Chapter 21
Heterologous Protein Expression in Psychrophilic Hosts

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21.1 Introduction

The vast number of candidate proteins generated from genome projects are creating enormous opportunities for biologists. 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 from common prokaryotic (*Escherichia coli*) and eukaryotic (yeast and CHO cells) hosts, these conventional systems have often proved to be unproductive due to the peculiar 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 proteins 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 the recombinant protein production, a rational experimental approach has consisted in lowering the

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cultivation temperature (Baneyx 1999), since this change has a pleiotropic consequence on the folding processes. There are many examples in literature describing the effectiveness of enhancing the solubility of a number of difficult proteins by this approach (Vasina and Baneyx 1997). Inclusion bodies formation is a process mainly driven by hydrophobic interactions which are directly dependent on temperature (Kiefhaber et al. 1991). Another interesting aspect linked to the growth of *E. coli* at suboptimal temperature is the partial elimination of heat-shock proteases, whose production is generally induced under over-expression conditions (Chesshyre and Hipkiss 1989), and the observed higher intracellular concentration of host-encoded chaperones at temperatures around 30°C (Mogk et al. 2002; Ferrer et al. 2003).

There are two main systems which optimize the recombinant protein production in *E. coli* at reduced temperatures. The first system takes advantage of the host response to temperature downshift, which induces the transcription and translation of the cold-shock genes while ceasing the expression of virtually all the other genes (Qing et al. 2004). The other one is based on the co-expression of the target protein with chaperones from a psychrophilic bacterium. The two chaperones (Cpn60 and Cpn10 from *Oleispira antarctica* RB8T) allow *E. coli* to grow at a reasonable rate at 4°C (Ferrer et al. 2004).

There is, however, a major drawback in the cultivation of *E. coli* at reduced temperatures: biomass production decreases with temperature, reducing the global process productivity, i.e. the total biomass produced divided by the process time. Therefore, the use of psychrophilic bacteria as alternative expression hosts is the compelling choice towards the exploitation of industrial processes at temperatures as low as 0°C.

To develop such a technology in cold-adapted bacteria, the availability of several genetic tools and structural/functional information are required, such as molecular signals for the autonomous DNA replication (origin of replication), marker genes enabling efficient selection schemes for the isolation of transformed psychrophilic cells, and gene-expression regulating sequences (promoters). These genetic tools should be derived from psychrophilic bacteria or should at least be able to work in the psychrophilic cellular context. In literature, there are only two reported examples of heterologous protein production in psychrophiles by using mesophile-derived replicons with a broad host-range profile.

In 1999, Remaut and coworkers reported the expression of the moderately thermolabile, eukaryotic luciferase (from *Photinus pyralis*) by cloning its coding gene into a pJB3-derived replicon (Blatny et al. 1997) under control of *E. coli*-derived transcriptional and translational signals, in an Antarctic strain growing at 15°C. The second example is the production of a β-galactosidase and the green fluorescent protein in the piezo-psychrophile *Photobacterim profundum* SS9 (Lauro et al. 2005). A broad-host range gene-expression vector was constructed by cloning the *E. coli* P_{BAD}/*araC* system from pJN105 (Newman and Fuqua 1999) into an RSF1010 (IncQ) derivative. The resulting plasmid was effective in the regulated production of the β-galactosidase in the cold-adapted bacterium, although the production yields resulted to be about one order of magnitude lower than what was obtained by the same system in *E. coli* (Lauro et al. 2005).

The isolation and molecular characterization of a true psychrophilic plasmid from the Antarctic Gram-negative bacterium *Pseudoalteromonas haloplanktis*

TAC125 (Tutino et al. 2001) have been instrumental in setting up several *E.coli*-psychrophile shuttle vectors for the efficient recombinant protein production. In the next sections, we will describe our "cold" gene-expression technology and some examples of its application to the production and secretion of "difficult" proteins.

21.2 The genetic system

In this section, we describe the bacterial host and all the genetic tools developed to achieve recombinant protein production. Features of psychrophilic gene-expression vectors set up are described in detail.

21.2.1 The psychrophilic 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 determined and carefully annotated (Medigue et al. 2005). Genomic and metabolic features of this Gammaproteobacterium, 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.

Amongst these relevant traits, it is worth mentioning that the bacterium seems to cope with the increased solubility of oxygen at low temperature by multiplying dioxygen scavenging while deleting whole pathways producing reactive oxygen species (ROS). In the latter direction is the unusual (if compared to other related bacteria, such as the vibrio or *Shewanella*) abolition of the ubiquitous molybdopterin-dependent metabolism and the deletion of all genes coding for enzymes using the molybdopterin cofactor. In general, *P. haloplanktis* TAC125 is remarkably well protected against ROS under cold conditions, a feature that could be very useful for the expression of foreign proteins at low temperatures.

Moreover, as seen in silico with its proteome composition (Medigue et al. 2005), it provides a way to resist the aging features involving asparagine cyclisation and deamidation. Indeed, *P. haloplanktis* TAC125 proteome reveals a concerted amino acid usage bias specific to psychrophiles: for instance, this proteome is enriched in asparagine residues compared to those from organisms growing at higher temperatures, making it an organism of choice for foreign protein production when deamidation ought to be put to a minimum (Weintraub and Manson 2004).

Being a eurypsychrophile, the duplication of *P. haloplanktis* TAC125 has been observed in the range 4–30°C, with an apparent optimal growth temperature at 20°C, where the observed duplication time in rich medium is 31 min (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 characterized.

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.

Moreover, the knowledge of *P. haloplanktis* TAC125 genome has made feasible the development of an efficient genetic scheme for the construction of knock-out mutants in specific psychrophilic genes (unpublished results from this laboratory). Setting up genomic mutants is the obligatory step towards either the study of the role of a given gene, if any, or the construction of genetically engineered bacterial strains, possibly displaying improved features as host for recombinant protein production and secretion.

21.2.2 The psychrophilic gene-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 psychrophilic molecular signals into a modified *E. coli* cloning vector. Figure 21.1

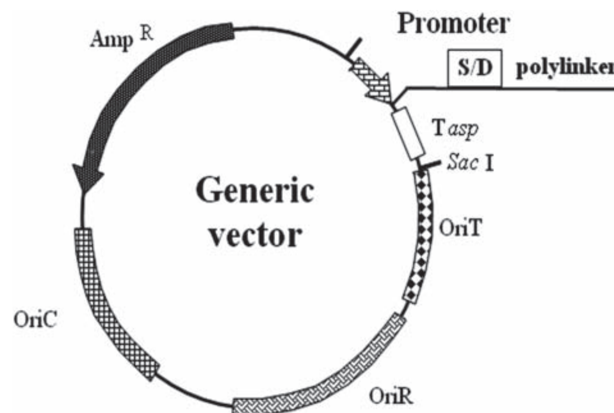

Fig. 21.1 Psychrophilic gene-expression vector

shows the scheme of a generic psychrophilic gene-expression vector 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 psychrophilic host, i.e. a shuttle vector. Another crucial mesophilic signal is represented by the OriT sequence, the conjugational DNA transfer origin from the broad host range plasmid pJB3 (Blatny et al. 1997). OriT is recognized by some proteins (encoded by *mob* gene cluster) involved in the plasmid DNA mobilization from the donors to the psychrophilic recipient cells during conjugation (Duilio et al. 2004a).

In the following sections, we will describe in more detail some structural and functional results concerning the psychrophilic origin of replication (OriR) isolated from the *P. haloplanktis* TAC125 pMtBL plasmid and the cold-adapted promoters used for the recombinant protein production.

21.2.2.1 Psychrophilic origin of replication

We reported the isolation and molecular characterization of the first plasmids isolated from Antarctic marine bacteria, pTAUp from the strain *Psychrobacter sp*. TAD1 (Tutino et al. 2000) and pMtBL from *P. haloplanktis* TAC125 (Tutino et al. 2001).

We focused our attention on the small multicopy plasmid pMtBL, since this episome turned out to be an interesting extra-chromosomal element displaying unique molecular features. First of all, its nucleotide sequence (4081 bp) is still not related to any entry stored in the databanks and it has been found to be transcriptionally silent. Because of being cryptic, the inheritance of pMtBL totally relied on the efficiency of its replication functions. This function can be linked to a region of about 850 bp by an in vivo assay. In fact, the pMtBL autonomous replication sequence (ARS) (OriR) was defined as the smallest *Alu*I portion of the psychrophilic plasmid able to actively promote the duplication of an *E. coli* vector (unable to replicate by itself) in the cold-adapted host *P. haloplanktis* TAC125. Interestingly, no incompatibility but a simple competition was observed between the endogenous wild type plasmid and OriR-containing shuttle vectors actually carrying the selection resistance gene (amp^R). It might be that other functions, possibly related to a specific plasmid partitioning mechanism, are responsible of the stable pMtBL inheritance at a low copy number in *P. haloplanktis* TAC125 transconjugants.

pMtBL-derived shuttle vectors were used to successfully transform several psychrophilic bacteria, belonging either to *Pseudoalteromonas* and *Psychrobacter genera*, or unclassified marine bacteria (Tutino et al. 2001) and the piezopsychrophile *Photobacterium profundum* SS9 (F. Lauro, personal communication). These data suggest that pMtBL ARS can be used as a broad-host-range origin of replication and functionally support some structural observations on the OriR sequence, which highlighted its similarity to the OriV origin of replication from the broad host range RK2 plasmid (Fang and Helinski 1991).

21.2.2.2 Psychrophilic transcription initiation signals

A necessary background to investigate the adaptation of gene-expression mechanisms is represented by the study of RNA polymerase and its cognate molecular signals. This knowledge is of utmost importance to control and modulate the recombinant protein production in any gene-expression system, and, therefore, also in a cold-adapted one.

Concerning the psychrophilic transcription initiation signals, despite the steady increase in the number of sequenced cold-adapted genes, there is only one example of systematic analysis of the sequence of promoter regions in psychrophilic bacteria. The structural/functional characterization of *P. haloplanktis* TAC125 promoters (Duilio et al. 2004b) 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. A promoter-trap library was constructed by cloning *P. haloplanktis* TAC125 genomic DNA fragments into a shuttle psychrophilic vector upstream of the promoter-less β-galactosidase gene from *P. haloplanktis* TAE79 (*Ph*TAE79*lacZ*) (Hoyoux et al. 2001). The recombinant vectors were mobilized into *P. haloplanktis* TAC125 cells by inter-generic conjugation (Duilio et al 2004a), and several dark blue colonies, possibly containing strong promoters, were selected from X-Gal containing agar plates (Duilio et al. 2004b).

The determination of the putative transcription start site was obtained by primer extension analysis and a promoter consensus sequence for *P. haloplanktis* TAC125 was proposed on the basis of a sequence comparison between the various active promoters (Fig. 21.2).

The identified consensus sequences resulted to be very similar to those of the *E. coli* σ^{70}-dependent promoter. Indeed, as far as this small promoter sample is concerned, the cold-adapted promoters seem to diverge from the mesophilic ones mainly in the 2nd and 5th positions of -35 box, where the occurrence of T and C have more frequently been observed (Lisser and Margalit 1993).

Data collected in this study highlight the strong similarity between the defined psychrophilic transcriptional signals with the *E. coli* counterparts. Furthermore, this resemblance is not limited to the −35/−10 sequences but it is also supported by their functionality in the mesophilic context, since the *E. coli* transcriptional machinery initiates the m-RNA synthesis exactly from the transcriptional start point used by *P. haloplanktis* TAC125. This functional characterization supports the observed high sequence similarity between the mesophilic and the psychrophilic RNA-polymerases (Medigue et al. 2005).

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, characterized by the constitutive production of the recombinant protein.

However, efficient production can sometimes be achieved only by fine tuning the recombinant gene expression. This goal can be reached by using regulated promoters and efficient induction strategies. Indeed, physical separation between bacterial growth phase and expression of the desired proteins can either improve

A

```
                 -35                              -10          +1

P2-1     AGAATGAACTATTACCCGATGCAGTGAG-TCTGTATATAGAG
P2-2     GTCATTGGTATAAAACCCC--TAGCATGTTAAAATGCACGCTAT
P3       AGGCTTGATCTGTTTTCGG---ATCTCCCTATAATGCGACCCCA
P4       CAAGTCGGTAAAACCGAGTAAACTATGCGTATTATATTATGC
P6       GCCGTGAGTTGTTTGTAAT----TGCTGATATGCCGTTTATGACTTA
P14      TTATTTCAATGAGTTGCATAA---CAGCCTAAAATTAAGT
P15      TCTCTAGTTTGCTAAGCACC--AAATGAGTTACATTGTAACTT
P17      GTGGTAGCTTTTGCCCAT---CAACATGGTATTAAAGTGTTT
P25      CTTTTACAATGGCAAAGTCA-----CCGGTAATACTGTG
```

B

```
Consensus
             -35                        -10                     +1
E.coli       TTGACA -- (16-18 bp) -- TATAAT -- (5-8 bp) --
PhTAC125     TRGRTW -- (14-19 bp) -- TATRAY -- (4-12 bp)--
```

Fig. 21.2 Promoter consensus sequence for *P. haloplanktis* TAC125 Alignment of promoter regions from *Ph*TAC125 (**A**) and identification of a cold-adapted consensus sequence (**B**). The determined transcriptional start sites are *boldface* and *underlined. Dashes* indicate gaps introduced to maximize the alignment. Nucleotides highlighted in *black* are those considered for the definition of the consensus sequence shown in panel B. It consists of nucleotides that are present in any given position in more than 51% of the sequences. In *brackets* the average distances (in base pairs) between the promoter consensus sequences are reported. R: A or G; Y: T or C; W: A or T. *E.coli* promoter consensus sequence was derived from Lisser and Margalit (1993)

the productivity of the entire system or it can also play an important role in the production of toxic proteins for the host cells.

Recently, using a differential proteomic approach, we isolated and characterized a two-component system. This regulatory system is responsible for the transcriptional regulation of the gene coding for an outer membrane porin, 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 results to be under L-malate control. This inducible system was effective in the production of both psychrophilic and mesophilic proteins in *P. haloplanktis* TAC125 (Papa et al. 2007).

21.2.3 Molecular signals for protein addressing

Although the production of recombinant protein in the host cytoplasm is the preferred strategy in 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 (due to the presence of reducing enzymes, such as thioredoxin and glutaredoxin) and the formation of disulphide bridges is confined in the periplasmic space, where the enzymes belonging to the Dsb family are located (Rietsch and Beckwith 1998). *P. haloplanktis* TAC125 genome analysis lead us to confirm the presence of at least one clear homologue for any *E. coli dsb* gene (from *dsbA* to *dsbG*) characterized so far. However, the psychrophilic bacterium notably diverges from the mesophilic counterpart since it possesses two adjacent chromosomal genes coding for two DsbA-like proteins (Madonna et al. 2006). Their transcriptional regulation mechanisms were investigated in detail. It turned out that both proteins are produced in vivo but at different expression rates during growth, suggesting their likely involvement in different but complementary cellular processes (Madonna et al. 2006).

Protein translocation from the cytoplasm to the periplasmic space can be achieved by three different routes: the Sec pathway (Matlack et al. 1998) which is a post-translational export; the Srp pathway (Luirink and Sinning 2004), which is a co-translational export and shares some components with the previous one; and the TAT pathway (Lee et al. 2006), which differs significantly from other pathways since it is able to translocate fully folded passengers. From the genome analysis, we know that *P. haloplanktis* TAC125 contains all the above mentioned export machinery (Medigue et al. 2005). Therefore, we set up a family of psychrophilic gene expression systems for the recombinant protein production in the periplasmic space. Gene fragments encoding two signal peptides from psychrophilic secreted proteins have been cloned under the control of constitutive promoters with different transcription efficiencies. Several chimerical proteins were generated, where the signal peptide was fused to the N-terminus of several mesophilic disulfide bond containing proteins, and their efficiency as periplasm addressing tag was tested. An example of successful use of these vectors for the soluble production of human nerve growth factor (hNGF) as reported in the last section of this chapter.

Furthermore, the final aim in recombinant protein production is to simultaneously reach a high specific recombinant protein production rate and a high product quality. One strategy to avoid quality problems and improve protein production process is to target the protein to the outer compartment of the host cell. This strategy avoids inclusion body formation and the majority of proteolytic proteins in the cytoplasm and achieves a primary purification reducing the costs of downstream process. In this context, the setting up of a gene expression system for the production and secretion of recombinant proteins in psychrophilic bacteria could conbine the effects of low temperatures on the recombinant product solubility with the advantages linked to extra-cellular protein targeting. Our research starting point was the study of the secretion of a psychrophilic protein: α-amylase from *P. haloplanktis* TAB23 (Feller et al. 1992). This exo-protein is produced and secreted as a larger precursor, with a long C-terminal propeptide that constitutes a structurally independent domain that neither exhibits any foldase function nor affects the amylase catalytic activity (Feller et al. 1998). Indeed, when the psychrophilic enzyme is produced by

recombinant cold-adapted bacteria (the source strain, *P. haloplanktis* TAC125 and *Psychrobacter sp*. TAD1) the propeptide is not mandatory for the α-amylase secretion (Tutino et al. 2002; Cusano et al. 2006a). Starting from the latter observation we set up a cold-adapted secretion system which makes use of the mature α-amylase as a carrier. This system was quite efficient, since several heterologous proteins were produced and secreted (Cusano et al. 2006b). A key aspect towards the optimisation of this secretion system is the identification of the specific α-amylase secretion pathway. Indeed, this information is instrumental in the construction of engineered cell hosts with an improved secretory potential. An in vivo complementation experiment was set up to identify the genes responsible for the α-amylase secretion in the cold-adapted bacteria chosen as host for the heterologous protein production (*P. haloplanktis* TAC125). By applying this strategy, a 37.5-Kb-long genomic fragment was selected, whose nucleotide sequence was determined and analyzed in silico. Surprisingly, the selected DNA portion does not code for any of the already characterized secretion pathways (Lee and Schneewind 2001). These results are strongly suggestive of a novel secretion machinery occurring in the Antarctic *P. haloplanktis* TAC125 strain. An extensive mutagenesis analysis to identify the psychrophilic functions specifically involved in the extra-cellular targeting is in progress.

21.3 Examples of heterologous protein expression in psychrophilic bacteria

21.3.1 **P. haloplanktis** ***TAE79 β-galactosidase and*** **Saccharomyces cerevisiae** ***α-glucosidase production***

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 the recombinant form in mesophilic hosts even at sub-optimal temperature conditions.When the β-galactosidase was produced in *E. coli* cells at 18°C, 20 mg of catalytically active enzyme was produced per liter of culture. Analogously, recombinant yeast α-glucosidase produced in *E. coli* aggregates in an insoluble form, the active soluble amount of protein being 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 (Fig. 21.3) as soluble and catalytically active enzymes. Structural and kinetic analysis of the recombinant proteins showed that both enzymes were nearly identical to their native counterparts. The absence of aggregated protein material might be due to the expression temperature that lowers hydrophobic interactions. However, since the optimal expression temperature determined for *P. haloplanktis* TAC125 is only marginally lower than that used for

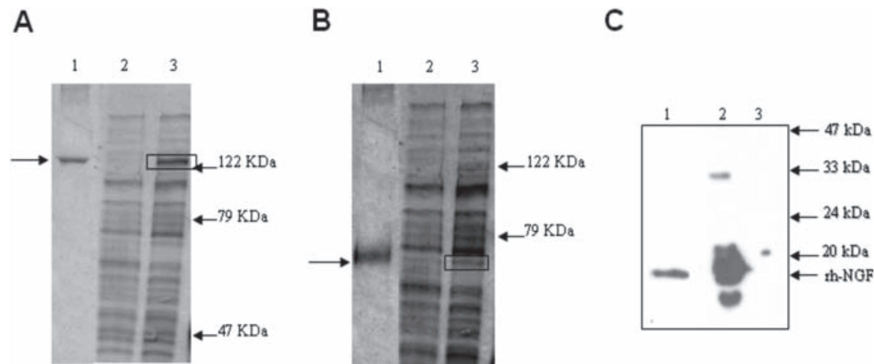

Fig. 21.3 Examples of recombinant protein production in *P. haloplanktis* TAC125 **A** Recombinant production of the thermally labile β-galactosidase from *Ph*TAE79 in *Ph*TAC125 cells. 7.5% SDS PAGE gel electrophoresis of protein extracts from *Ph*TAC125 cells harboring P(PSHAb0363) and grown in minimal medium in the absence (lane 2) and in the presence (lane 3) of L-malate, in comparison with β-galactosidase from *Ph*TAE79 used as control (lane 1). The recombinant protein is indicated by an *open box*. **B** 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 commercial α-glucosidase from yeast used as control (lane 1). The recombinant protein is indicated by an *open box*. **C** rh-NGF production and cellular localisation in recombinant *P. haloplanktis* TAC125. Western blotting analysis of periplasmic (lane 2) and cytoplasmic (lane 3) fractions of 4°C grown *P. haloplanktis* TAC125-pPM13*psDngf* recombinant cells. Polyclonal anti-h-NGF antibodies were used for immunodetection. As positive control, 50ng of rm-NGF proteins was loaded in lane 1

protein production in *E. coli* (15°C compared to 18°C), other factors must have an effect in preventing aggregation.

Experimental conditions for optimal protein production in the cold-inducible expression system were also defined. Low concentrations of L-malate and long induction times are effective for maximal protein production. Under optimal expression conditions, recombinant β-galactosidase is produced with high yields (620–720 mg l^{-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 α-glucosidase 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.

21.3.2 *hβ-NGF Production*

Another example of "difficult" proteins is the mature form of human nerve growth factor (hNGF), a neurotrophin which found promising applications as a therapy agent in several neurological disorders such as Alzheimer's disease (Lad et al. 2003).

hNGF is translated as prepro-protein, where the presequence mediates translocation into the endoplasmic reticulum, while the prosequence seems to facilitate the folding of the mature part, at least from the *E. coli* inclusion bodies (Rattenholl et al. 2001), and it is removed by a cellular specific protease to give the mature hNGF form. 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 (hNGF) in *P. haloplanktis* TAC125 and investigated the production and the cellular localization of the recombinant protein. The protein produced at 4°C was soluble and efficiently translocated in the host periplasmic space (Fig. 21.3). A gel exclusion chromatography also indicated that the protein was largely in a dimeric form, the quaternary structure required for its biologic activity (Harmer et al. 2003). Vigentini and coworkers investigated the influence of growth conditions and cultivation operational strategies on final biomass concentration and on rhNGF production to optimize the use of *P. haloplanktis* TAC125 as a novel host for recombinant protein production.

21.3.3 Secretion of several heterologous proteins

Cusano et al. (2006b) described the setting up and use of a "cold" gene-expression system implemented for the secretion of recombinant proteins in *P. haloplanktis* TAC125. As previously mentioned, such a system combines the advantages of extra-cellular protein secretion with the positive low temperature effect on the recombinant product solubility. 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 synthesised as a preproenzyme, made of: (1) a Sec-dependent signal peptide; (2) the mature enzyme; (3) a flexible spacer; and (4) a structurally independent C-terminal propeptide. The C-terminal propeptide is removed by the action of a host-secreted protease which recognizes and cleaves the -Ala-Ser-(↓)Ser-Thr- sequence contained in the flexible spacer. This event occurs when the precursor reaches the extra-cellular medium (Feller et al. 1998). It was demonstrated that the C-terminal propeptide is not mandatory for the *P. haloplanktis* TAB23 α-amylase recombinant secretion either in the source strain or in *P. haloplanktis* TAC125 (Tutino et al. 2002). 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 (Fig. 21.4). Three chimeric proteins, obtained by fusing intra-cellular proteins to the psychrophilic exoenzyme, were produced in *P. haloplanktis* TAC125 and their secretion was analyzed. The results demonstrated that the cold-adapted secretion

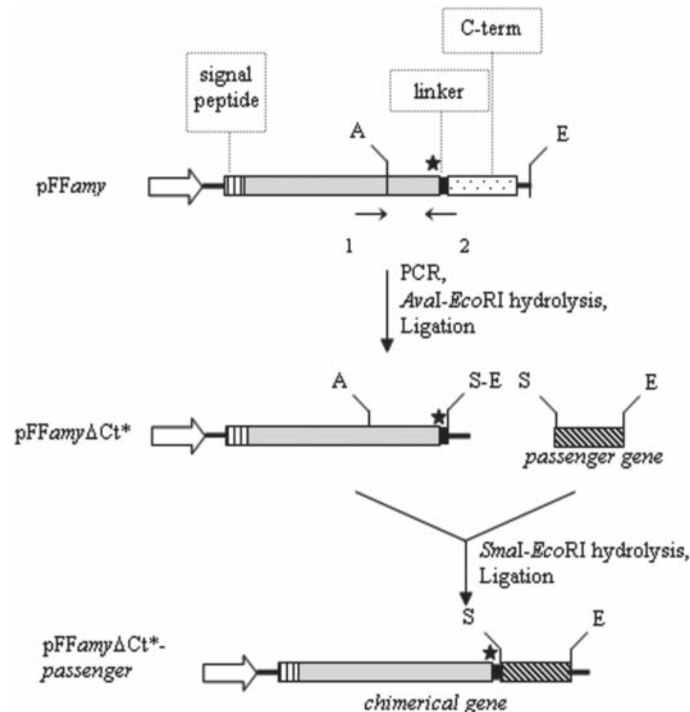

Fig. 21.4 Cold gene-expression system implemented for the secretion of recombinant proteins in *P. haloplanktis* TAC125 *White arrow*, *P. haloplanktis* TAC125 *aspC* promoter; signal peptide, sequence encoding *P. haloplanktis* TAB23 α-amylase signal peptide; C-term, α-amylase C-terminal propeptide 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 presence of a sequence encoding the amino acid motif -Ala-Ser-Ser-Thr-, recognized and cleaved by a *P. haloplanktis* TAC125 secreted protease

system is efficient since all tested chimeras were translocated with a secretion yield always above 80%. Furthermore, reported activity data indicated that the system also allows the correct disulphide bond formation of chimera components (Cusano et al. 2006b).

21.4 Conclusions

In this chapter, we briefly summarized the recent achievements in recombinant protein production in psychrophilic bacteria. Over the last decade, we significantly enhanced the number of set ups of reliable genetic systems for the recombinant gene expression in an Antarctic marine Gram-negative bacterium. Our results clearly demonstrated that the production of recombinant proteins in

cold-adapted bacteria is not only a mature and reliable technology, but it is also a successful strategy to overcome the product solubility problems sometimes 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. The next challenge towards their industrial application is to develop an efficient fermentation scheme to up-scale the recombinant protein production in automatic bioreactors.

However, there are other foreseeable applications of the cold-adapted gene-expression system, one of which is its use as a host for the screening of metagenomics libraries from psychrophilic environments (see Chap. 18). Indeed, it is widely accepted that up to 99.8% of microbes present in many environments are not readily cultivable (Streit and Schmitz 2004). Metagenomics try to overcome this bottleneck by cloning and expressing environmental-derived large DNA fragments in bacterial hosts (routinely, in *E. coli*). This strategy makes the discovery of novel biocatalysts easily achievable, best suited for specific industrial applications (Lorenz and Eck 2005). The prerequisite is that the observed metagenes are expressed in the heterologous host and the resulting products are stable. In this context, metagenomes from cold environments may encode thermallabile enzymes, whose expression rate would be greatly enhanced by using a psychrophilic bacterial host system.

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