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METHOD PAPER

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Pichia pastoris is a valuable host for the expression of genes encoding membrane proteins from the hyperthermophilic Archeon *Pyrococcus abyssi*

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Abstract We present here the experimental strategies, first results and identified bottlenecks of a structural genomics initiative on membrane proteins of the hyperthermophilic archaea *Pyrococcus abyssi*. Five ORFs coding for putative membrane proteins have been cloned and expressed in the methylotrophic *Pichia pastoris* expression system, using two different constructs, with or without the signal sequence α-mating factor of *Saccharomyces cerevisiae*. A c-myc epitope and 6 His codons were added at the 3′-end of the targeted genes to allow immunodetection of the recombinant proteins and to facilitate their further purification. We have selected at least one producer clone for each protein of interest and for almost every construction. All the membrane proteins were produced in Erlenmeyer flasks culture and in fed-batch cultivation for large-scale preparation. The proteins were detected in the membrane fractions of *P. pastoris*. Production efficiencies were relatively low in both production conditions but the quantities of biomass obtained during fed-batch cultivation have allowed us to collect sufficient amount of material for further purification. The proteins were extracted, solubilized and partially purified. Large-scale purification will be necessary for further structural work.

Keywords *Pichia pastoris* · Structural genomics · Membrane protein · Archaea · *Pyrococcus abyssi*

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Introduction

The hyperthermophilic archaeon *Pyrococcus abyssi* was isolated from a deep-sea hydrothermal vent at 2,000 m depth in the North Fiji Basin (southwest pacific) (Erauso et al. 1993). The genome of *P. abyssi* GE5 was completely sequenced in 1998 (Genome project at Genoscope, GenBank Refseq NC000868). *P. abyssi* is presently used as model organism to study chromosome plasticity and DNA machineries (Myllykallio et al. 2000; Makino et al. 2001; Cohen et al. 2003). Because of its deep-sea origin, it has opened a novel field of research for microbiologists who were fascinated by extreme environments and the origin of life (Forterre et al. 2003, 2005). Physiological, enzymological studies and key metabolic features were undertaken to understand the bases of thermotolerance of hyperthermophiles (Adams 1999), resulting in biotechnological applications and development.

Very little is known about the structure and biochemical mechanisms of membrane proteins from hyperthermophilic organisms such as *P. abyssi*. Hyperthermophilic archaea are able to grow in extreme habitats (high temperature and high pressure). Their membrane proteins should have particular and very interesting functional and structural characteristics that confer cell stability under such conditions.

We report here the first results of a structural genomics initiative on membrane proteins from the hyperthermophilic Archaea *P. abyssi*. Structural genomics studies require the development of systematic cloning, production and purification strategies in order to obtain enough recombinant protein for their 3D structure characterisation. A number of worldwide initiatives have been launched, but these are mainly targeting soluble proteins (Lesley et al. 2002; Quevillon-Cheruel et al. 2003). The aim of our present project is to develop experimental strategies for the production and purification of membrane proteins for crystallization

purposes. As a pilot project, we chose five ORFs encoding for putative membrane proteins of hyperthermophilic Archaea *P. abyssi*.

Structural resolution of membrane proteins presents many bottlenecks. Production of large amounts of chemically and conformationally homogenous protein samples is necessary for successful crystallization and structure determination. Membrane proteins are usually not abundant. Therefore enhanced expression must be obtained using plasmid-based heterologous expression. However, overproduction is very often toxic for the cell (toxic effect exerted by functionally expressed proteins and/or massive protein insertion in the membrane). Moreover solubilization in a detergent is necessary for their purification. The presence of detergent as well as the biophysical characteristics of these proteins makes crystallization a difficult task.

The methylotrophic yeast *Pichia pastoris* has been widely and successfully used for high-level heterologous expression of proteins from various sources: bacteria, fungi, invertebrates, vertebrate, humans, plants and virus. Intracellular, secreted proteins and to a much lower extent integral membrane proteins were expressed (Hollenberg and Gellissen 1997; Cereghino and Cregg 2000; Macauley-Patrick et al. 2005). The *P. pastoris* expression system has several advantages. When the encoding genes are placed under control of the highly inducible alcohol oxidase-1 (AOX1) promoter the level of recombinant proteins produced can reach more than 30% of the total protein content. Moreover, the AOX1 promoter is repressed in the presence of glucose, non-induced in presence of other different substrates (sorbitol, ethanol, glycerol), and its activity is maximal in the presence of methanol. It has therefore been possible to reduce potential heterologous protein toxicity by uncoupling the growth phase from the induction of protein production phase. *P. pastoris* is also very well-adapted to high cell density cultures in fermentor. In this way, up to 100 g/l of dry cell weight can be swiftly produced on a non-inducible substrate prior to the addition of methanol, which will be metabolize very slowly (Cereghino et al. 2002). Futhermore the recombinant elements are genetically stable in large-scale fermentation (Macauley-Patrick et al. 2005).

Although the *P. pastoris* system was successfully used for the production of many soluble proteins, only recently heterologous production of membrane proteins was reported for this expression host (Weiss et al. 1995). Recent publications reported on expression of several candidates of different protein families: (1) the large family of G-protein coupled receptors (GPCRs) (Weiss et al. 1995, 1998; Talmont et al. 1996; Sarramegna et al. 2002a, b; De Jong et al. 2004), (2) the family of multidrug resistance protein (MRP transporter) (Cai et al. 2001), and (3) the family of electrogenic pumps Na^+, K^+-ATPases (Cohen et al. 2005). Recombinant MRP transporter and GPCRs were correctly produced and inserted in *P. pastoris* membranes sometimes at similar levels and in another cases at a concentration level up to ten times higher than those reported for transfected mammalian cells or insect cells (Weiss et al. 1995; Sarramegna et al. 2002a, 2003).

Here we describe cloning, production and purification of membrane proteins of *P. abyssi* in *P. pastoris*. Heterologous expression was performed using pPICZ vectors containing the AOX1-methanol inducible promoter. Overproduction of such proteins in a heterologous system and subsequent purification of the protein to homogeneity is a prerequisite for biophysical and structural studies as well as functional and pharmacological studies to advance in understanding the structure–function relationships.

Materials and methods

Bacterial, yeast strains and media

Escherichia coli strain Top10F' (Invitrogen) was used for the propagation of recombinant plasmids. *E. coli* transformants were selected on low salt LB plates with 25 μg/ml zeocin.

The wild-type Mut^+ *P. pastoris* strain X-33 was used as the receptor strain for expression. This strain was routinely grown on YPD medium. The buffered glycerol-complex medium (BMGY) and buffered methanol-complex medium (BMMY) were used for expression studies in microtiter plates and Erlenmeyer flasks. *P. pastoris* transformants were selected on YPDS plates with 100 μg/ml zeocin. The composition of all these media is the same as indicated in the Invitrogen instruction manual (Easyselect™ Pichia expression kit) except BMGY contained 4% glycerol. Large-scale cultures used the synthetic medium (SM) and SM for fed-batch culture (SMFB) in a bioreactor (Laborde et al. 2004).

Construction of the expression plasmids and transformation

The ORFs were amplified from the genomic DNA of *P. abyssi* using different primers and cloned into the plasmids pPICZB and pPICZαA or -αC (Table 1).

These primers were synthesized on the basis of the DNA sequence (Genbank accession number NC 000868) with additional nucleotides introducing the restriction sites *Eco*RI, *Xba*I, *Cla*I or *Not*I (underlined characters) in order to splice the PCR products into the multicloning sites of the vectors and to achieve directional cloning in frame with the α-factor prepro-signal sequence from *S. cerevisiae* (for pPICZαA and pPICZαC only) and the myc epitope and polyhistidine tag.

The PCR was performed with the high fidelity *Pfu* polymerase (Promega) using a GeneAmp PCR system 9700 from Applied Biosystems. Amplifications were performed with 5 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 4.5 min and followed by 20 cycles of 95°C for

Table 1 Plasmids and primers used

Plasmid, ORFs	Description/sequence of the primers	Source or reference
Plasmids		
pPICZB	*AOX*1 promoter, *AOX*1 transcription termination, C-terminal c-myc epitope and polyhistidine tag, zeo^R (in *E. coli* and *P. pastoris*), pUC ori	Invitrogen
pPICZαA and αC	*AOX*1 promoter, *AOX*1 transcription termination, C-terminal c-myc epitope and polyhistidine tag, zeo^R (in *E. coli* and *P. pastoris*), pUC ori, α-factor secretion signal from *Saccharomyces cerevisiae*	Invitrogen
ORFs		
PAB0107	5′-CCGGAATTCATGGATGGGTATTTCAACAAAGC-3′ 5′-GCTCTAGATACTTGCAGCACTTAGCTAG-3′	This study
PAB2010	5′-CCATCGATGAAGGCATACACTCTGG-3′ 5′-GCTCTAGATAGCGAAGGGTCCTTTTCTGG-3′	This study
PAB0965	5′-CCGGAATTCATGC TGGCGATTGG CCTC-3′ 5′-GCTCTAGATAGTACCATCGATAATATCTCTTTGAGCC-3′	This study
PAB0677	5′-CCGGAATTCATGTACGAGGAG TACGTAAAGA AGAG-3′ 5′-GCTCTAGACATACCCTTCCCTCCATCTTCAGC-3′	This study
PAB0724	5′-CCGGAATTCATGAATAGGAACTTCTGGCTCTTCG-3′ 5′-ATAGTTTAGCGGCCGCATAATCTTCGGGAGGAACCCCAGG-3′	This study

The underlined characters in the sequence of the primers used for PCR amplification of the five ORF selected indicates the restriction sites *Eco*RI, *Xba*I, *Cla*I or *Not*I

30 s, 60°C for 30 s, 72°C for 2.5 or 4.5 min. The PCR products were analyzed on agarose gels. The expected DNA strands were purified using the Wizard PCR Preps DNA purification systems (Promega) and digested by the restriction enzymes *Eco*RI and *Xba*I (ORFs PAB0107, PAB0965, and PAB0677), *Cla*I and *Xba*I (ORF PAB2010) or *Eco*RI and *Not*I (ORF PAB0724). The fragments were ligated into the pPICZ vectors (pPICZαA or pPICZαC, pPICZB, Invitrogen) previously digested with the same set of restriction endonucleases. To eliminate non-recombinant plasmids, the ligation mixtures were heated 10 min at 65°C to inactivate the ligase and digested by the restriction enzyme *Sfi* I at 50°C.

After transformation of *E. coli* TOP10F' with the ligated-plasmids by electroporation, transformants were selected on low salt LB plates with 25 μg/ml zeocin. Plasmid DNA were extracted using a Qiagen plasmid midi kit and sequenced to check that no nucleotide modifications occurred during the PCR amplification and to confirm the correct integration of the ORFs into the plasmids.

Approximately 10 μg of recombinant plasmids were linearized with *Pme*I and used to transform competent cells of *P. pastoris* by electroporation. This resulted in the genomic integration of the construct by homologous recombination. Transformants were selected on YPDS plates with 100 μg/ml zeocin. After selection, *P. pastoris* transformants were grown 24–48 h at 30°C in 96 well microtiter plates containing YPD with zeocin 100 μg/ml. Glycerol was added to a final concentration of 25% and the microtiter plates were frozen at −80°C. Selection of multicopy-integrated plasmids transformants was performed by selection on YPD with increasing amounts of zeocin. The test was achieved for 4 × 96 clones for each plasmid derivative.

DNA manipulations

Molecular procedures were carried out as described by Sambrook and Russel (2001).

Cell growth and induction of protein synthesis

Screening clones for expression by colony-immunoblots

To test for expression, transformants were grown 24 h at 30°C in 96 well-microtiter plates containing YPD with Zeocin 100 μg/ml. After growth, the transformants were transferred on BMMY agar plates with a replica plater and incubated at 30°C to induce expression. Methanol was added to the lid of the inverted plates twice a day. Colony hybridization was carried out by transferring the bacteria from the plate to a nitrocellulose filter (Hybond-C extra, Amersham) by capillarity (Laborde et al. 2004). Immunoblot analysis was performed using alkaline phosphatase-conjugated antibodies [anti-myc-AP and anti-His (C-term)-AP] at a final concentration of 1/2,000 (v/v) as described by Invitrogen.

Expression of the selected clones

About 1 ml of YPD broth with Zeocin 100 μg/ml was inoculated with the selected clone for 20–24 h at 28°C with vigorous shaking (250 rpm). Cells were then transferred in 25 ml of BMGY and grown 20–24 h at 28°C with vigorous shaking (250 rpm) until the $OD_{600\ nm}$ was 100–150. Cells were centrifuged, resuspended in BMMY broth and grown for 3 days as described above. Methanol (0.5%) was added twice a day.

Scaling up of cultures

Large-scale cultivation was performed in a bioreactor (1.8 l maximal capacity) using a fed-batch procedure as described by Shrestha et al. (2004) except the pH was regulated to 5.5. Cell growth was measured by measuring OD600 using a Hitachi U1100 spectrophotometer. One unit corresponds to 0.15 g of dry cell weight per litre. Residual methanol was analyzed by a FFJA-HPLC column (Waters) using an isocratic mode of 0.8 ml per min of orthophosphoric acid (5 mM). Detection of methanol was performed by refractometry (IOTA2, Precision Instruments) twice a day.

Separation of the secreted, cytoplasmic- and membrane-associated proteins

Pichia pastoris cells were collected by centrifugation and the pellet was resuspended in an ice-cold buffer containing 50 mM imidazole pH 7.5, 1 mM $MgCl_2$, 250 mM sorbitol, 1 mM PMSF and a complete protease inhibitor cocktail (Roche) at 2 ml of buffer/g of cells. The cells were mixed with glass beads (425–600 µm, Sigma, 1 g/g of cells) and disrupted for 8 min using a Glass Bead Beater (Retsch MM2000). For larger scale preparations, cells were lysed by passage through a French pressure cell at 20,000 psi.

Unbroken cells and beads were removed by two successive centrifugation steps (10 min at 2,300*g* and 4°C). The supernatant was further centrifuged at 16,000*g*, 4°C for 1 h. The membrane pellet was washed in an ice-cold buffer containing 10 mM imidazole pH 7.5, 1 mM $MgCl_2$, 1 mM PMSF and a complete protease inhibitor cocktail (Roche) and resuspended in this buffer.

To analyse the secreted protein composition, the BMMY broth separated from the cells was mixed with ice-cold acetone 80% (v/v) to precipitate the proteins.

Solubilization and purification of the membrane proteins

Solubilization tests were performed with Triton X100 in a 20 mM phosphate buffer pH 8. If necessary, asolectine or cholesteryl hemisuccinate (CHS) was added to improve solubilization of the protein. Incubation with the detergent was performed 1 h at 4°C with a helical wheel and the preparation was centrifuged at 16,000*g*, 4°C for 1 h. The supernatant containing the solubilized protein was separated from the pellet. The pellet was resuspended in the same buffer and same volume as the detergent-solubilized proteins.

The detergent-solubilized proteins (500 µl) were purified with Ni-NTA spin columns under native conditions (Ni-NTA Spin handbook Qiagen). The buffers used contained additional 0.5% Triton X100 and 0.2% CHS was added if necessary.

Stability of the purified proteins to high temperatures

The Ni-NTA eluted-proteins were incubated for 5 min at 50, 60, 70 80 or 90°C, cooled 10 min on ice and centrifuged at 16,000*g*, 4°C for 1 h to eliminate heat-unstable proteins. The supernatant containing the heat-stable proteins were analyzed on silver stained SDS-PAGE.

SDS-PAGE, western-immunoblots and protein assay

SDS-PAGE was performed as described by Laemmli (1970). The gels were either stained with Coomassie brilliant blue, silver (SilverXpress, Invitrogen) or blotted to nitrocellulose membrane (Hybond-C extra, Amersham) using a Mini Trans-blot electrophoretic transfer cell (Bio-Rad). Immunoblot analysis was performed using alkaline phosphatase-conjugated antibodies (anti-myc-AP) as described by Invitrogen at a final concentration of 1/2,000 (v/v).

Protein content was determined using the Bio-Rad protein assay. Bovine serum albumine was used as a standard.

Results

Cloning of putative membrane protein-encoding genes from *Pyrococcus abyssi*

Targets encoding for putative membrane proteins were selected according to different criteria:(1) small size proteins (< 50 kDa), (2) a clear presence of transmembrane regions and (3) possessing different putative functions (Table 2). The hydrophobic profiles of the target proteins using transmembrane region prediction protocols showed the alternation of hydrophobic and hydrophilic regions typical of integral membrane proteins (data not shown). The number of membrane spanning regions predicted varied from three to ten according to the protein. Primary sequence analysis of our targets revealed homologies with different kinds of putative transporter proteins. Putative eukaryotic and/or bacterial signal anchor and signal sequences were predicted (Table 2).

All chosen ORFs were amplified by PCR and successfully cloned into the pPICZ vectors in *E. coli*. The selected recombinant plasmids were sequenced to check that no nucleotide modifications occurred during the PCR amplification and to confirm the correct integration of the ORFs into the plasmids. Vectors containing in frame the *S. cerevisiae* α-mating factor signal sequence (89 amino acids), the target sequence and both a c-myc epitope and a six histidines tag (21 amino acids) were obtained for the five ORFs studied (pPICZαA-PAB0107, pPICZαC-PAB2010, pPICZαA-PAB0965, pPICZαA-PAB0677, pPICZαA-PAB0724). Another version of the same vectors was obtained without the

Table 2 Characteristics of the five selected ORFs

	ORF size (bp)	Predicted protein size (kDa)[a]	Number of predicted transmembrane region[b]	Putative function[c]	Predicted signal sequence or anchor[d]
PAB0107	555	20.4	5	Predicted membrane-bound metal-dependent hydrolase	No
PAB2010	1,140	40.8	10	Voltage gated chloride channel family protein	Eukaryotic signal anchor
PAB0965	567	20.9	3	Protein of unknown function Family of hypothetical archaeal proteins (DUF531)	No
PAB0677	1,032	36.7	9	Fe^{3+} ABC transport system family protein	Eukaryotic signal anchor /Bacterial signal peptide
PAB0724	1,257	46	9–10	Putative carbohydrate transport system family protein	Eukaryotic signal peptide

[a]Using MWCALC (http://www.infobiogen.fr/)
[b]According to Hofmann and Stoffel (1993)
[c]Using Basic Local Alignment Search Tool (BLASTp) (http://www.ncbi.nlm.nih.gov/)
[d]Using SignalP 3.0 Server (Bendtsen JD et al. 2004)

α-mating factor signal sequence (pPICZB-PAB0107, pPICZB-PAB2010, pPICZB-PAB0965, pPICZB-PAB0677, pPICZB-PAB0724). Fusion of the c-myc epitope and histidines tag at the C-terminus was used to facilitate purification and immunological detection of the expressed proteins.

The different constructs were introduced at the chromosomal *AOX1* locus of the methyltrophic yeast *P. pastoris* by homologous recombination. In *P. pastoris* it is known that during integration single or multiple insertions occurs (Higgins and Cregg 1998). The expression level is influenced by the number of copies of the gene introduced into the genome. It is admitted that multiple copies of the expression plasmid integrated into the genome results in an increase in zeocin resistance via a gene dosage effect. The resistance phenotype provides indirect information about the number of gene copy integrated. In order to check that single and multiple integrations of our recombinant plasmids occurred among the selected *P. pastoris* transformants, we tested the transformants on YPD plates containing zeocin 100, 500 and 1,000 μg/ml. Depending on the plasmid, around 50–65, 20–40, and 7–10% of the tested clones were, respectively, resistant to Zeocin 100, 500 and 1,000 μg/ml. These results confirmed that for all the constructions we had transformants with different numbers of recombinant plasmids integrated and potentially different copies of the gene of interest.

Screening clones for expression by colony- and western-immunoblots

Randomly picked transformants were transferred on BMMY agar plates and expression was tested after 24, 48 and 72 h of induction with methanol using immunological detection. All the clones were able to grow in presence of methanol as the sole carbon source. Immunoblot analysis was performed using alkaline phosphatase-conjugated antibodies [anti-myc-AP and anti-His (C-term)-AP]. Only few positive clones were detected except for pPICZB-PAB0965 (Table 3).

The absence of immunological detection is probably due to insufficient protein production levels (caused by problems related to plasmid integration, mRNA unstability, codon usage, proteolytic activity). Misfolding, aggregation or unsuccessful localization of the neosynthesized product is also possible. Most of the time, the number of *P. pastoris* transformants producing the membrane protein of interest is not mentionned in publications. To our knowledge, the few publications reporting this type of information was from Karlsson et al. (2003) and Cai et al. (2001). In their work, Karlsson et al. (2003) overexpressed one of the major integral proteins from spinach leaf plasma membranes in *P. pastoris*. They reported the selection of three positive clones among ten independent transformants. For Cai et al. (2001) a frequency of 10–20% of clones producing a multidrug-resistance protein is mentionned. It is difficult to know if the low number of producing clones we selected is due to the nature of the protein, the archaeal origin of the protein and/or the screening method used.

When anti-His (C-term)-AP antibodies were used for detection, a high background was observed on the nitrocellulose membrane (data not shown). It was not the case when the anti-myc-AP antibodies were used. Further immunological analysis was performed with anti-myc-AP antibodies.

Expression tests were then carried in Erlenmeyer flask with the clones expressing at the highest level. The expression test was carried out for 3 days and 2 ml samples were picked every 24 h to analyse the proteins produced during methanol induction. Global analysis by SDS-PAGE and western-immunoblot of membrane, cytoplasmic and secreted proteins was done for each clone of interest after methanol induction. Recombinant proteins were only detected after immunoblotting of the various fractions. These results indicate that the proteins are not produced in high enough amounts to be detected by SDS-PAGE or not capable of binding sufficient

Table 3 Number of positive clones detected by colony-immunoblotting (+) among all the *P. pastoris* clones tested for all constructions

	pPICZB-PAB0107	pPICZB-PAB2010	pPICZB-PAB0965	pPICZB-PAB0677	pPICZB-PAB0724
Nb clones +	1	1	92	1	2
Nb clones tested	45	192	96	96	96
	pPICZαA-PAB0107	pPICZαC-PAB2010	pPICZαA-PAB0965	pPICZαA-PAB0677	pPICZαA-PAB0724
Nb clones +	0	2	2	3	7
Nb clones tested	45	96	96	96	96

Alkaline phosphatase-conjugated antibodies (anti-myc-AP) were used for revelation

coomassie dye to be detectable. Quantitative immunodetection would be needed to distinguish these alternatives. The immunoreactive proteins were detected in the membrane fractions after induction with methanol. No immunoreactive protein was detected among the secreted proteins in the media or in the cytoplasmic fraction. When the pPICZα recombinant vectors were used for heterologous expression, different variants of the protein were detected in the membrane fractions. These species certainly correspond to different forms of the protein: a protein with the expected molecular mass corresponding to the mature protein and another form with a higher apparent molecular mass. These recombinant proteins could be glycosylated. However, only one putative *N*-glycosylation site (N-X_{aa}-S/T motif) was found in PAB0965 and PAB0677. Moreover the length of oligosaccharide chains added in *P. pastoris* is low (8–14 mannoses residues). The differences of molecular size observed were higher than those expected if N-linked glycosylation have occurred. A 0-linked glycosylation has very little probability to occur in *P. pastoris*. This result suggests that part of the protein expressed was not correctly processed and contained additional amino acids. Variability in the amino terminus is commonly seen with heterologous proteins produced by *P. pastoris* using the α-factor prepro leader (Cereghino and Cregg 2000).

Heterologous expression with the pPICZB-recombinant vectors allowed the detection of unique form of the proteins. The immunoreactive proteins PAB0107, PAB0965, PAB2010 were detected after 24, 48 and 72 h of induction whereas PAB0677 and PAB0724 were detected after 48 and 72 h of methanol induction (data not shown). The apparent molecular mass of all the immunoreactive proteins was consistent with the expected molecular mass (Table 2). So we decided to continue this work with the pPICZB-derivatives.

High-cell density cultivation procedures

Each producer clone carrying the pPICZB construction was then cultivated using the fed-batch procedure for large-scale production. Typical kinetic growth curves and membrane protein production for batch cultures achieved in fermentor are represented only for one producer clone: *P. pastoris* X33 pPICZB-PAB2010 (Fig. 1). The kinetic data obtained for the other clones are summarized in Table 4.

Western-immunoblot analysis of membrane protein production for each clone is shown in Fig. 2. As observed with Erlenmeyer cultivation conditions, the size of the immunoreactive proteins detected corresponds to the mature form of the proteins.

In fed-batch mode, the exponential feeding strategy allowed cells to grow at a constant specific growth rate by using methanol as a growth-limiting nutrient and thus, caused minimum perturbation on cellular carbon metabolism. As shown in Fig. 1, the specific growth rate was successfully controlled at a constant value around 0.015 h^{-1} during methanol induction. This growth profile was reproduced for 3 out of 6 producer clones (Table 4): for the control strain X33-pPICZB and the two strains producing the proteins PAB2010 and PAB0677. In this case, about 80 g/l of dry weight was obtained after 3 days of methanol induction, and the final biomass values were comparable to the theoretical one. The slow methanol addition allowed sufficient time for the *P. pastoris* clones to utilize all methanol supplied even if in some cases temporary accumulation occurred.

For the other proteins produced in high cell densities cultures (PAB0107, PAB0965 and PAB0724), the kinetic curves showed two different steps during the induction phase. In the first step, the biomass increased exponentially with an estimated specific growth rate of 0.013–0.028 h^{-1} against a theoretical value of 0.015 h^{-1}. In the second phase, the specific growth rate dropped abruptly to values lower than 0.01 h^{-1}. This phenomenon was not due to the accumulation of toxic methanol concentration in the medium. When accumulation of methanol was noticed (3.5–17 g/l according to the clone), methanol feeding was stopped until methanol was entirely consumed. This growth profile seems to be related to the nature of the produced protein rather than to the accumulation of the recombinant proteins. We can observe that there is no correlation between the growth profile and the recombinant protein production efficiency: PAB0965, −0677 and −0724 proteins seem to be produced at higher levels than the PAB0107 and −2010 proteins (Fig. 2).

Whatever the fed-batch cultivation tested, the estimated growth yields were very low in comparison with the expected value (Table 4). This observation could be easily explained by the fact that in bioreactor cultiva-

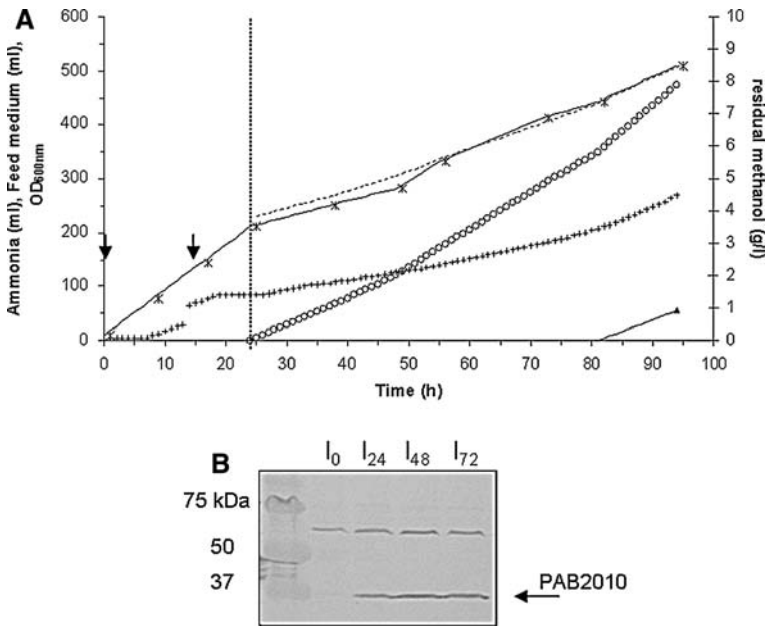

Fig. 1 Kinetic evolution of biomass (*asterisk*, $OD_{600\ nm}$) in a fed-batch culture in fermentor (**a**) using SM medium and analysis of the membrane proteins produced after methanol induction (**b**). Pulses of SM medium [with 4% (w/v) glycerol] are represented by *arrows*. The *vertical dashed line* represents the beginning of the induction of protein production by the methanol feeding (*open circle*). Residual methanol (*filled triangle*), ammonia consumption (+) are measured. Alimentation flow of SMFB medium [with 98.7% (v/v) of methanol] is defined in order to maintain a theoretical specific growth rate at 0.015 h^{-1} and a biomass yield $Y_{X/MEOH}$ at 0.3 g g^{-1}. Theoretical growth curve is also shown (*dotted line*) Kinetics of protein production during the methanol induction phase were analysed by western-immunoblots using anti-myc-AP antibodies. I_o, I_{24}, I_{48} and I_{72} correspond to, respectively, 0, 24, 48 and 72 h of induction. *P. pastoris* strain used: X33-pPICZB-PAB2010. Conditions of *cultivation*: 28°C, pH 5.5, 1 vvm, 1,500 rpm

Table 4 Kinetic data under two different culture conditions used to produce the control strain *Pichia pastoris* X33-pPICZB and the producer clones of the five different membrane proteins

	Fed-batch cultivation				Batch cultivation
	Final X (g/l)	Residual MeOH[a] (g/l)	$Y_{X/MEOH}$ (g/g)	μ induction[b] (h^{-1})	Final X[c] (g/l)
Theoretical values	78.0	0	0.300	0.015	20.0
pPICZB	95.0	0	0.250	0.020	11.0
pPICZB-PAB0107	64.5	0	0.164	0.0283 0.0011	21.0
pPICZB-PAB2010	76.5	1.0	0.123	0.0130	14.5
pPICZB-PAB0965	63.0	3.5	0.110	0.0133 0.0042	11.5
pPICZB-PAB0677	84.0	9.0	0.130	0.0135	9.2
pPICZB-PAB0724	65.1	17.0	0.130	0.0138 0.0008	11.3

The cell concentration (X) and biomass yield (Y) are indicated
[a]Concentration in residual methanol was not determined in Erlenmeyer flasks. The indicated value does not correspond to the finale value but to the maximum one
[b]In several cases, two different growth phases were observed and so, two different specific growth rates were calculated
[c]Specific growth rates were not easy to estimate in Erlenmeyer flasks where cell flocculation occurred, probably because of two reasons: the lack of oxygen during the methanol induction phase and the discontinuous addition of inducer twice a day during the growth

tion, aeration probably triggered evaporation of a large part of added methanol.

The western-immunoblots applied on 20 µg of total protein of the membrane fraction showed similar results for Erlenmeyer and fed-batch cultivation conditions except all the proteins were detected after 24 h of induction during fed-batch cultivation (data not shown). In all cases, the fed-batch method consistently enhanced the final recombinant cell concentrations from three to nine times in comparison with batch cultures (Table 4). Sufficient quantities of recombinant cells were collected from fed-batch cultures to investigate the further steps of extraction and purification. Small-scale extraction and purification protocols were performed.

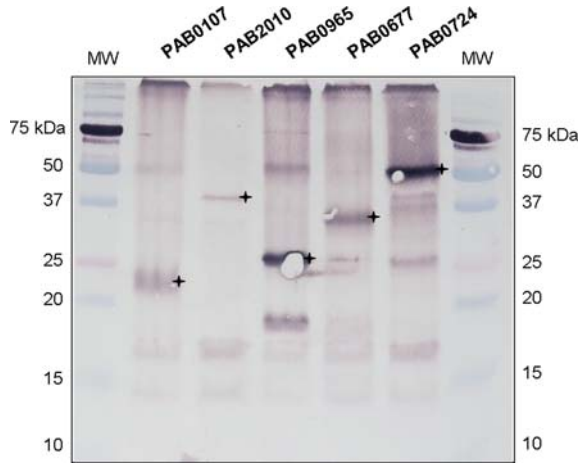

Fig. 2 Western-immunoblot analysis of membrane proteins produced in fed-batch culture. The membrane extracts (20 µg of proteins) from pPICZB-clones expressing PAB0107 (20.4 kDa), PAB2010 (40.8 kDa), PAB0965 (20.9 kDa), PAB0677 (36.7 kDa) and PAB0724 (46 kDa) were loaded on a 15% SDS-PAGE and transferred to nitrocellulose for immunoblotting. Alkaline phosphatase conjugate (anti-myc-AP) were used for revelation. The calculated molecular mass is deduced from the nucleotide sequence. Additional 2.5 kDa due to the c-myc epitope and six histidines tag has to be added. Position of the proteins is indicated with (*plus*). The prestained molecular weight (Precision plus protein standards, dual color, Sigma) is used as a standard (*Lanes* MW)

Solubilization and purification

Different solubilization protocols were tested on the membrane fractions to solubilize the recombinant proteins. The non-ionic detergent triton X100 was used at different final concentrations (0.5, 1 and 2%) and different detergent to protein ratio of 5:1 and 10:1 (w/w). Solubilization of PAB2010, PAB0677 and PAB0724 was successful with both 0.5 and 1% Triton ratios. To minimize the concentration of detergent, solubilization was further performed with 0.5% Triton at a detergent to protein ratio of 5:1.

PAB0107 and PAB0965 could not be solubilized under these conditions. Prior urea/alkaline stripping of the membranes or addition of asolectine during the solubilization experiment did not improve detergent solubilization. However, the addition of 0.2% CHS improved the solubilization of these two proteins and immunoreactive proteins could be detected in this condition (Fig. 3). For PAB0965 the immunoreactive signal is low. For the other proteins, no significant CHS effect was noticed (Fig. 3).

The detergent-solubilized proteins were loaded on Ni^{2+} metal chelate columns and elution was performed with a buffer containing 250 mM imidazole. Analysis of the flow-through (loading of the sample, washing steps and elution step) by Western immunoblot showed that a large part of the protein PAB0107 was found in the flow-through after loading of the sample on the column. This result demonstrates that part of the protein was not able to bind to the column. The other proteins were all retained on the columns and eluted with high concentration of imidazole. The eluted proteins were analyzed on silver-stained SDS-PAGE (Fig. 4). All the target proteins were partially purified. For PAB0107, a faint immunoreactive band is detected. Again CHS was not necessary to solubilize PAB2010, PAB0677 and PAB0724 after elution.

Without biochemical assays, it is difficult to evaluate the purification yields of these proteins. We can roughly estimate the total amount of protein we can purify from the 1 l fed-batch cultures we obtained taking in account the volumes used at each step of preparation. According to Fig. 4, the final proteins are almost pure. Considering that we have almost 500 ng of pure protein by well on SDS-PAGE after small-scale extraction and Ni-NTA purification, we potentially have from 10 to 30 mg of protein in 1 l of suspension cells cultivated in fed-batch conditions. Even if the purification efficiency drops when scaling up the procedure, yields are in principle sufficient to obtain enough amounts of material for structural studies.

Stability to heat treatment

Proteins from hyperthermophilic organisms tend to be characterized by extreme structural stability and can function at elevated temperatures. As the optimal growth temperature of *P. abyssi* is 96°C at atmospheric pressure (Erauso et al. 1993), the stability of the partially purified proteins was tested from 50 up to 90°C (data not shown). The most heat stable protein was PAB0965: the protein remained soluble at all tested temperature. For PAB2010, PAB0677 and PAB0724, the proteins were stable up to 70°C. However, the protein quantities decreased slightly from 50 to 70°C in the case of PAB2010 and PAB0677. For PAB0107 no protein was detected. As *P. abyssi* is able to grow up to 100°C, one would expect that its proteins would be stable at 90°C. However, such thermal stability is observed when these proteins are in their natural environment that is, within the membranes of *P. abyssi*. The requirement of specific lipids in the surrounding of these proteins is certainly needed and might explain the difference of heat stability between the proteins tested.

The thermal stability of these proteins is also very useful to improve the purification. According to these results a prepurification step at a minimal temperature of 50°C depending of the protein could be use prior Ni-NTA purification to eliminate contaminant proteins.

Discussion

We showed that using *P. pastoris* as an expression host, it was possible to produce membrane proteins from a hyperthermophilic archaea *P. abyssi*. Five different genes encoding putative membrane proteins with different functions were selected and subcloned in pPICZ vectors. To increase the probability of achieving

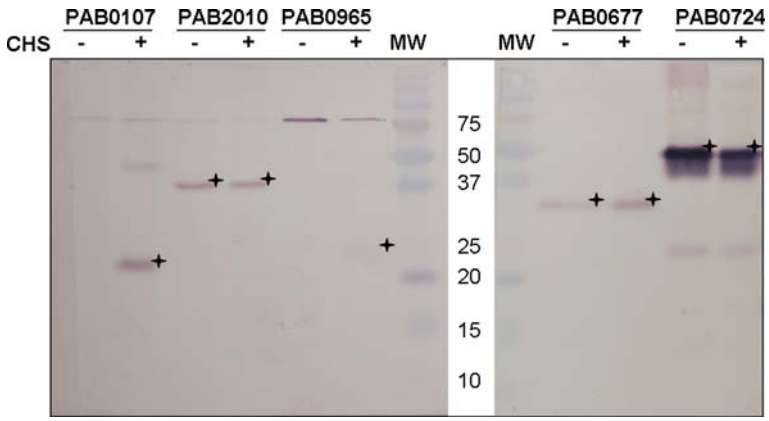

Fig. 3 Western-immunoblot analysis of membrane proteins solubilized with triton X100 in presence or not of CHS. Solubilization experiments were performed with 0.5% Triton in absence (−) or presence (+) of 0.2% CHS on membrane extracts from pPICZB-clones expressing PAB0107, PAB2010, PAB0965, PAB0677 and PAB0724. Position of the proteins is indicated with (*plus*). Three μg of proteins were loaded on a 15% SDS-PAGE. Alkaline phosphatase conjugate (anti-myc-AP) were used for revelation. The prestained molecular weight (Precision plus protein standards, dual color, Sigma) is used as a standard (*Lanes* MW)

expression of the membrane proteins, two plasmids were tested: pPICZ and pPICZα containing the *S. cerevisiae* α-mating factor prepro signal sequence. Previous studies reported successful expression with membrane proteins cloned into *P. pastoris* vectors in presence of the *S. cerevisiae* α-mating factor signal sequence (Weiss et al. 1995; Talmont et al. 1996; de Jong et al. 2004). However, expression was also reported in absence of the signal sequence (Cai et al. 2001; Karlsson et al. 2003; Cohen et al. 2005). In our case, expression was obtained with both plasmids. Moreover the pPICZB-expressed proteins were associated to the membrane fraction. This result suggested that the α-MF factor was not necessary to produce the membrane proteins from *P. abyssi* in *P. pastoris*. The presence of putative eukaryotic signal peptide in some proteins suggest that these proteins could be targeted to the plasma membrane. However, as observed by Sarramegna et al. (2002b), recombinant membrane protein integration could also occur in endoplasmic reticulum or Golgi apparatus membranes. Integration in the membrane would need to be checked by in situ localization experiments using immunofluorescence.

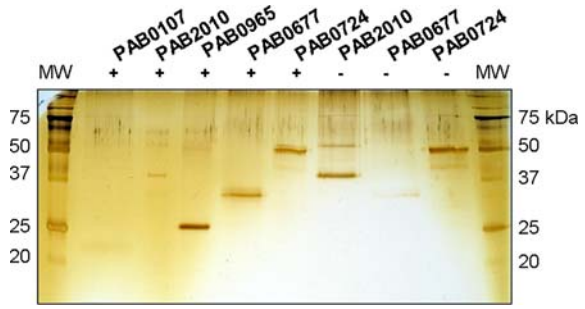

Fig. 4 Partial purification of the proteins with Ni-NTA columns. Solubilization experiments with Triton 0.5% were performed in presence (+) or absence (−) of CHS on membrane extracts from pPICZB-clones expressing PAB0107, PAB2010, PAB0965, PAB0677 and PAB0724. The solubilized proteins were loaded on a Ni-NTA spin column and eluted with imidazole. The eluted proteins (500 ng) were loaded on a 15% SDS-PAGE and the gel was silverstained. The prestained molecular weight (Precision plus protein standards, dual color, Sigma) is used as a standard (*Lanes* MW)

In general the effect of gene copy number on expression level in *P. pastoris* was shown to be unpredictable. There are numerous examples in which a single copy of the expression cassette is sufficient for optimal production. In other cases, multiple copies allowed high level expression or in the contrary induced dramatic effects (Higgins and Cregg 1998). Sometimes an optimal gene copy number is required (Weiss et al. 1998). According to these observations we decided to test single- and multiple-copy transformants for expression. It was very easy with *P. pastoris* to select a large number of single- and multiple-copy transformants in a single experiment. Moreover the colony-immunoblots procedure allowed fast and easy screening of *P. pastoris* transformants and selection of membrane protein-producer clones. Unfortunately, few producer clones were selected (except for pPICZB-PAB095). So it was not possible to determine any correlation between the gene copy number and the expression level.

The clones selected were able to produce the membrane proteins both in Erlenmeyer and high cell densities conditions. Scale-up was possible without loss of production yields. The level of recombinant proteins produced in the membrane had no drastic consequence on host physiology and on cellular carbon metabolism. In some cases, perturbations of growth kinetics at high cell densities culture were observed. This phenomenon was probably correlated to the nature of the protein produced.

One of the major problems to study membrane proteins is that they are often unstable once extracted from the lipid bilayer. During solubilization experiments with triton, no problem was noticed with the proteins PAB2010, PAB0677 and PAB0724 whereas PAB0107

and PAB0965 were unstable. Therefore the solubilization experiment had to be improved for these two proteins. First urea/alkali treatment of the membranes was tested. Such treatment has been shown to enhance solubilization of integral membrane proteins by removing peripheral membrane proteins and proteins adhering to the membranes (Werten et al. 2001). This method was used successfully for the purification of an aquaporin from spinach overexpressed in *P. pastoris* (Karlsson et al. 2003). Unfortunately this method did not work with PAB0107 and PAB0965. Another strategy used to stabilize membrane proteins consists of adding lipid molecules**.** Many membrane proteins have specific lipid and sterol requirements, involved in the folding and stability of the protein as well as their function (Opekarova and Tanner 2003). Such molecules can be useful additives for stabilizing membrane proteins in detergent solution. The dependence of activity on detergent and lipids like asolectine was demonstrated for the P-type ATPase from *Methanococcus jannaschii* (Morsomme et al. 2002). Addition of CHS during the solubilization experiments increased the recovery and stability of the human adenosine A2a receptor (Weiss and Grisshammer 2002). In our experiment CHS was used successfully for solubilization of PAB0107 and PAB0965.

We showed here for the first time that membrane proteins from *P. abyssi* could be successfully expressed in *P. pastoris* in sufficient amounts for purification. Since activity assays for these membrane proteins are lacking the correct folding of the proteins is not certainly guaranteed. We showed that the membrane proteins produced in *P. pastoris* were stable at high temperatures (except PAB0107) suggesting that they may be correctly folded.

Pichia pastoris is a valuable expression host for archaeal membrane proteins. The yield of protein produced was low but by increasing biomass we could potentially obtain enough protein to initiate structural work. Scale up of protein extraction will be needed for further biophysical characterization. Mass spectrometry will also be performed to determine the size of the proteins produced and see if glycosylation, cleavage of the putative signal peptides occurred.

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