HETEROLOGOUS GENE EXPRESSION IN COLD-ADAPTED MICRO-ORGANISMS

Duilio A., Tutino M.L., and Marino G.
University of Naples, Federico II, Italy

Keywords: cold-adapted bacteria, plasmid, shuttle vector, gene expression, Pseudoalteromonas haloplanktis, Psychrobacter sp. TA144

Contents

1. Introduction
2. Heterologous Protein Production in Bacteria other than Escherichia coli
2.1. Use of Gram-Positive Bacteria
2.2. Use of Cold-Adapted Bacteria
3. Cold-Adapted Bacteria Transformation
3.1. Use of BHR Plasmids
3.2. Use of Naturally Cold-Adapted Plasmids
3.2.1. Screening for Cold-Adapted Replicons
3.2.2. Psychrobacter sp. TA144 and its Plasmids
3.2.3. Pseudoalteromonas haloplanktis TAC 125 and pMtBL Plasmid
4. Construction of Cold Genetic Systems and their Cold Host Profile
5. The α -Amylase Example of Heterologous Protein Production in Cold-Adapted Bacteria
6. Conclusions and Future Perspectives.
Acknowledgements
Glossary
Bibliography
Biographical Sketches

Summary

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 the role played by hydrophobic interactions as mainly responsible of driving partially folded protein intermediates to stick and subtracting them from the productive folding pathway. 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. Two

genetic systems were set up, based on the replication functions (ARS, autonomous replication sequence) of two natural cold-adapted plasmids isolated from Antarctic bacteria. The ability of these cloning vectors to replicate in several psychrophilic hosts was analyzed and it turned out that they are quite broad host range systems, since the number of Antarctic bacteria which they are able to replicate in is steadily increasing.

Combining the functional elements described above (i.e. cold ARS and promoter), we successfully over-produced a cold α-amylase from *Pseudoalteromonas haloplanktis* A23, since our results demonstrate that the cold-adapted enzyme was not only produced but also efficiently secreted by the recombinant *Ph*TAC 125 cells.

1. Introduction

The development of recombinant DNA techniques opened a new era for protein production both for basic research and industrial application. Recombinant protein expression in transformed microorganisms is by far the simplest genetic approach to guarantee an unlimited supply of rare, high-value proteins that are not accessible using conventional protein isolation techniques. The Gram-negative bacterium *Escherichia coli* is usually the principle host of choice for the recombinant protein production and there are many reasons that justify this large success. Its physiology and metabolism have been extensively characterized, so that its ability to grow rapidly and at high cell density could be achieved easily even in quite inexpensive substrate. Furthermore, there are many genetic tools readily available for its manipulation. In fact, an increasingly large number of cloning vectors, strong and/or regulated transcriptional promoters, and mutant host strains have been selected in the past years, with the final aim of setting up efficient and versatile gene expression systems. As a result, the synthesis of heterologous polypeptides in *Escherichia coli* has become a matter of routine.

Actually, the final yield of heterologous recombinant protein of interest is dependent on several factors to be carefully taken into account. However, to efficiently produce active biomolecules, a high rate of protein synthesis is necessary, but it is by no means sufficient. If a newly synthesized polypeptide is recognized by the proteolytic machinery of the cell, its net or steady-state accumulation will be very low because of degradation. Proteolysis of heterologous proteins in bacteria is conceptually like a primitive immune system, and as such deserves far more experimental work; the mechanisms by which *E. coli* recognizes “self ” are completely unknown and no generic methods exist for blocking all host proteases. At the moment, investigators try to overcome the proteolysis problem by producing heterologous protein in the available *E. coli* protease mutants, although many proteins are reported to be unstable in every strain. Furthermore, some heterologous proteins are extremely toxic for *E. coli*. In such cases, the induction of heterologous genes is shortly followed by cessation of protein synthesis. Finally, and more importantly, the polypeptide chain has to fold into the correct native three-dimensional structure. Almost from the beginning of the recombinant DNA technology era, it became apparent that many cloned gene products could not attain their correct three-dimensional conformation in *E. coli*. In many cases, instead of the native protein, the recombinant polypeptides were found to be sequestered within large refractile aggregates known as inclusion bodies; subsequent studies of

recombinant protein expression in *E. coli* showed that inclusion body formation is the rule rather than the exception.

The misfolding and aggregation of polypeptide chains into insoluble bodies is a serious problem in both biotechnology and basic research; several experimental approaches have been developed to recover the protein products from the inclusion bodies in an active form. As an example, active proteins can be recovered from inclusion bodies through a cycle of denaturant-induced solubilization of the aggregates followed by removal of the denaturant under conditions that favor refolding. Even very complex proteins containing prosthetic groups or multiple disulfide bonds have been produced with a reasonable yield in this manner. Such success stories not withstanding, refolding is still largely an art and the relevant processing costs can be quite significant.

Attempts have been made to produce recombinant proteins in a soluble form by coexpression of a class of host protein called “chaperones," that assist folding by preventing the aggregation. Despite numerous experimental efforts, positive results are scarce although in certain cases, a positive effect of co-expression of molecular chaperones or other folding enhancers such as thiol-disulphide-isomerases or peptidyl-prolyl-isomerases has been reported. More recently, the influence of various fusion protein partners for the expression of recombinant proteins in soluble form in *E. coli* has been studied. The choice of protein partners is based on their favorable cytoplasmic solubility characteristics as predicted by a statistical solubility model. Examples of fusion partners that have been touted as solubilizing agents include thioredoxin, gluthatione S-transferase, maltose-binding protein, Protein A, ubiquitin, and DsbA. Although widely recognized and potentially of great importance, this solubilizing effect remains poorly understood. In particular, it is not known whether the solubility of many different polypeptides can be improved by fusing them to a highly soluble partner or whether this approach is only effective in a small fraction of cases. For these reasons, there has been considerable interest in the development of techniques for the direct expression of correctly folded proteins in bacteria other than *E. coli*.

2. Heterologous Protein Production in Bacteria other than *Escherichia coli*

Many bacteria possess a broad diversity of metabolic activities and might therefore be promising hosts for cloning and expression studies. Indeed some genes can only be functionally expressed in a particular physiological background, e.g., when an enzyme is integrated into a specific biochemical pathway. In the following paragraphs, the most significant results obtained by using alternative hosts will be described

2.1. Use of Gram-Positive Bacteria

As we have pointed out above, there are many good reasons why *E. coli* is a popular host for heterologous gene expression. However, a single organism is unlikely to be suitable for every application and the choice of host bacteria is primarily influenced by the application at hand. Many bacteria may serve as alternative hosts to *E. coli*; however, the availability of tools for their genetic manipulation generally dictates the extent of their utilization.

In particular, the problems of proteolytic degradation in *E. coli* cytoplasm could be solved by setting up a suitable expression system by which the recombinant product is directed towards secretion. Gram-positive bacteria, and in particular several species of *Bacillus*, naturally secrete large amounts of enzymes, such as amylase and proteases, and are used for commercial production. The development of the genetic tools of *Bacillus subtilis* has made this an attractive alternative host for recombinant protein production. The drawback of the use of *B. subtilis* is that this bacterium produces endogenous extracellular proteases, whose activity could affect the amount and the quality of the heterologous products. More recently, *Bacillus brevis* has been preferred, since it has the same secretion capacity of *B. subtilis* but has no detectable extra-cellular proteases.

2.2. Use of Cold-Adapted Bacteria

Beside the obvious impossibility to achieve the large scale production of thermolabile proteins at the normal *E. coli* growth temperature, sometimes it is observed an incorrect folding of the nascent polypeptides resulting in their aggregation and accumulation as insoluble inclusion bodies. Misfolding is a particularly vexing problem often encountered in the expression of mammalian proteins, especially those that are composed of multiple subunits, have several disulfide bonds, or contain prosthetic groups. The study of inclusion bodies formation highlighted the role played by hydrophobic interaction as mainly responsible of driving partially folded protein intermediates to stick and subtracting them from the productive folding pathway.

To minimize these undesirable effects, many experimental approaches have been explored with some success, one of which consists in lowering the expression temperature up to the physiological 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 process, 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 uneconomic. Starting from the above considerations, the use of naturally "cold-adapted" bacteria as hosts for protein production at low temperature (even at around 0 °C) might represent a rational alternative to expression in mesophiles. However, the optimization of a reliable expression system is based on the selection of appropriate host cells and also on a number of genetic elements, such as promoters, genes, and plasmids. The choice of each component can then be critical for the successful expression of the gene of interest; the following section reports the most significant results obtained by using different cold-adapted bacteria transformation tools.

3. Cold-Adapted Bacteria Transformation

Since no genetic tools were available for the cold-adapted bacteria transformation, two main approaches were possible: a) the use of already known broad host range plasmids, isolated from mesophilic Gram-negative bacteria; b) the use of naturally cold-adapted replicons for setting up genetic/expression systems. In the following paragraphs, the

most significant results obtained by using these alternative approaches will be described, taken into account the successful reported examples of heterologous protein production in cold-adapted bacteria.

3.1. Use of BHR Plasmids

The use of nonenteric bacteria for basic and applied molecular research has extended the need for well-characterized vector system for such organisms. In practice these problems are solved either by developing specific vector systems for each species of interest or by taking advantage of already available broad-host-range replicons. The latter approach has the advantage that a few such systems can be studied more extensively, and the accumulate knowledge can then be utilized with many species. Vectors based on the broad-host-range RK2 and RSF1010 replicons represent the most frequently used system. RK2 and RSF1010 belong to different incompatibility groups, and these replicons can therefore be maintained together in the same cell.

RK2 is functional in more than 29 Gram-negative bacterial species and is also able to replicate also in some gram-positive organisms. The minimal RK2 replicon consists of the origin of vegetative replication *(ori*V) and the gene encoding an essential initiator protein (*trf*A) that binds to iterons in *ori*V. RK2 encodes two operons containing the *par*DE and *par*CBA genes, respectively, which are involved in the maintenance of RK2 plasmids or heterologous replicons in diverse bacterial populations. Furthermore the copy number of RK2 plasmids is estimated to be five to seven per chromosome in *E. coli*

In order to develop an expression system in cold-adapted bacteria, a replicon having broad-host-range properties was build up. In this study, the gene coding for the moderately thermolabile eukaryotic luciferase (from *Photinus pyralis*) was cloned into a pJB3-derived replicon (from the natural RK2 plasmids) and expressed, under control of *E. coli*-derived transcriptional and translational signals, in an Antarctic strain grown at 15°C. This work has demonstrated the feasibility of this approach since the protein was successfully produced.

-
-
-

Bibliography

Baneyx F. (1999). Recombinant protein expression in *Escherichia coli*. *Current Opinions in Biotechnology* **10**, 411–421. [In this review, several problems relating to transcription, translation, proteolysis, inclusion body formation, and cell growth during the recombinant protein expression in *E. coli* are discussed.]

Billman-Jacobe H. (1996). Expression in bacteria other than *Escherichia coli. Current Opinions in Biotechnology* **7**, 500–504. [Properties and peculiar use of bacterial gene expression systems alternative to *Escherichia coli* are summarized in this review, taking into account the actually available genetic tools for the manipulation of these hosts.]

Georgiou G. and Valax P. (1996). Expression of correctly folded proteins in *Escherichia coli. Current Opinions in Biotechnology* **7**, 190–197. [This review describe various strategies that could be applied for the production of active recombinant proteins in *Escherichia coli.*]

Rudolph R. and Lilie H. (1996). *In vitro* folding of inclusion body proteins. *FASEB Journal* **10**, 49–56. [Protein folding largely remains an experimental trade and folding protocols still have to be developed on a case-by-case basis This review describe various strategies for the *in vitro* inclusion body protein folding.]

Remaut E., Bliki C., Iturizza M., and Keymeulen K. (1999). Development of regulatable expression systems for cloned genes in cold-adapted prokaryotes. *Biotechnological Applications of Cold-Adapted Organisms* (ed. R. Margesin and F. Schinner), pp. 1–16. Berlin: Springer-Verlag. [This textbook chapter describes several results on the development of an expression system in cold-adapted bacteria using a mesophilic replicon displaying broad-host-range properties.]

Speed M.A., Wang, D.I.C., and King J. (1996). Specific aggregation of partially folded polypeptide chains: the molecular basis of inclusion body composition. *Nature Biotechnology* **14**, 1283–1287. [This study on the specific interactions implicated in the inclusion bodies formation indicates that protein aggregation is driven by specific interaction between partially folded polypeptides rather than by nonspecific co-aggregation.]

Tutino M.L., Duilio A., Fontanella B., Moretti M.A., Sannia G., and Marino G. (1999). Plasmids from Antarctic bacteria. *Cold-Adapted Organisms: Ecology, Physiology, Enzymology and Molecular Biology* (ed. R. Margesin and F. Schinner), pp. 335–348. Berlin: Springer-Verlag. [This is a textbook chapter reporting some results on the molecular characterization of plasmid from Antarctic bacteria.]

Tutino M.L., Duilio A., Moretti M.A., Sannia G., and Marino G. (2000). A rolling-circle plasmid from *Psychrobacter sp.* TA144: evidence for a novel Rep subfamily. *Biochemical and Biophysical Research Communications* **274**, 488–495. [This article report results on the molecular characterization of two plasmids from the Antarctic *Psychrobacter sp.* strain TA144 strain.]

Tutino M.L., Duilio A., Parrilli E., Remaut E., Sannia G., and Marino G. (2001). A novel replication element from an Antarctic plasmid as a tool for the expression of proteins at low temperature. *Extremophiles* **5**, 257–264. [This paper reports the identification of the origin of replication of a novel Antarctic plasmid and its use to set up an expression system for the production of the cold-adapted α-amylase.]

Biographical Sketches

Gennaro Marino received a doctorate degree in Chemistry in 1964 from the University of Naples. In 1966, he was appointed as Research Associate at the Chemistry Department of the same University. In 1967 and 1971, he was EMBO fellow at the Institute of Chemistry of Natural Products in Gif-sur-Yvette and at the Organic Chemistry Department, University of Liverpool, respectively. From 1973 to 1980, he was Associate Professor of Biochemistry, University of Naples. Since 1980, he has been Professor of Biological Chemistry and Fermentation Biotechnologies, Department of Organic Chemistry and Biochemistry. He is member of the *Accademia Nazionale delle Scienze detta dei XL* (Italian National Academy of Sciences) since 1997. His research interests span from the application of mass spectrometry to proteins, and now to proteomes, to the molecular basis of extremophiles adaptation.

Angela Duilio received a BSc degree in Biological Science in 1981 from the University of Naples Federico II. In 1987, she got a PhD degree in Biochemistry with a thesis focusing on the study of the structure organization and gene expression of tRNA gene clusters containing the primers of MULV

reverse transcriptase. From 1988 until 1996, she was Research Associate at the Department of Biochemistry and Medical Biotechnology, Faculty of Medicine, University of Naples Federico II. Her main research interests concerned the studies on the regulation of tissue-specific and age-related expression of rat brain genes. In 1997, she was appointed Assistant Professor at the Department of Organic and Biological Chemistry, Faculty of Science, University of Naples Federico II. Her recent research projects focused on the investigation of cold adapted microorganisms, paying particular attention to the mechanisms of heterologous gene expression in Antarctic bacteria.

Maria Luisa Tutino received a BSc degree in Biological Science in 1992 from the University of Naples Federico II in Italy. In 1998, she got a PhD degree in Biochemistry and Molecular Biology with a thesis focusing on the cloning, recombinant production, and transcription regulation of the operon responsible for the tryptophan biosynthetic pathway in the hyperthermophilic archaeon *Sulfolobus solfataricus*. In 1998, she was appointed Research Associate at the Department of Organic Chemistry and Biochemistry, Faculty of Science, University of Naples Federico II. In the context of her interest on the molecular adaptation displayed by extremophilic microorganisms, from 1997 she focused her research interests on some Antarctic Gram-negative bacteria. Enzyme adaptation to the catalysis at low temperature was investigated by studying the aspartate aminotransferase from *Pseudoalteromonas haloplanktis* TAC 125. Furthermore, the isolation, cloning, and molecular characterization of three plasmids, isolated from Antarctic bacteria, paved the way to the setting up of genetic/expression recombinant vectors for the heterologous gene expression in cold-adapted bacteria.