Extremophiles (2001) 5:257–264
Digital Object Identifier (DOI) 10.1007/s007920100203

ORIGINAL PAPER

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A novel replication element from an Antarctic plasmid as a tool for the expression of proteins at low temperature

Received: December 14, 2000 / Accepted: March 23, 2001 / Published online: June 30, 2001

Abstract Genetic manipulation of Antarctic bacteria has been very limited so far. This article reports the isolation and molecular characterization of a novel plasmid, pMtBL, from the Antarctic gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC 125. This genetic element, 4,081 bp long, appeared to be a multicopy cryptic replicon with no detectable transcriptional activity. By an in vivo assay, the pMtBL autonomous replication sequence was functionally limited to an *Alu*I plasmid fragment of about 850 bp. This novel cold-adapted replication element showed quite a broad host range profile; it was cloned into a mesophilic genetic construction, obtaining a cold-adapted expression vector that was able to promote the production of *P. haloplanktis* A23 α-amylase in a psychrophilic bacterium. This study represents the first report of successful recombinant production of a cold-adapted protein in an Antarctic host.

Key words Antarctic bacteria · Cold-adapted plasmid · Gene expression · pMtBL · *Pseudoalteromonas haloplanktis* · Shuttle vector

Communicated by G. Antranikian

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Presented at the Third International Congress on Extremophiles, Hamburg, 2000

Introduction

In recent years there has been growing interest in cold-adapted microorganisms, mainly because of the biotechnological potential of their enzymes (Gounot 1991; Russell 1992). Indeed, many comparative analyses have highlighted the unusually high activity of psychrophilic enzymes at low temperatures. Understanding the structural and functional features that underlie these properties is a prerequisite for exploitation of their biotechnological applications (for a recent review, see Gerday et al. 2000).

Considerable progress has been made in the structural biology of cold-adapted enzymes. Our knowledge of the genetics and molecular biology of cold-loving microorganisms, however, remains largely fragmental. Although a few genes from psychrophilic bacteria have been isolated and characterized, limited information is available on bacterial genetic elements such as plasmids or viruses (Dahlberg et al. 1997; Sobecky et al. 1997, 1998).

The isolation and molecular characterization of plasmids from cold-adapted bacteria should provide powerful experimental tools in the study of basic molecular processes and their adaptation to low temperatures. These plasmids provide easy model systems for the elucidation of fundamental mechanisms such as DNA replication, recombination, and repair. Furthermore, knowledge of the fine structure of a plasmid opens prospects for setting up cloning/expression systems in cold-adapted bacteria. In particular, the construction of such genetic systems would greatly enhance the opportunities for genetic manipulation of the bacterial hosts, with the aim of clarifying the physiological processes that allow them to thrive in such critical thermal conditions. Also, an expression system in cold-adapted bacteria may circumvent the problem of limited stability of the product that is sometimes encountered during heterologous production of psychrophilic proteins in mesophilic hosts (Gerike et al. 1997; Feller et al. 1991). The further application of such genetic systems may not be limited to the overproduction of heat-labile products but could be extended to the bioengineering of industrial processes involving genetically engineered cold-adapted bacteria.

We have reported the cloning, sequencing, and molecular characterization of a small plasmid, pTAUp, from the Antarctic gram-negative *Psychrobacter* sp. strain TA144. This plasmid duplicates in vivo via a rolling-circle mechanism. The gene coding for its replication initiator protein (*Psy*Rep) contains enough information for promoting the replication of an *Escherichia coli* vector in another Antarctic strain, TAD 1 (Tutino et al. 2000).

In this article, we report the isolation and molecular characterization of a small cryptic plasmid, designated pMtBL, from *Pseudoalteromonas haloplanktis* TAC 125. Its origin of replication was functionally identified and used as a tool for the construction of a shuttle vector able to replicate in several species of cold-adapted bacteria.

To investigate the possibility of obtaining recombinant proteins from these cold-adapted hosts, we used the psychrophilic α-amylase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* A23 as the model enzyme to be produced. This enzyme was chosen for several reasons: (i) it is heat labile (Feller et al. 1994); (ii) the psychrophilic α-amylase is a secreted protein; and (iii) the selected host *P. haloplanktis* TAC 125 lacks the endogenous amylase and belongs to the same species as the source strain of the amylase. Our results demonstrated that the cold-adapted enzyme was not only produced but also efficiently secreted as a larger precursor by the recombinant *Ph*TAC 125 cells and correctly cleaved to release the mature enzyme in the culture supernatant.

Materials and methods

Enzymes and reagents

Restriction enzymes, T4 DNA ligase, alkaline phosphatase, T4 polynucleotide kinase, DNA polymerase Klenow fragment, and *Taq* DNA polymerase were supplied by Boehringer (Boehringer-Roche, Basel, Switzerland), Amersham (Amersham-Pharmacia Biotech, Milan, Italy), Promega (Madison, WI, USA), or New England Biolabs (Beverly, MA, USA). Enzyme assay conditions were those suggested by each manufacturer.

DNA fragment purification was carried out with the QUIAEX II kit from Quiagen (Hilden, Germany). Acrylamide and agarose were purchased from Sigma (St. Louis, MO, USA).

Bacterial strains and microbiological techniques

The Antarctic bacterial strains were isolated from seawater in the vicinity of the Dumont d'Urville Antarctic station (66°40' S, 40°01' E) during several summer campaigns of the Expeditions Polaires Française in Terre Adélie. All the strains (kindly provided by Prof. C. Gerday, University of Liege, Belgium) are gram negative and were grown in aerobic conditions at 4° and 15°C in TYP broth (16 g/l yeast extract, 16 g/l bacto tryptone, 10 g/l marine mix) or Luria-Bertani (LB) broth (Sambrook et al. 1989) at pH 7.5.

Escherichia coli DH5α [*supE*44, Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1] was used as host for the gene cloning. *E. coli* strain S17-1(λpir) (*thi, pro, R⁻ M⁺ recA* [RP4 : 2-TC^R : Mu⁻ : Km^R : Tn7, Tp^R, Sm^R]λpir) (Tascon et al. 1993) was used as the donor in interspecific conjugation experiments. *E. coli* cells were routinely grown in Terrific broth (Sambrook et al. 1989) containing 100 μg/ml of ampicillin if transformed.

Plasmid isolation and sequencing

Pseudoalteromonas haloplanktis TAC 125 cells were grown at 15°C to an optical density (OD) at 600 nm of 8; chloramphenicol was then added at a final concentration of 135 μg/ml and incubation was continued for a further 16 h. The cells were collected by centrifugation and lysed using the alkaline lysis procedure as described in Sambrook et al. (1989). Supercoiled pMtBL DNA was isolated by ultracentrifugation on a CsCl gradient.

The pMtBL plasmid was linearized by either *Xba*I or *Pst*I digestion and cloned in pGEM 4z opened at the corresponding site. DNA from the two subclones was purified by ultracentrifugation on CsCl gradient and fully sequenced on both strands by the TaqFS Dye-Terminator kit (Perkin Elmer, Norwalk, CT, USA) using the Applied Biosystems automatic sequencer model 373A (Perkin Elmer). The sequence has been assigned the EMBL data bank entry number AJ224742.

Sequence analysis

The nucleotide sequence was searched for homologues stored in the nucleotide sequence data banks and for the presence of putative ORFs. The deduced amino acid sequences were used as templates for similarity searches in the SWISS PROT data bank by using the BLAST and FASTA programs (http://www.expasy.ch/). The distribution of direct and inverted repeated sequences was analyzed by using the REPEAT program (PCGENE) under standard conditions of stringency.

Construction of a conjugative pGEM-derived plasmid

An *E. coli* conjugative plasmid was constructed by cloning into pGEM 7zf (Promega) the conjugational DNA transfer origin (OriT) from the broad host range plasmid pJB3 (kindly provided by Dr. S. Valla, Trondheim, Norway), an RK2 derivative plasmid (Blatny et al. 1997).

Digestion of pJB3 by the endonucleases *Bgl*II and *Sca*I resulted in the generation of two fragments 3,912 bp and 874 bp in length; the latter was purified from an agarose gel and then subjected to *Taq*I hydrolysis, thus generating a *Taq*I/*Bgl*II DNA fragment of 429 bp containing the OriT. This fragment was cloned into pGEM 7zf previously digested by *Cla*I/*Bam*HI. From the newly synthesized pGEM 7z-derivative, OriT was excised by *Sma*I and *Sac*I

digestion and inserted into pGEM 4zf into the corresponding cloning sites, generating the vector pGEM-T.

Construction of a pMtBL library in pGEM-T

Two micrograms of pMtBL plasmid were partially hydrolyzed with 0.4 U of *Alu*I enzyme for 45 min at 37°C. The average dimension of the statistically generated fragments was between 1 and 2 kb. The fragments were inserted into the compatible *Sma*I site of the pGEM-T vector, generating a pMtBL statistic library that was used to transform *E. coli* strain S17-1(λpir)-competent cells.

Mating experiments

The pMtBL statistic library was mobilized from *E. coli* S17-1(λpir) cells (donor strain) into *P. haloplanktis* TAC 125 by interspecific conjugation. Aliquots (100 µl) of logarithmic cultures of the donor and the recipient strains were mixed and spotted as a drop onto a TYP plate. After 16 h of mating at 15°C, the cells were resuspended in 200 µl of TYP medium. Psychrophilic transconjugants were selected by plating serial dilutions at 4°C on TYP plates containing 100 µg/ml ampicillin. Indeed, the mesophilic donors are unable to grow as a colony at 4°C. The recombinant cold-adapted bacteria were grown in liquid medium containing 100 µg/ml ampicillin. The extrachromosomal DNA was recovered and analyzed for the presence of pMtBL-derived fragments. Of 100 clones screened, two recombinant vectors containing the smallest pMtBL insert were sequenced. Both were identical and contained a 842-bp-long plasmid fragment (from position 1,917 to 2,758, pMtBL ARS) (Fig. 1). This vector was called *clone Q*.

To investigate if the transformation of the TAC 125 cells with *clone Q* induced the loss of the endogenous pMtBL, extrachromosomal DNA extracted from cold-adapted transformed cells was used as template for a polymerase chain reaction (PCR) by using pMtBL-specific oligonucle-

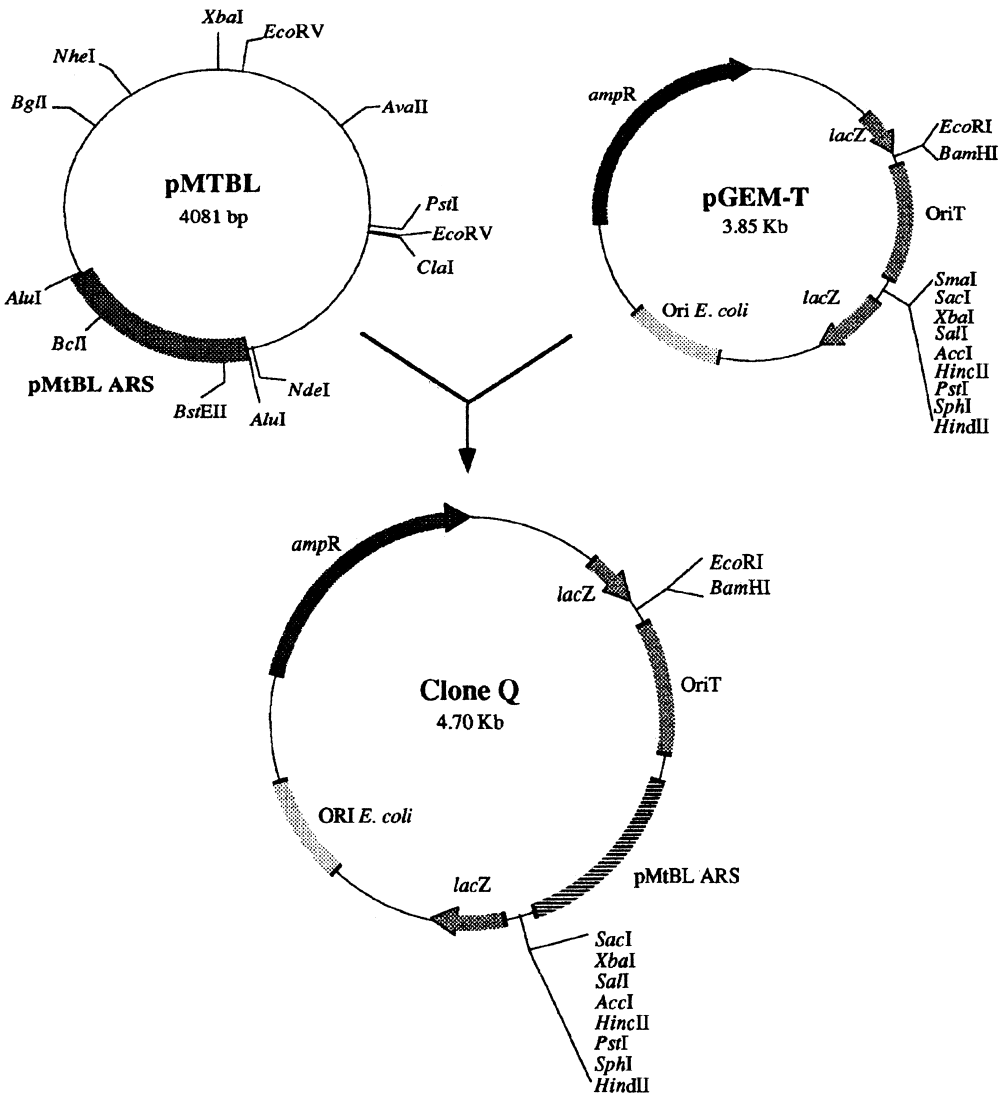

Fig. 1. Map and construction of the *clone Q* shuttle vector. Details of the construction are described in the text

otides as primers. The amplification was performed in a mixture containing 50 ng of template, 50 pmole of each oligonucleotide primer, 1.8 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-Cl, pH 8.3, 0.1% gelatin, and 200 µM deoxynucleotide triphosphate (dNTP) in a final volume of 50 µl. The mixture was incubated at 95°C for 10 min, after which 1.25 units *Taq* DNA polymerase was added. Then, 35 cycles of amplification (consisting of at 95°C, 1.5 min at 55°C, and 1 min+5 s/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 to complete DNA synthesis. The primer pair used was A4 (5′-ATGAGCTGGGCTATATGC-3′, nucleotides 1,401–1,419) and B6 (5′-AGAGCAAATAACCGAGTCT-3′, nucleotides 1,921–1,940). The amplified fragments were analyzed on a 1% agarose gel.

Construction of a cold expression system for the production of the cold α-amylase in *P. haloplanktis* TAC 125

The double *Eco*RI/*Bam*HI hydrolysis of *clone Q* vector resulted in the production of a fragment (about 1.2 kb long), containing the pJB3-derived OriT and the pMtBL-derived ARS, that was called a "T/R box" or a "cold replication cassette." This fragment was purified and subjected to a fill-in reaction to generate blunt ends. The recombinant vector pαH12, used for the *P. haloplanktis* TAB 23 α-amylase production in *E. coli* (Feller et al. 1998), was linearized by *Xba*I digestion, blunt ended by Klenow DNA polymerase treatment, and ligated with the T/R box previously prepared, generating the pA6 vector that was mobilized into *Ph*TAC 125 cells by interspecific conjugation. The production and secretion of the heterologous α-amylase by the recombinant cold-adapted cells was monitored by following the appearance of amylase activity in the supernatant of cultures grown at 4°C and 15°C in TYP medium containing 200 µg/ml ampicillin. Quantification of enzyme activity and Western blotting analysis of culture supernatants were carried out as described by Feller et al. (1998).

Results

Cloning, sequencing, and nucleotide sequence analysis of pMtBL from *Pseudoalteromonas haloplanktis* TAC 125

Five bacterial Antarctic strains (Table 1) were analyzed for the presence of extrachromosomal elements. Two of these, TAE 79 and TAC 125, contained small plasmids, whereas no extrachromosomal elements were detected in the other three (data not shown). Of the two novel plasmids identified, the replicon contained in *P. haloplanktis* TAC 125, called pMtBL, was selected for further molecular characterization because it appeared to be present at a higher copy number (about 20–50 copies/cell) and could be amplified by extreme chloramphenicol treatment. The plasmid was isolated by standard techniques, and preliminary restriction analysis revealed the presence of at least two unique restriction sites (*Xba*I and *Pst*I), which were used for the pMtBL cloning in two alternative forms.

The comparison of the data obtained by fully sequencing both strands of these two subclones resulted in the pMtBL nucleotide sequence (stored in the EMBL data bank under the accession number AJ224742). Southern analysis, using the whole plasmid as a probe, confirmed the episomal location of pMtBL because no hybridization signals were detected with the TAC 125 genomic DNA (data not shown).

To obtain insight into the nature of the pMtBL genetic element and to search for biological functions, we compared the 4,081-bp-long nucleotide sequence with entries stored in the EMBL data bank. However, none of the database sequences showed any significant similarity to that of the psychrophilic plasmid. Moreover, none of the putative open reading frames in pMtBL showed significant identity to the known protein sequences stored in the SWISS PROT data bank. Furthermore, Northern analysis of the plasmid transcriptional activity provided no evidence for functional genes on pMtBL (data not shown). The plasmid therefore can be considered a cryptic genetic element.

In vivo identification of pMtBL ARS and construction of a shuttle vector

To identify the minimal pMtBL region responsible for its autonomous replication (ARS), an in vivo assay was based on the expected ability of pMtBL ARS to promote the replication of an *E. coli* vector in the cold-adapted host *P. haloplanktis* TAC 125. Because growth of all the Antarctic strains reported in Table 1 was inhibited by ampicillin, the ampicillin resistance determinant was used for the direct selection of psychrophilic transformants.

A conjugative *E. coli* vector was constructed by cloning a pJB3-derived fragment (Blatny et al. 1997), containing OriT, into the pGEM 4z plasmid (Table 2). Preliminary experiments demonstrated that the resulting vector, pGEM-T, could be successfully mobilized between two

Table 1. Antarctic bacterial strains used in this work

Strain	Identification	Plasmid	Reference
A23	*Pseudoalteromonas haloplanktis*	No	Feller et al. (1992)
TAC 125	*Pseudoalteromonas haloplanktis*	Yes	Birolo et al. (2000)
TAE 56	Not determined	No	C. Gerday collection
TAE 79	*Pseudoalteromonas haloplanktis*	Yes	C. Gerday collection
TAD 1	*Psychrobacter* sp.	No	Di Fraia et al. (2000)

E. coli strains using as donor the S17-1(λpir) strain, for example (Tascon et al. 1993), which contains the *mob*- and *tra*-encoded functions of RK2.

A statistic collection of pMtBL fragments was cloned in pGEM-T and used to transform S17-1(λpir)-competent cells, generating a pMtBL library that was subsequently mobilized into TAC 125. The transconjugants were grown at 4°C on solid medium containing ampicillin. The mesophilic donor cells are unable to form colonies at this low temperature. Ampicillin-resistant colonies actively growing under these conditions are therefore TAC 125 cells that have received a recombinant vector containing the pMtBL ARS.

The plasmid fragments contained in about 100 colonies coming from this screening were analyzed, and the smallest insert (spanning the *Alu*I sites at positions 1,917 and 2,758) was defined as the pMtBL ARS (see Fig. 1). The vector pGEM-T containing the identified cold ARS was defined as *clone Q*.

Molecular properties of the pMtBL-derived shuttle vector

A plasmid DNA preparation from TAC 125 transconjugants (Fig. 2; lanes 1 and 2) was used to transform *E. coli* DH5α-competent cells, and the vector recovered from the mesophilic host was analyzed by restriction analysis (Fig. 2; lanes 3 and 4). As shown in Fig. 2, no rearrangements or modifications of *clone Q* were observed after its transfer from *E. coli* to *P. haloplanktis* TAC 125 and back.

To determine whether the presence of *clone Q* in TAC 125 caused the loss of the endogenous episome, plasmid DNA was prepared from cold-adapted transconjugants and used as template for a PCR amplification of a pMtBL-specific sequence, outside the ARS region. As shown in Fig. 2 (lane 5), the synthesis of a specific fragment of the expected size (lane 6) indicated that the replication of the endogenous plasmid is only slowed down but not inhibited by the active duplication of the shuttle vector, which of course confers the ampicillin resistance. A Southern analysis of the total DNA from cold-adapted transconjugants ruled out the possibility of pMtBL integration in the chromosome (data not shown).

The ability of *clone Q* vector to transform and to replicate in other Antarctic bacterial strains was further investigated by conjugation experiments. The plasmids pGEM-T and *clone Q* were mobilized in each of the cold-adapted strains listed in Table 1 . All the bacteria were able to stably maintain *clone Q* but not pGEM-T. This result confirms the ability of the pMtBL ARS to promote the autonomous DNA replication of a mesophilic cloning vector in at least two different Antarctic bacterial species.

Table 2. Plasmids used in this work

Plasmid	Description	Reference
pJB3	Broad host range cloning vector	Blatny et al. (1997)
pGEM-T	pGEM 4z derivative containing the OriT sequence from pJB3 (position 4,360 to 4,790)	This work
Clone Q	pGEM-T containing a pMtBL ARS (position 1,917 to 2,758)	This work
paH12	PUC12 derivative containing the *P. haloplanktis* a-amylase gene	Feller et al. (1998)
pA6	pαH12 derivative containing the T/R box	This work

Production of the cold α-amylase in *P. haloplanktis* TAC 125

To investigate if it was possible to produce recombinant proteins in cold-adapted hosts, the expression vector pαH12, used for the production in *E. coli* of the psychrophilic α-amylase from *P. haloplanktis* A23, was modified by inserting the cold replication cassette. The resulting vector, pA6, could be mobilized by conjugation and was able to actively replicate in TAC 125 cells. The production and secretion of the heterologous α-amylase by the recombinant cold-adapted cells were demonstrated by the appearance of the protein and its amylase activity in the cell-free supernatant of cultures grown at 4° and 15°C (Fig. 3).

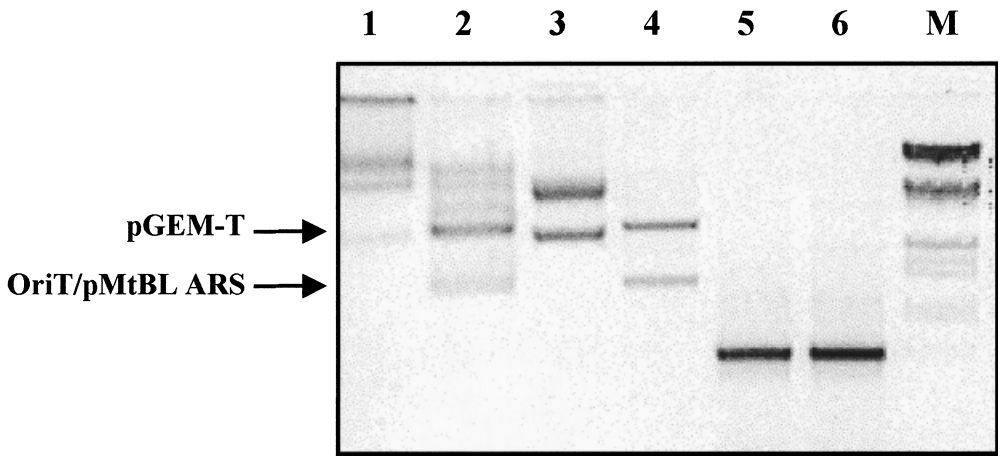

Fig. 2. Agarose gel electrophoresis of *clone Q* isolated from *Ph*TAC 125 transconjugants (*lane 1*, uncut; *lane 2*, *Eco*RI/*Bam*HI digested), and from recombinant *Escherichia coli* DH5α (*lane 3*, uncut; *lane 4*, *Eco*RI/*Bam*HI digested). PCR amplification of a pMtBL-specific fragment using as template the preparation loaded in *lane 1* (*lane 5*), or a purified pMtBL sample (*lane 6*). *M*, molecular size marker

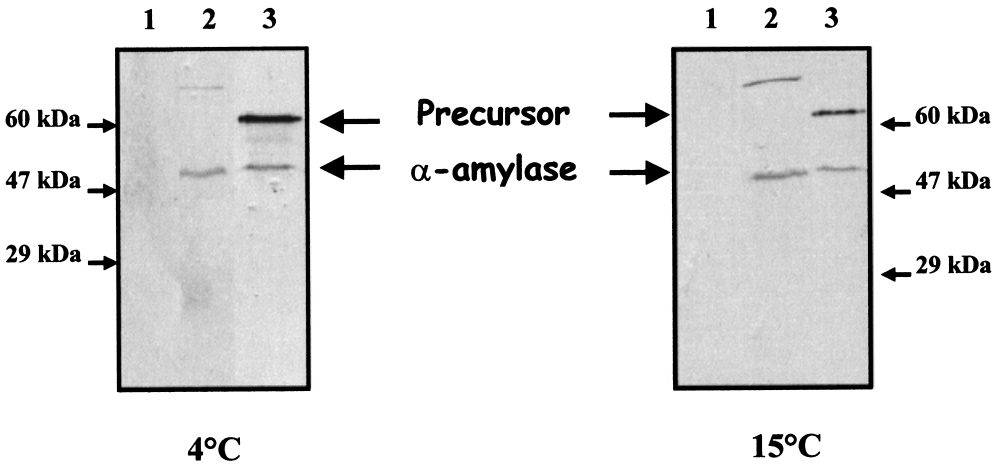

Fig. 3. Recombinant α-amylase production in *Ph*TAC 125/pA6 cells. Western blot of cell-free supernatants from cultures of *Ph*TAC 125 wild type (*lanes 1*), *Ph*A23 (*lanes 2*), and *Ph*TAC 125/pA6 grown at 4°C (*left*) and 15°C (*right*). Molecular weight markers are indicated by *small arrows*. The antigen detection was carried out by using rabbit IgG anti-α-amylase; secondary antibodies were alkaline phosphatase-conjugated antirabbit IgG

Interestingly, in TAC 125/pA6 recombinant cells the cold α-amylase is secreted as a larger precursor and is cleaved to give a mature enzyme, essentially in the same way as happens in the A23 wild-type strain (Feller et al. 1998). Indeed, Western blot analysis of samples taken at all growth stages (Fig. 4) confirmed the occurrence of a maturation kinetic in the TAC 125 extracellular medium. Furthermore, neither the precursor nor the mature enzyme was detected in TAC 125/pA6 cell pellets (data not shown).

Interestingly, the growth temperature had a substantial effect on protein production and secretion by the TAC 125/pA6 strain. The α-amylase was recovered at about 1.1 mg/l culture when expression was carried out at 4°C, whereas the yield was considerably lower (0.3 mg/l culture) at 15°C.

Discussion

The molecular genetics of psychrophilic bacteria has been poorly investigated. There are several reasons for this, including the lack of studies on genetic exchange systems and the absence of suitable episomal vectors. The isolation of endogenous plasmids from these extremophiles, which could be used for the development of cloning systems, should overcome some of these difficulties.

In a previous study, we reported the isolation and the molecular characterization of the first cold-adapted plasmid, pTAUp, from the Antarctic gram-negative *Psychrobacter* sp. strain TA144. This plasmid duplicates in vivo by a rolling-circle mechanism, and several functional and structural features of the Rep initiator protein led us to propose the existence of a novel subfamily of RC replicons (Tutino et al. 2000).

To further investigate the diversity of psychrophilic genetic elements, we focused our attention on a small multicopy plasmid, pMtBL, from the gram-negative *P. haloplanktis* TAC 125 strain. This episome turned out to be an interesting extrachromosomal element because it displays unique molecular features. First of all, its nucleotide sequence is not related to any entry stored in the EMBL data bank and it appears to be transcriptionally silent. Although the pMtBL sequence (4,081 bp) contains several putative or "likely" open reading frames, none of them was found to be expressed in the tested conditions. These considerations suggest the fascinating hypothesis that this psychrophilic plasmid represents a sort of genetic relict, what remains of a larger element possibly mobilized in *Ph*TAC 125 by horizontal gene transfer and fated to be functionally mute. Being cryptic, the inheritance of pMtBL relies totally on the efficiency of its replication functions. This function can be bound to a region of about 850 bp by an in vivo assay (see Fig. 1). In fact, the pMtBL ARS 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 *Ph*TAC 125.

Interestingly, no incompatibility but rather a simple competition was observed between the endogenous wild-type plasmid and the pMtBL-derived shuttle vector (*clone Q*) actually carrying the selection resistance gene (amp^R). It might be that other functions, possibly related to a specific plasmid-partitioning mechanism, are responsible for the stable pMtBL inheritance at a very low copy number in TAC 125/*clone Q* transconjugants (see Fig. 2).

The use of conjugation to transform the Antarctic bacteria with the *clone Q* vector allowed the definition of its host range. Indeed, this shuttle vector was able to replicate and to be stably maintained in the cold-adapted gram-negative strains listed in Table 1. We infer from these data that pMtBL ARS can be used as a broad host range origin of replication. This observation further supports the previously reported idea of a shuffling origin of this psychrophilic plasmid.

Another aim of this study was the exploitation of recombinant protein production in cold-adapted bacteria. In fact, almost all the psychrophilic enzymes characterized so far display a moderate to extreme thermal instability (Gerday et al. 2000). Therefore, their production from recombinant mesophilic expression hosts was hitherto performed at temperatures suboptimal for growth (generally 15°–18°C for *E. coli*). The development of an expression system in cold-adapted bacteria would open new possibilities to enhance

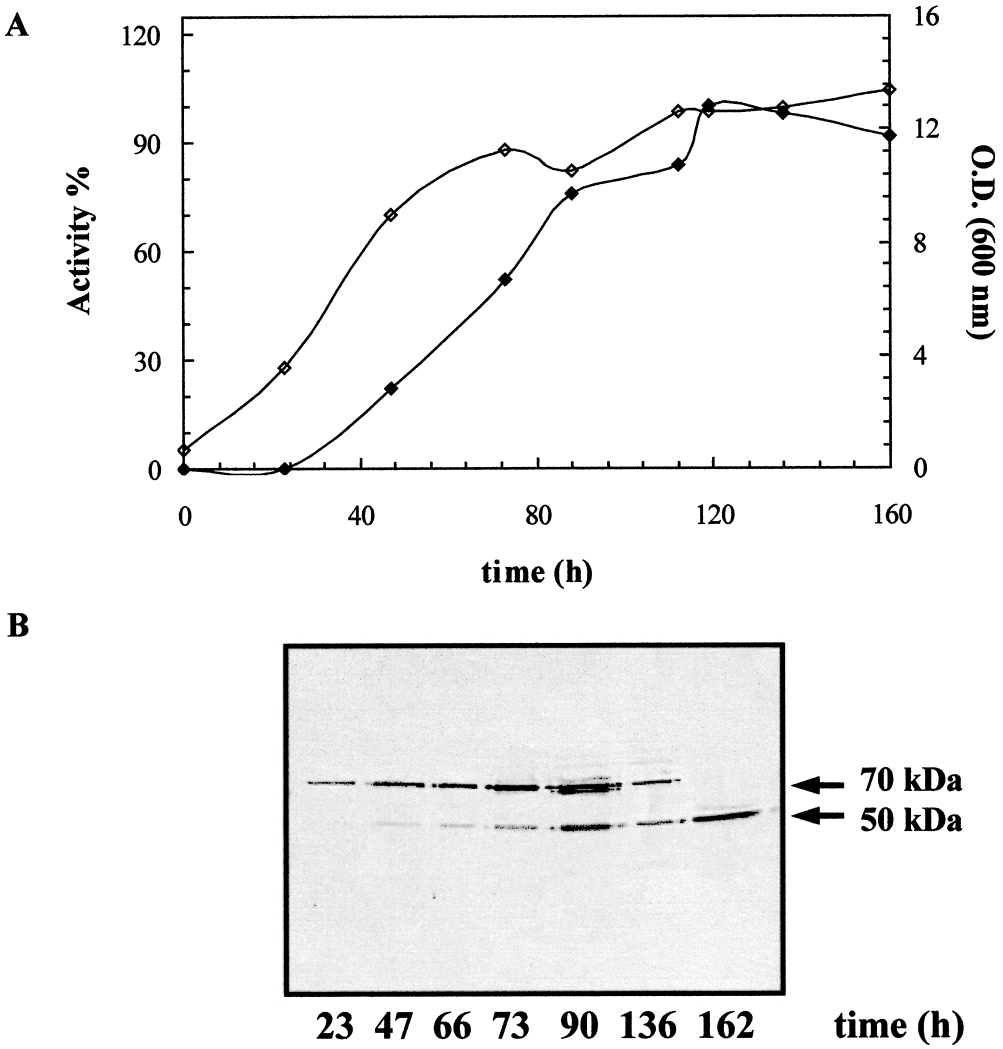

Fig. 4A,B. Growth of *Ph*TAC 125/pA6 recombinant cells and cold α-amylase secretion and maturation. **A** Growth of the recombinant psychrophilic bacterium at 4°C (*open diamonds*) and α-amylase activity in the culture supernatant (*solid diamonds*). **B** Western blot of cell-free supernatant samples corresponding to the culture shown in **A**. Sampling time (*x-axis*) and molecular mass (*arrows on right*) are indicated. Conditions for antigen detection were the same as in Fig. 3

the production yield and quality of thermolabile biocatalysts. Earlier work has demonstrated the feasibility of this approach (Remaut et al. 1999). In this study, the gene coding for the moderately thermolabile eukaryotic luciferase (from *Photinus pyralis*) was cloned into a pJB3-derived replicon and expressed, under control of *E. coli*-derived transcriptional and translational signals, in an Antarctic strain growing at 15°C.

In the present study, we investigated the production of a true cold-adapted enzyme in a genetically engineered Antarctic bacterium. To this end, we cloned the "replication cassette" (OriT/pMtBL ARS) from the *clone Q* shuttle vector into the mesophilic recombinant vector pαH12 (kindly provided by Dr. G. Feller, University of Liege, Belgium). The resulting construction, pA6, is a cold-adapted shuttle expression plasmid in which the gene coding for the *Ph*A23 α-amylase precursor was still subjected to the mesophilic *lac*Z promoter transcription regulation. The transformed strain *Ph*TAC 125/pA6 produces and also efficiently secretes the recombinant α-amylase. The protein accumulates in the culture medium and is also subjected to proteolytic processing, as occurs in the source strain (Feller et al. 1998). A detailed comparison of native and recombinant enzyme properties is presently under investigation (work to be published).

In conclusion, the described expression system represents the first example of heterologous protein production based on a true cold-adapted replicon. Although α-amylase production from the pMtBL-derived cold expression system was significantly lower (about two orders of magnitude) than that obtained in *E. coli* at 18°C, it is easily foreseen that optimization of several parameters, such as the regulation and efficiency of transcription and translation initiation, can considerably improve the efficiency of the system. The inherent replicative stability of the pMtBL-derived replicon in cold-adapted hosts is likely to be of great value in the development of stable expression systems for high-level production of thermolabile proteins at temperatures as low as 4°C.

Acknowledgments We are grateful to G. Feller for his support, frequent exchange of ideas, and valuable advice. This work was supported by grants of the European Union (program COLDZYME, contract ERB BIO4 CT96 0051; program EUROCOLD, contract ERB BIO4

CT95 0017; program COLDNET, contract ERB FMRX CT97 0131), of Ministero dell'Università e della Ricerca Scientifica (Progetti di Rilevante Interesse Nazionale 1999), and of Consiglio Nazionale delle Ricerche (CNR contract 97.01138.PF49, Progetto Finalizzato Biotecnologie), and by grants 1.5567.98N and G005097 of the Fonds moor Wetenschappelijk Onderzoek-Vlaanderen.

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