figure 1** The genetic system for recombinant protein secretion in *P. haloplanktis* TAC125 extra-cellular medium. *White arrow*, *P. haloplanktis* TAC125 *aspC* promoter; signal peptide, sequence encoding *P. haloplanktis* TAB23 α-amylase signal peptide; C-term, α-amylase C-terminal propeptide encoding sequence; linker, α-amylase linker encoding sequence; A, *AvaI*; E, *EcoRI*; S, *SmaI* restriction endonuclease sites; *black arrows,* PCR primers. The *black star* indicates the *P. haloplanktis* TAC125 exo-protease cleavege site.

## 4. Recombinant protein production in *P. haloplanktis* TAC125

### 4.1 Cytoplasmic production: *P. haloplanktis* TAE79 β-galactosidase and *Saccharomyces cerevisiae* α-glucosidase

Two "difficult" proteins were produced to test performances of the cold expression system inducible by L-malate (Papa *et al*., 2007). These proteins (the psychrophilic β-galactosidase from *P. haloplanktis* TAE79 and the *S. cerevisiae* α-glucosidase) were chosen because they can hardly be expressed in mesophilic hosts even at sub-optimal temperature conditions. When the β-galactosidase was produced in *E. coli* cells at 18°C, 20mg of catalytically active enzyme was produced per litre of culture. Analogously, recombinant yeast α-glucosidase produced in *E. coli* aggregates in an insoluble form and the active soluble amount of protein was less than 1% of the total production (Le Thanh and Hoffmann, 2005). Both recombinant psychrophilic β-galactosidase and yeast α-glucosidase were produced in *P. haloplanktis* TAC125 (Figure 2A and B) as soluble and catalytically active enzymes at 15°C. Structural and kinetic analysis of the recombinant proteins showed that both enzymes were nearly identical to their native counterparts. Experimental conditions for optimal protein production in the cold expression system were also defined. Under optimal expression conditions, recombinant β-galactosidase is produced with high yields (620-720 $mgL^{-1}$), indicating that the inducible system can be very effective in the expression of psychrophilic proteins that are usually poorly produced in mesophilic hosts. A significantly lower production yield is observed for yeast a-glucosidase (27 $mgL^{-1}$) possibly due to the different codon usage between the eukaryotic and bacterial organisms. Nevertheless, the cold expression system yielded a satisfactory amount of this protein in a soluble and active form.

### 4.2 Periplasmic production: *h-β-NGF*

Another example of "difficult" protein is the mature form of the human nerve growth factor (h-NGF), a neurotrophin which found promising applications as a therapy agent in several neurological disorders such as Alzheimer's disease (Lad *et al*., 2003). Therapeutical applications require the expression and purification of a large amount of functional protein. However, the recombinant production of this protein exhibits several problems in the conventional host *E. coli,* due to its tendency to form insoluble aggregates either when produced as prepro-protein or as mature form (Dicou *et al*., 1989; Rattenholl *et al.*, 2001). Vigentini *et al*., (2006) reported the expression of the mature form of human NGF gene in *P. haloplanktis* TAC125 and investigated the production and the cellular localisation of the recombinant protein. The protein constitutively produced at 4°C was soluble and efficiently translocated in the host periplasmic space (Figure 2C). A gel exclusion chromatography also indicated that the protein was largely in a dimeric form, the quaternary structure required for its biological activity (Harmer *et al.*, 2003).

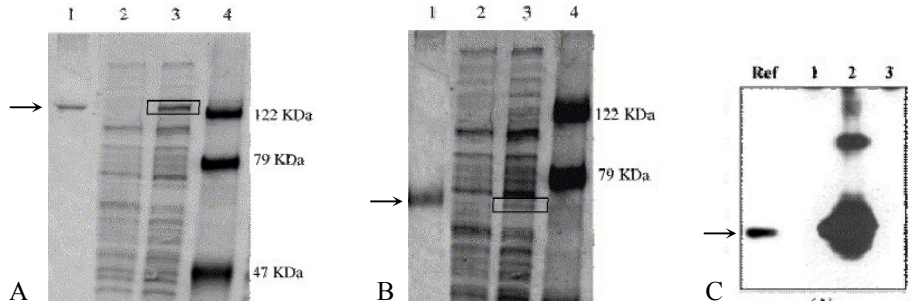


**Figure 2** Recombinant protein production in *P. haloplanktis* TAC125. **A** Recombinant production of the thermally labile β-galactosidase from *Ph*TAE79 in *Ph*TAC125 cells. 7,5% SDS PAGE gel electrophoresis of protein extracts from *Ph*TAC125 cells harboring p(PSHAb0363) and grown in minimal medium at 15°C in the absence (lane2) and in the presence (lane3) of L-malate, in comparision with β-galactosidase from *Ph*TAE79 used as control (lane1). The recombinant protein is indicated by an *open box.* **B** Recombinant production of the mesophilic α-glucosidase from *Saccaromyces cerevisiae* in *Ph*TAC125 cells. 7,5% SDS PAGE gel electrophoresis of protein extracts from *Ph*TAC125 cells harboring pUCRPGLUCP1 and grown in minimal medium at 15°C in the absence (lane2) and in the presence (lane3) of L-malate, in comparision with commercial α-glucosidase from yeast used as control (lane1). The recombinant protein is indicated by an *open box.* **C** rh-NGF production and cellular localisation in recombinant PhTAC125. Western blotting analysis of periplasmic (lane2) and cytoplasmic (lane3) fractions of 4°C grown PhTAC125-pPM13*psDngf* recombinant cells. Polyclonal anti-h-NGF antibodies were used for immunodetection. As positive control, 50ng of rm-NGF proteins was loaded in lane1.

### 4.3 Extra-cellular secretion of several heterologous proteins

One strategy to improve the protein production process in to target the protein to the outer compartment of the host cell. This strategy avoids inclusion body formation and the majority of the proteolytic proteins in the cytoplasm and achieves a primary purification reducing the costs of downstream processes. Cusano *et al*., (2006b) described the setting up and utilisation of a cold gene expression system in *P. haloplanktis* TAC125 implemented for the specific secretion of recombinant proteins in the extra-cellular medium by the use of the psychrophilic α-amylase as a secretion carrier. Three chimerical proteins, obtained by fusing intra-cellular proteins to the psychrophilic exo-enzyme, were produced in *P. haloplanktis* TAC125 and their secretion kinetic was evaluated. The results demonstrated that the cold adapted secretion system is extremely efficient since all tested chimeras were correctly localised in the extra-cellular medium with a secretion yield always above 80% (Table1). Furthermore, reported activity data indicated that the system also allows the correct disulphide bond formation of chimera components (Cusano *et al.,* 2006b).

**Table 1**

Secretion yield of chimerical proteins in recombinant *P. haloplanktis* TAC125 cells

| vector | α-amylase (UI/ml) | | α-amylase | alkaline phosphatase | **secretion yield**[a] |
|---|---|---|---|---|---|
| | p | em | em(%) | em(%) | |
| pFF*amyΔ*Ct-*dsbA* | 0.12 ± 0.01 | 3.58 ± 0.03 | 97 | 4 | **93** |
| pFF*amyΔ*Ct-*trpC* | 0.07 ± 0.02 | 3.65 ± 0.03 | 98 | 5 | **93** |
| pFF*amyΔ*Ct-*blaM* | 1.23 ± 0.03 | 6.21 ± 0.03 | 83 | 1 | **82** |

Data are average results of three independent experiments. The volume of periplasmic fraction was made the same as corresponding extra-cellular medium to allow a comparison. UI, international units; p, periplasmic extract; em, extra-cellular medium fraction; em(%), percentage of the activity in extra-cellular medium fraction of the total activity (periplasmic plus extra-cellular medium fractions). [a] The difference between the em (%) of amylase activity and em (%) of alkaline phosphatase activity.

## 5. Conclusions

Over the last decade the number of set ups of reliable genetic systems for recombinant gene expression in cold adapted hosts was significantly enhanced. Our results demonstrated that the production of recombinant proteins in psychrophilic bacteria is not only a mature and reliable technology but it is also a successful strategy to overcome the product solubility problems often occurring in conventional systems such as in *E. coli*. In this context, *P. haloplanktis* TAC125 and the gene expression systems set up have a valuable biotechnological potential as non-conventional systems for the production of "difficult" proteins.

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